State-of-the-Art in Research on Medical Countermeasures Against Biological Agents

(Etat de la recherche sur les contre-mesures médicales de lutte contre les agents biologiques)

The Final Report of Task Group 186 represents a first-of-its-kind International compendium on the state-of-the-art in Medical Biological Defence.
State-of-the-Art in Research on Medical Countermeasures Against Biological Agents

(Etat de la recherche sur les contre-mesures médicales de lutte contre les agents biologiques)

The Final Report of Task Group 186 represents a first-of-its-kind International compendium on the state-of-the-art in Medical Biological Defence.
The NATO Science and Technology Organization

Science & Technology (S&T) in the NATO context is defined as the selective and rigorous generation and application of state-of-the-art, validated knowledge for defence and security purposes. S&T activities embrace scientific research, technology development, transition, application and field-testing, experimentation and a range of related scientific activities that include systems engineering, operational research and analysis, synthesis, integration and validation of knowledge derived through the scientific method.

In NATO, S&T is addressed using different business models, namely a collaborative business model where NATO provides a forum where NATO Nations and partner Nations elect to use their national resources to define, conduct and promote cooperative research and information exchange, and secondly an in-house delivery business model where S&T activities are conducted in a NATO dedicated executive body, having its own personnel, capabilities and infrastructure.

The mission of the NATO Science & Technology Organization (STO) is to help position the Nations’ and NATO’s S&T investments as a strategic enabler of the knowledge and technology advantage for the defence and security posture of NATO Nations and partner Nations, by conducting and promoting S&T activities that augment and leverage the capabilities and programmes of the Alliance, of the NATO Nations and the partner Nations, in support of NATO’s objectives, and contributing to NATO’s ability to enable and influence security and defence related capability development and threat mitigation in NATO Nations and partner Nations, in accordance with NATO policies.

The total spectrum of this collaborative effort is addressed by six Technical Panels who manage a wide range of scientific research activities, a Group specialising in modelling and simulation, plus a Committee dedicated to supporting the information management needs of the organization.

- AVT Applied Vehicle Technology Panel
- HFM Human Factors and Medicine Panel
- IST Information Systems Technology Panel
- NMSG NATO Modelling and Simulation Group
- SAS System Analysis and Studies Panel
- SCI Systems Concepts and Integration Panel
- SET Sensors and Electronics Technology Panel

These Panels and Group are the power-house of the collaborative model and are made up of national representatives as well as recognised world-class scientists, engineers and information specialists. In addition to providing critical technical oversight, they also provide a communication link to military users and other NATO bodies.

The scientific and technological work is carried out by Technical Teams, created under one or more of these eight bodies, for specific research activities which have a defined duration. These research activities can take a variety of forms, including Task Groups, Workshops, Symposia, Specialists’ Meetings, Lecture Series and Technical Courses.

The content of this publication has been reproduced directly from material supplied by STO or the authors.

Published August 2016

Copyright © STO/NATO 2016
All Rights Reserved


Single copies of this publication or of a part of it may be made for individual use only by those organisations or individuals in NATO Nations defined by the limitation notice printed on the front cover. The approval of the STO Information Management Systems Branch is required for more than one copy to be made or an extract included in another publication. Requests to do so should be sent to the address on the back cover.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xvi</td>
</tr>
<tr>
<td>HFM-198 List of Authors and Other Task Group Members</td>
<td>xvii</td>
</tr>
<tr>
<td>Executive Summary and Synthèse</td>
<td>ES-1</td>
</tr>
</tbody>
</table>

## Chapter 1 – Generic Threat Assessment and Relative Risk: Biological Threat Agents and Weapons

1.1 Introduction and Definitions                                          1-1
1.2 The Technical Probability of Agent Use                                 1-2
1.3 The Dummies                                                           1-3
1.4 The State Actor                                                        1-3
1.5 The Non-State Actor                                                    1-3
1.6 Dissemination                                                          1-4
1.7 The Risk Posed by the Agents                                           1-5
1.8 The Actor                                                              1-6
1.9 Identifying and Choosing Agents                                        1-7
1.10 Procuring Seed Stock                                                  1-9
1.11 Characterisation                                                      1-9
1.12 Isolate, Culture and Confirm Properties                               1-10
1.13 Harvest and Store                                                     1-13
1.14 Weaponization                                                         1-13
1.15 Dissemination and Effect                                              1-14
1.16 Technical Probability – Non-State                                    1-15
1.17 Technical Probability – State                                         1-16
1.18 Interplay Between Targeting, Agent Choice and Intent                 1-17
1.19 A Final Word on Consequences                                          1-17
1.20 References                                                            1-18

## Chapter 2 – Genome-Based “Omics” Tools to Develop Vaccines Against Biothreat Agents

2.1 Overview                                                              2-1
  2.1.1 Literature, Database, and Web Search                                2-1
2.2 Genome-Based “Omics” Tools for Vaccine Development                    2-2
  2.2.1 Introduction                                                       2-2
  2.2.2 The Effect of “Omics” Methods in Vaccine Generation and Production 2-3
    2.2.2.1 Bacterial Vaccine Production                                    2-3
    2.2.2.2 Synthetic Biology and Viral Vaccines                          2-5
2.2.3 The Effect of “Omics” Methods in Vaccine and Immune Response: Vaccinomics
   2.2.3.1 The Unified Immune Response Network
   2.2.3.2 Population Genetic Considerations for Targeting Vaccine Development
   2.2.3.3 Immunogenetics (Summarized from Poland et al.)
   2.2.3.4 Pathogen Evolution
   2.2.3.5 Microbiome and Vaccine Immunity

2.2.4 The Effect of “Omics” Methods on Vaccine Evaluation: Correlates of Protection and Immunity
   2.2.4.1 Immunomics (Summarized from Six et al.)

2.2.5 Conclusions

2.3 References

Chapter 3 – Challenges and Technological Advances in Vaccine Development for Biodefense
3.1 Introduction
3.2 Rationale Vaccine Design: Antigen Discovery Technologies
   3.2.1 Reverse Vaccinology
   3.2.2 Structural Vaccinology
3.3 Vaccine Delivery and Adjuvants
3.4 Ex Vivo Human Mimetics
3.5 Future of Biodefense Vaccines
   3.5.1 Challenge of Evolving Biological Threats
   3.5.2 Streamlining Vaccine Development
3.6 References

Chapter 4 – Use of Adjuvants for Enhancement of Vaccine Potency
4.1 Introduction
4.2 Aluminum Salt Adjuvants
4.3 Emulsion Adjuvants
4.4 Alternative Adjuvant Systems
4.5 References

Chapter 5 – State-of-the-Art Therapeutic Medical Countermeasures for Biological Threat Agents
5.1 Introduction
5.2 Pathogen-Targeted Small Molecule Therapeutics
   5.2.1 Anti-Virals
      5.2.1.1 Poxviruses
      5.2.1.2 Filoviruses
      5.2.1.3 Venezuelan Equine Encephalitis Virus (VEEV)
   5.2.2 Anti-Bacterials
      5.2.2.1 Conventional Anti-Bacterials
      5.2.2.2 Modifications of Conventional Antibiotics
5.2.2.3 Novel Anti-Bacterial and Anti-Virulence Chemistries and Targets

5.2.3 Anti-Toxins
  5.2.3.1 Botulinum Neurotoxin
  5.2.3.2 Ricin
  5.2.3.3 B. anthracis Toxins

5.3 Host-Targeted Small Molecule Therapeutics
  5.3.1 Viral Therapeutics
  5.3.2 Toxin Therapeutics

5.4 Immunotherapeutics
  5.4.1 Adaptive Immune Therapies
    5.4.1.1 Virus Countermeasures
    5.4.1.2 Toxin Countermeasures
    5.4.1.3 Bacterial Countermeasures
  5.4.2 Innate Immune Therapies

5.5 Anti-Microbial Peptides

5.6 Nucleic Acid Sequence-Based Therapeutics
  5.6.1 Viral Therapeutics
  5.6.2 Bacterial Therapeutics

5.7 Conclusion

5.8 References

Chapter 6 – Laboratory Identification of Biological Threats

6.1 Introduction

6.2 The Laboratory Response
  6.2.1 Role of the Military Clinical and Field Laboratories
  6.2.2 Military Field Laboratories
    6.2.2.1 Army
    6.2.2.2 Navy
    6.2.2.3 Air Force
  6.2.3 Defense Laboratory Network (DLN)
  6.2.4 Identification Levels
    6.2.4.1 Civilian
    6.2.4.2 Military
    6.2.4.3 Allies

6.3 Identification Approaches
  6.3.1 Specimen Collection and Processing
  6.3.2 Culture-Based Microbiological Methods
    6.3.2.1 Automated Identification Systems
    6.3.2.2 Antibiotic and Anti-Microbial Susceptibility Testing
  6.3.3 Microbial Culture versus Rapid Methods
  6.3.4 Integration of In Vivo and In Vitro Diagnostic Tests
  6.3.5 Immunodiagnostic Methods
    6.3.5.1 Enzyme-Linked Immunosorbent Assay (ELISA)
    6.3.5.2 Electrochemiluminescence
    6.3.5.3 Flow Cytometry
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.13.2 Oral CMX001 (Brincidofovir)</td>
<td>7-18</td>
</tr>
<tr>
<td>7.13.3 Oral Arestvyr™ (ST-246)</td>
<td>7-19</td>
</tr>
<tr>
<td>7.13.4 Effect of Administration of ST-246 on Vaccine Protection</td>
<td>7-20</td>
</tr>
<tr>
<td>7.14 Smallpox Today?</td>
<td>7-20</td>
</tr>
<tr>
<td>7.15 References</td>
<td>7-21</td>
</tr>
</tbody>
</table>

**Chapter 8 – Filoviruses: Potential Vaccines, Therapeutics, and Drug Targets** 8-1

### 8.1 Introduction

8.1.1 Filoviridae

8.1.2 Pathogenesis and Host Immune Response

8.1.3 Animal Models

8.1.4 Medical Countermeasure Strategies

8.2 Supportive Care

8.3 Vaccines and Therapeutics in Clinical Trials

8.3.1 Vaccines

8.3.2 Therapeutics

8.4 Vaccines and Therapeutics Effective in Non-Human Primates

8.4.1 Vaccines Effective in NHPs

8.4.1.1 Adenovirus Vector Vaccines

8.4.1.2 *Rhabdoviridae* Vector Vaccines – rVSV

8.4.1.3 VEE Replicon Particles (VRPs)

8.4.1.4 Paramyxovirus-Based Vaccines

8.4.2 Therapeutics Effective in NHPs

8.4.2.1 Recombinant Human-Activated Protein C

8.4.2.2 RNA Interference

8.4.2.3 Stable Nucleic Acid-Lipid Particles

8.4.2.4 Immunotherapy

8.5 Vaccines and Therapeutics Effective in Small Animal Models and *In Vitro*

8.5.1 Vaccines Effective in Small Animal Models

8.5.1.1 *Rhabdoviridae* Vectored – Rabies Virus (RABV) Vaccines

8.5.1.2 Ebola Immune Complex (EIC) Produced in Nicotiana

8.5.1.3 ZEBOVGP-Fc Fusion Protein

8.5.2 Therapeutics Effective in Mouse Models

8.5.2.1 PMOs Targeting VPS4

8.5.2.2 Mannose-Binding Lectin

8.5.2.3 Ebola-Specific Monoclonal Antibodies

8.5.2.4 Small Molecule Inhibitors

8.5.3 Therapeutics Effective *In Vitro*

8.5.3.1 Niemann-Pick C1

8.5.3.2 HSP-90 Inhibitors

8.5.3.3 Δ-peptide Immunoadhesins

8.5.3.4 C-peptides

8.5.3.5 Alkylated Porphyrins

8.5.3.6 Benzodiazepine Small Molecule Compounds

8.6 Potential Future Therapeutic Targets

8.6.1 Targeting Pathogenesis
8.6.2 Targeting of the Host Immune Response 8-17
  8.6.2.1 Interferons 8-17
  8.6.2.2 Innate Immune Response Interference 8-17
  8.6.2.3 Inhibition of Apoptosis 8-18
8.6.3 Targeting Virus-Host Interactions 8-18
  8.6.3.1 Assembly and Budding 8-18
  8.6.3.2 Ubiquitination 8-18
  8.6.3.3 Protein Transport 8-19
  8.6.3.4 Entry Inhibitors 8-19

8.7 Acknowledgements 8-20
8.8 Conflict of Interest 8-20
8.9 References 8-20

Chapter 9 – Alphaviruses: Review of Medical Countermeasures 9-1
  9.1 Introduction 9-1
  9.2 The Encephalitic Alphaviruses 9-1
  9.3 Clinical Disease and Pathology 9-2
  9.4 Animal Models 9-3
  9.5 Host Immune Response 9-3
  9.6 Therapeutics 9-4
    9.6.1 Antibodies 9-4
    9.6.2 Interferons 9-4
  9.7 Prophylaxis 9-5
    9.7.1 Immune Modulators 9-5
    9.7.2 Vaccines 9-5
  9.8 Summary 9-7
  9.9 References 9-7

Chapter 10 – Anthrax and Bacillus Anthracis 10-1
  Abstract 10-1
  10.1 Introduction 10-2
  10.2 Bacterial Virulence and Anthrax Pathogenesis 10-3
  10.3 Vaccines 10-9
    10.3.1 Vaccine Immunity 10-9
    10.3.2 Filtered Cell-Free Culture Supernatant Vaccines 10-10
    10.3.3 Recombinant PA-Based Vaccines 10-10
    10.3.4 PA Combined with Other Bacillus Components 10-13
    10.3.5 Microbial Vaccine Strategies 10-14
      10.3.5.1 Bacterial Expression Systems 10-14
      10.3.5.2 Viral Expression Systems 10-15
    10.3.6 DNA Vaccines 10-15
  10.4 Diagnosis 10-16
  10.5 Therapy 10-17
    10.5.1 Antibiotics 10-17
    10.5.2 Non-Antibiotic and Combination Therapeutics 10-18
Chapter 14 – Coxiella Burnetii

14.1 Introduction
14.2 Zoonosis
  14.2.1 Transmission
  14.2.2 The Outbreak in the Netherlands
  14.2.3 Clinical Manifestations
  14.2.4 Military Relevance of Q Fever
  14.2.5 Genomics
  14.2.6 Molecular Typing
  14.2.7 Virulence Determinants
  14.2.8 Animal Models
14.3 Diagnosis
14.4 Medical Countermeasures
  14.4.1 Anti-Microbial Treatment
  14.4.2 Vaccines
14.5 Conclusions
14.6 Acknowledgements
14.7 References

Chapter 15 – A Review of Medical Countermeasures, Protection and Treatment, Against the Brucella Species

Abstract
15.0 Executive Summary
15.1 Introduction
15.2 Preventative Measures
  15.2.1 Antibiotics
  15.2.2 Anti-Brucella Antibodies
  15.2.3 Vaccines Against Brucella Species
    15.2.3.1 Killed Cells as Vaccine Candidates
    15.2.3.2 Live Attenuated Cells as Vaccine
    15.2.3.3 Sub-Unit Components as Vaccine Candidates
15.3 Treatments After Infection
  15.3.1 Antibiotics
  15.3.2 Anti-Brucella Antibodies
  15.3.3 Vaccines
15.4 Future Directions for Research and Development of Medical Countermeasures Against Brucellosis
  15.4.1 Sub-Unit Vaccines
  15.4.2 Immunomodulators
  15.4.3 Liposome Encapsulated Antibiotics and Vaccines
  15.4.4 Antibody Therapy
  15.4.5 Serum Components Other Than Antibodies
15.5 References
Chapter 20 – Biological Surety

20.1 Introduction

20.2 Biological Surety

20.2.1 Control of Biological Select Agents and Toxins

20.2.2 Registration for Possession, Use, and Transfer of Biological Select Agents and Toxins

20.2.3 Security Risk Assessment

20.2.4 Biological Select Agents and Toxins Inventory and Accountability

20.2.5 Centralized Management of Long-Term Biological Select Agents and Toxins

20.2.6 Biological Select Agents and Toxins Inventory Audits

20.2.7 Biological Select Agent and Toxin Transfers

20.2.8 Reporting Theft, Loss, or Release of Biological Select Agents and Toxins

20.2.9 Identifying Select Agents and Toxins

20.2.10 Restricted Experiments

20.2.11 Biosafety

20.2.12 Personnel Reliability

20.2.12.1 Initial Interview

20.2.12.2 Personnel Records Screening

20.2.12.3 Personnel Security Records Screening

20.2.12.4 Medical Evaluation

20.2.12.5 Drug Testing

20.2.12.6 Certifying Official’s Final Evaluation and Briefing

20.2.13 Biosecurity

20.2.13.1 Biological Select Agents and Toxins Security

20.2.13.2 Physical Security

20.2.13.3 Personnel Security

20.2.13.4 Operational Security

20.2.13.5 Information Security

20.2.14 Incident Response and Emergency Management

20.2.14.1 Theft, Loss or Release

20.2.14.2 Inventory Discrepancies

20.2.14.3 Security Breaches

20.3 Summary

20.4 References
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1-1</td>
<td>Overview of the Process of Creating a Biological Weapon</td>
</tr>
<tr>
<td>Figure 1-2</td>
<td>Actor Constraints</td>
</tr>
<tr>
<td>Figure 1-3</td>
<td>Choice of Agents</td>
</tr>
<tr>
<td>Figure 1-4</td>
<td>Procuring Seed Stock</td>
</tr>
<tr>
<td>Figure 1-5</td>
<td>Characterizing Chosen Agent</td>
</tr>
<tr>
<td>Figure 1-6</td>
<td>Determining Agent Properties</td>
</tr>
<tr>
<td>Figure 1-7</td>
<td>Storage Constraints</td>
</tr>
<tr>
<td>Figure 1-8</td>
<td>Impacting Factors on Weaponisation</td>
</tr>
<tr>
<td>Figure 1-9</td>
<td>Effect in Target</td>
</tr>
<tr>
<td>Figure 1-10</td>
<td>Casualties vs. Treatment Capacity</td>
</tr>
<tr>
<td>Figure 6-1</td>
<td>Orthogonal Diagnostic Testing Uses an Integrated Testing Strategy where More than One Technology, Technique, or Biomarker is Used to Produce Diagnostic Results, which are then Interpreted Collectively</td>
</tr>
<tr>
<td>Figure 6-2</td>
<td>The Network of Military Laboratories with Connections to Federal and State Civilian Response Systems Provides Unparalleled Depth and Resources to the Biological Threat Response</td>
</tr>
<tr>
<td>Figure 6-3</td>
<td>The Typical Infection and Response Time Course Begins with the Initial Pathogen Encounter and Leads to the Formation and Maintenance of Active Immunological Memory (IgM and IgG) where Serological Detection is Useful</td>
</tr>
<tr>
<td>Figure 6-4</td>
<td>Military Identification Levels Correspond with Both the Technology Employed and the Facility Doing the Laboratory Analysis</td>
</tr>
<tr>
<td>Figure 6-5</td>
<td>Representation of Common Enzyme-Linked Immunosorbent Assay (ELISA) Formats</td>
</tr>
<tr>
<td>Figure 6-6</td>
<td>Illustration of a Typical Hand-Held Immunoassay</td>
</tr>
<tr>
<td>Figure 6-7</td>
<td>Generic Overview of PLA Reactants and Assay</td>
</tr>
<tr>
<td>Figure 6-8</td>
<td>Overview of Real-Time PCR Reactants and Reaction Conditions</td>
</tr>
<tr>
<td>Figure 10-1</td>
<td>A Schematic Diagram Paired with a Transmission Electron Micrograph Illustrating the Layers of a \textit{B. anthracis} Spore</td>
</tr>
<tr>
<td>Figure 10-2</td>
<td>Functional Domains of Anthrax Toxin Components, Protective Antigen (PA), Lethal Factor (LF), and Edema Factor (EF)</td>
</tr>
<tr>
<td>Figure 18-1</td>
<td>Structural and Functional Similarities Among Ribosome Inactivating Proteins (RIPs)</td>
</tr>
<tr>
<td>Figure 18-2</td>
<td>The Ricin A-Chain Catalyzes the Hydrolysis of an Adenine in the Ricin-Sarcin Loop</td>
</tr>
<tr>
<td>Figure 18-3</td>
<td>RTA Catalyzed Depurination Reaction</td>
</tr>
</tbody>
</table>
Figure 18-4  Histological Sections of Lungs from CD1 Mice Exposed to Ricin by Aerosol Showing Perivascular Edema and Pulmonary Epithelial Necrosis, H&E Stain; 25X Original Mag; Pulmonary Epithelial Cell Necrosis, H&E Stain, 100X Original Mag

Figure 18-5  Lungs from a NHP Exposed to Ricin by Aerosol Exposure

Figure 18-6  Ricin Vaccines have been Derived from the A-Chain of the Toxin

Figure 19-1  Colorized Scanning Electron Micrograph of *Staphylococcus aureus*

Figure 19-2  Staphylococcal Enterotoxin B Structure

Figure 20-1  Key Elements of the Federal Select Agent Program and Biological Surety Program
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1-1</td>
<td>Effect of Dissemination of Something Like Anthrax on Disease Development – Time-Scale</td>
</tr>
<tr>
<td>Table 1-2</td>
<td>Competence and Facility Requirements</td>
</tr>
<tr>
<td>Table 1-3</td>
<td>Evaluation of Agent Properties as Biological Threat Agents (Aerosol Release)</td>
</tr>
<tr>
<td>Table 5-1</td>
<td>CDC Recommended Anti-Bacterials for Therapeutic Use Against Select Biodefense Agents</td>
</tr>
<tr>
<td>Table 5-2</td>
<td>Representative List of Compounds in Development that Fall Within Conventional Antibiotic Classes</td>
</tr>
<tr>
<td>Table 5-3</td>
<td>Representative List of Novel Anti-Bacterial and Anti-Virulence Compounds in Development</td>
</tr>
<tr>
<td>Table 6-1</td>
<td>Regulated Biological Select Agents and Toxins</td>
</tr>
<tr>
<td>Table 6-2</td>
<td>Presumptive and Confirmation Methods</td>
</tr>
<tr>
<td>Table 6-3</td>
<td>Specimen Collection for Select Biological Warfare Agents</td>
</tr>
<tr>
<td>Table 6-4</td>
<td>International and Domestic Standards for Shipping</td>
</tr>
<tr>
<td>Table 6-5</td>
<td>Viral Hemorrhagic Fever (VHF) Culture Information</td>
</tr>
<tr>
<td>Table 6-6</td>
<td>Automated Identification Systems for Biological Threat Agents</td>
</tr>
<tr>
<td>Table 6-7</td>
<td>Standard Antibiotic Susceptibility Testing for Biological Threat Agents</td>
</tr>
<tr>
<td>Table 6-8</td>
<td>Comparison of Immunodiagnostic Methods</td>
</tr>
<tr>
<td>Table 6-9</td>
<td>Biological Warfare Agent Disease Characteristics</td>
</tr>
<tr>
<td>Table 7-1</td>
<td>Real-Time PCR Assays Proven to be Useful for a Specific Identification of Variola Virus</td>
</tr>
<tr>
<td>Table 18-1</td>
<td>Case Report: Ricin Poisoning Causing Death After Ingestion of Herbal Medicine</td>
</tr>
<tr>
<td>Table 18-2</td>
<td>Biochemical Methods for Ricin Detection and Their Limits of Sensitivity</td>
</tr>
<tr>
<td>Table 20-1</td>
<td>Timeline of International Rules and Treaties to Limit or Ban Chemical and Biological Weapons Use</td>
</tr>
<tr>
<td>Table 20-2</td>
<td>Permissible Toxin Amounts</td>
</tr>
</tbody>
</table>
HFM-186 List of Authors

Julia E. BIGGINS (Chapter 8)
USAMRIID, Division of Virology, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: Julia@IntegratedBiotherapeutics.com

Joel A. BOZUE (Chapter 10)
USAMRIID, Division of Bacteriology, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: joel.a.bozue.civ@mail.mil

Paula BRYANT (Chapters 3, 8)
J9-Chemical and Biological Technologies Department
Translational Medical Division
8725 John J. Kingman Road
Fort Belvoir, VA 22060
UNITED STATES
Email: paula.bryant@nih.gov

America M. CERALDE (Chapter 20)
Alternate Surety Officer and Chief Personnel Reliability Program Branch
USAMRIID, Biosurety Division
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: america.m.ceralde.civ@mail.mil

John W. CHERWONOGRODZKY (Chapter 15)
BioTechnology Section
DRDC – Suffield Research Centre
P.O. Box 4000, Station Main
Medicine Hat, Alberta T1A 8K6
CANADA
Email: jcherwono@shaw.ca

Christopher K. COTE (Chapter 10)
USAMRIID, Division of Bacteriology, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: christopher.k.cote.civ@mail.mil

Jon M. DAVIS (Chapter 18)
Lieutenant Colonel, Medical Service Corps
Office of the Assistant Secretary of Defense for Nuclear, Chemical and Biological Defense Program 3050, Defense Pentagon
Washington, DC 20301-3050
(formerly, Chief, Division of Integrated Toxicology
USAMRIID, 1425 Porter Street, Frederick, MD 21702-5011)
UNITED STATES
Email: Jon.Davis@hhs.gov

Samuel S. EDWIN (Chapter 20)
Responsible Official and Surety Officer Chief, biosurety Division
USAMRIID, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: samuel.s.edwin.civ@mail.mil

William FLORENCE (Chapter 3)
Defense Threat Reduction Agency J9-Chemical and Biological Technologies Department
Translational Medical Division
8725 John J. Kingman Road
Fort Belvoir, VA 22060
UNITED STATES
Email: clint.florence@taurigroup.com

Brian M. FRIEDRICH (Chapter 8)
USAMRIID, Division of Virology, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: bfriedrich@live.com

John C. GORBET (Chapter 18)
Medical Service Corps Chief, Department of Aerosol Services
USAMRIID, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: john.c.gorbet.mil@mail.mil
Hermann MEYER (Chapter 7)
Bundeswehr Institute of Microbiology
Head of BSL 3 Laboratory
Neuherbergstrasse 11
D-80937 Muenchen
GERMANY
Email: hermann1meyer@bundeswehr.org

Timothy D. MINOGUE (Chapter 6)
USAMRIID, Diagnostic Systems Division,
Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: timothy.d.minogue.civ@mail.mil

Garry MOREFIELD (Chapter 4)
VaxForm
116 Research Drive
Bethlehem, PA 18015
UNITED STATES
Email: garry.morefield@vaxform.com

Les P. NAGATA (Chapter 9)
BioTechnology Section
DRDC – Suffield Research Centre
P.O. Box 4000, Station Main
Medicine Hat, Alberta T1A 8K6
CANADA
Email: lnagata@ualberta.ca

David NORWOOD (Chapter 6)
USAMRIID, Diagnostic Systems Division,
Fort Detrick
Frederick, MD 21702-5011
UNITED STATES
Email: david.a.norwood.civ@mail.mil

Gene G. OLINGER (Chapter 8)
USAMRIID, Division of Virology, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: gene.olinger@nih.gov

Gustavo PALACIOS (Chapter 2)
USAMRIID, Molecular and Translational Sciences,
Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: gustavo.f.palacios.ctr@mail.mil

Virginia I. ROXAS-DUNCAN (Chapters 17, 18, 20)
Chief, Select Agent Management Branch
Biosurety Division
USAMRIID, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: virginia.i.duncan.civ@mail.mil

Aruna SAMPATH (Chapter 5)
Science Applied International Corporation (SAIC)
Contractor to the U.S. Department of Health and
Human Services
Office of the Assistant Secretary for Preparedness
and Response, Biomedical Advanced Research
and Development Authority
Washington, DC
UNITED STATES
Email: sampathA1@ebsi.com

Mariano SANCHEZ-LOCKHART (Chapter 2)
Molecular and Translational Sciences
USAMRIID, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: mariano.sanchez-lockhart.ctr@mail.mil

Randal SCHOEPP (Chapter 6)
USAMRIID, Diagnostic Systems Division,
Fort Detrick
Frederick, MD 21702-5011
UNITED STATES
Email: randal.j.schoepp.civ@mail.mil

Leonard A. SMITH (Chair, Chapters 17, 18, 19)
Senior Research Scientist (ST) for Medical
Countermeasures Technology
Office of the Chief Scientist
USAMRIID, Fort Detrick
1425 Porter Street
Frederick, MD 21702-5011
UNITED STATES
Email: Leonard.A.Smith@comcast.net
Other Task Group Members

Janet Martha BLATNY
Forsvarets forskningsinstitutt
FFI Norwegian Defence Research Establishment Division Protection
P.O. Box 25, Instituttveien 20
N-2027 Kjeller
NORWAY
Email: janet-martha.blatny@ffi.no

Rashid A. CHOTANI
Chemical Biological Medical Systems
64 Thomas Johnson Drive
Frederick, MD 21702-4300
UNITED STATES
Email: rashid.chotani@amedd.army.mil

Jacques MATHIEU
IRBA/CRSSA French Army Biomedical Institute
Head of the Dept. of Microbiology
24 Avenue des Maquis du Grésivaudan
F-38702 La Tronche Cedex
FRANCE
Email: jdmathieu@crssa.net

Dominique VIDAL
IRBA
24, Avenue des Maquis du Grésivaudan
F-38702 La Tronche Cedex
FRANCE
Email: domvidal@gmail.com

David WILLIAMS
Chemical Biological Medical Systems (CBMS)
64 Thomas Johnson Drive
Frederick, MD 21702-4300
UNITED STATES
Email: dave.williams2@amedd.army.mil
State-of-the-Art in Research on Medical Countermeasures Against Biological Agents
(STO-TR-HFM-186)

Executive Summary

The threat of biological agents used against NATO forces and/or the potential for exposure to emerging and re-emerging infectious diseases among deployed forces remains a critical concern of all military Commanders. Preparedness against these disease causing agents requires a full-spectrum understanding of their mechanisms of action, rapid diagnosis of exposure and infection, and how to most effectively counter their effects – either pre- or post-exposure. Many biological agents (Biological Select Agents and Toxins (BSAT) as listed on the U.S. Centers for Disease Control and Prevention (CDC) Category A, B, and C lists) are still without licensed/approved vaccines and most do not have effective therapies to intervene in the pathogenesis of those diseases. Many of these diseases have a very low incidence of natural occurrence and are of little concern to the civilian research, development, and medical care communities. Even where these diseases are being examined within the civilian and academic communities, their diagnosis and treatment is addressed from a “hospital” perspective. While pre-exposure immunization remains the most effective prevention measure, recent history has demonstrated the difficulty in developing and obtaining regulatory approval of vaccines. In the absence of active immunization, rapid diagnosis of exposure and/or recognition of clinical manifestation of disease is the essential first step towards effective treatment. Addressing all of these factors pre-deployment and in a forward deployed setting is critical to sustained military operations.

NATO RTG/HFM-186 provided a threat and relative risk assessment in their Technical Report considering 15 of the most dangerous and deadly viruses (smallpox virus, viral hemorrhagic fever filoviruses, encephalitis alphaviruses), bacteria (causative agents for anthrax, plague, tularemia, Q-fever, brucellosis, typhus, melioidosis and glanders), and toxins (botulinum neurotoxin, SEB and ricin) that could pose significant peril to NATO forces and their medical readiness to function when needed. The Task Group reviewed cutting-edge technologies and platforms for the advancement of vaccines, therapeutic interventions and diagnostic tests for identifying these biological threat agents. The Task Group examined and reported on the current state-of-the-art in research on medical countermeasures against each of the biological threat agents. Although much progress has been achieved in understanding the mechanism of action(s) of these pathogens, as well as in the development of laboratory tests to identify them, much more work is required to produce license vaccines to protect against them and license therapeutics to intervene in the disease pathogenesis where vaccines and vaccinations are absent. Realization that the biological threat is real is the first step towards preparedness. Continued commitment along with the requisite investments in research, development, test and evaluation of medical countermeasure products against the biological threat agents discussed in this Technical Report, as well as other emerging and re-emerging infectious disease agents, will be required if NATO forces are to be “ready and prepared”.

STO-TR-HFM-186 ES - 1
Etat de la recherche sur les contre-mesures médicales de lutte contre les agents biologiques
(STO-TR-HFM-186)

Synthèse

La menace d’agents biologiques utilisés contre les forces de l’OTAN et/ou le potentiel d’exposition à des maladies infectieuses émergentes ou ré-émergentes au sein des forces déployées demeure une vive inquiétude pour tous les commandants militaires. L’état de préparation à ces agents pathogènes requiert une compréhension totale de leurs mécanismes d’action, un diagnostic rapide de l’exposition et de l’infection et une connaissance de la façon d’en contrer les effets les plus efficacement, avant ou après l’exposition. De nombreux agents biologiques (agents et toxines biologiques sélectionnés (BSAT) inscrits sur les listes A, B et C des Centers for Disease Control and Prevention aux États-Unis) ne disposent toujours pas de vaccins autorisés / approuvés et la plupart d’entre eux n’ont pas de traitement efficace agissant sur la pathogénie. Nombre de ces maladies ont une très faible incidence naturelle et présentent un faible intérêt pour les communautés civiles de la recherche, du développement et de la médecine. Même si les maladies sont examinées dans les communautés civile et universitaire, leur diagnostic et leur traitement sont abordés d’un point de vue « hospitalier ». Alors que l’immunisation avant exposition demeure la mesure de prévention la plus efficace, l’histoire récente a prouvé combien il était difficile de développer de nouveaux vaccins et de les faire approuver par les voies réglementaires. En l’absence d’immunisation active, le diagnostic rapide de l’exposition et/ou la reconnaissance de la manifestation clinique de la maladie sont la première étape essentielle vers un traitement efficace. Dans le cas des opérations militaires prolongées, il est capital de traiter tous ces facteurs avant le déploiement et dans le cadre d’un déploiement avancé.

Le TG-186 du HFM de l’OTAN a fourni une évaluation de la menace et du risque y afférent dans son rapport technique, en étudiant 15 des virus (virus de la variole, filovirus de la fièvre hémorragique virale, alphavirus des encéphalites), bactéries (agents étiologiques de l’anthrax, la peste, la tularémie, la fièvre Q, la brucellose, le typhus, la méloïdose et la morve) et toxines (neurotoxine botulique, entérotoxine staphylococcique B et ricine) les plus dangereux et les plus mortels pouvant représenter un risque important pour les forces de l’OTAN et leur état de préparation médicale en cas de besoin. Le groupe de travail a étudié les technologies et les plateformes de pointe pour l’avancement des vaccins, interventions thérapeutiques et tests diagnostiques d’identification de ces agents de menace biologique. Le groupe de travail a examiné et fait un compte rendu sur l’état de la recherche au sujet des contre-mesures médicales applicables à chacun des agents de menace biologique. Bien que de grands progrès aient été faits en ce qui concerne la compréhension des mécanismes d’action de ces pathogènes, ainsi que le développement d’essais en laboratoire pour les identifier, bien d’autres travaux sont nécessaires pour d’une part, produire les vaccins autorisés qui protégeront les personnes et d’autre part, autoriser des traitements qui interviendront dans la pathogénie de la maladie en cas d’absence de vaccin et de vaccination. Le fait de comprendre que la menace biologique est réelle constitue la première étape vers la préparation. Si l’on veut que les forces de l’OTAN soient préparées à toute éventualité, il faut faire preuve d’un engagement constant et investir dans la recherche, le développement, les essais et l’évaluation de produits de contre-mesure médicale qui luttent contre les agents de menace biologique traités dans le présent rapport technique et contre d’autres agents pathogènes infectieux émergents et ré-émergents.
Chapter 1 – GENERIC THREAT ASSESSMENT AND RELATIVE RISK: BIOLOGICAL THREAT AGENTS AND WEAPONS

LtCol Per Leines Lausund, DVM MPH, Maj. Knut Amund Grani, DVM and Professor Per-Einar Granum, PhD
Forsvarets forskningsinstitutt FFI
Norwegian Defence Research Establishment, Kjeller
NORWAY

Corresponding Author (Lausund): veterinary.surgeon@gmail.com

1.1 INTRODUCTION AND DEFINITIONS

Biological threat agents are agents that cause disease or damage to humans, other animals, plants or materiel, to (mis-)quote the NATO definition. Here, we are concerned with the threat and risks that humans may be exposed to if or when a biological threat agent (this publication covers those agents on our list) is used as a weapon or terrorist tool. At our meeting in Norway in May 2010, we achieved consensus on a list of 15 agents that we in the RTG/HFM-186 believe are the most probable threat agents and the ones we should concern ourselves with. This chapter, with an assessment of the relative risk and threat associated with biological threat agents is limited to these, though other agents may be dealt with the same way in order to expand the list.

A common definition of the term “risk” is as an expression of the probability and consequences of an event. When discussing the relative merits of the agents on the list, this event may be seen as “intentional use” expressed as the probability based on the technical challenges that must be met for each agent. The term “risk” is thus used to characterise the product of the probability of intentional use based on technical feasibility, as defined by the underlying factors that are evaluated, and the expected consequences of such use.

The term “threat” usually expresses a product of intent and capability to create an adverse event. The dimension of the threat is usually decided by the degree of intent and the actual capacity of the actor. Capacity is understood as the quantification of capabilities. In order to evaluate the relative threats posed by agents on the list one would have to delve deeper into the minds of potential perpetrators, which is outside the scope of this publication. However, in order to make an attempt at describing the relative threat that could be related to these agents, two dummy entities are used as anonymous examples of a state and non-state actor respectively, and are used to have a baseline “intent” for the purposes of threat description. Detailed capacities and intentions for both are described and defined later in this chapter. The use of disease in the sabotage role is outside the scope of the document.

When the phrase “Biological Weapon (BW)” is used in this document it must be interpreted as describing a completed attack system using a biological agent as filling, and including all the necessary components in a piece of ordinance (casing, fuse, control mechanisms, safeguards, dissemination or dispersal mechanism/system, etc., and with a militarily accepted efficiency and predictability when used) that has been tested and approved as functional. This process and the development of a product are extremely complicated, expensive and time-consuming, and it would not be expected that a non-state actor could develop such a system unnoticed in today’s environment. Of the known groups, the Aum Shinrikyo in Japan [1] is the one that was closest to producing an actual weapon, but did not have the necessary testing and proofing, and as such they failed.
The non-state actor is assumed to have lesser capabilities and capacities than a state, and also to be unable to produce a biological weapon as described above. The degree of refinement and sophistication of a device used by a non-state actor will of course vary with the abilities, skills, knowledge and opportunities of the actor, but the common phrase used here for this kind of device is BTA or “Biological Terrorism Agent” to cover both agent and delivery mechanisms associated with it.

1.2 THE TECHNICAL PROBABILITY OF AGENT USE

Several factors influence the probability of a specific agent being used in or to develop a Biological Weapon (BW) or a Biological Terrorism Agent (BTA). The list of factors is also a list of requirements and challenges to be met during the selection, acquisition, development and use of a biological agent. In most cases, the difference between BW and BTA will be in how well these challenges and requirements can and need to be met. The description of the technical probability of agent use will not differentiate between these two facets, but will be covered in the evaluation of the threat the different agents pose when in the hands of a state or non-state actor based on their level of technical proficiency and their knowledge and skills.

Factors:

- Availability of agent, either in culture collections or in nature, or from diagnostic samples.
- Identifiability, i.e. how well-suited the agent is to definitive identification:
  - Differing strains; and
  - Differing pathogenicity/virulence in sub-strains.
- Culture, describing the degree of skills and conditions necessary to culture or replicate agent.
- Isolation of agent, the difficulty or easy with which it can be “lifted” from a mixed culture.
- Virulence describes the ability of the agent to cause disease when the optimal sub-strain is chosen.
- Stability, describing how well the agent retains its properties with regards to virulence, viability, etc., \textit{in vitro}:
  - Including development in the presence of immunity (modifications to circumvent); and
  - Routes of infection.
- EoP, or Ease of Production, where end result is usable quantities of agent suitable for weaponization or use.
- EoH, or Ease of Handling, describing how easily the agent can be handled from acquisition and production through dissemination.
- Morbidity expectations.
- Mortality expectations (in the absence of countermeasures).
- TTE, or Time To Effect, denoting probability of a release causing effects within a desired and predictable time-frame.
- RoE, or Reliability of Effect, including environmental survivability during dissemination.
- Possibility of genetic or other modification.
1.3 THE DUMMIES

Two categories of actors will be described and represented by dummies in this document: the state and non-state actors. The non-state actor is assumed to operate without dependable state support.

1.4 THE STATE ACTOR

The dummy used to exemplify a state actor has the following characteristics:

- A burgeoning biotechnological industrial base, with established production of fermentation-based and gene modified products.
- A scientific base for the biotechnology industry in the country and an active R&D establishment working in the fields of microbiology and molecular genetics, and with at least one Biosafety Level (BSL)-3Ag facility with access to laboratory animals, available with the necessary security arrangements in place to be able to conduct agent tests clandestinely.
- A military or military controlled scientific establishment working in parallel with, and drawing knowledge and experience from, the civil science and R&D establishment.
- A civil and military medical service able to afford what the regime deems to be sufficient protection of the country’s population and armed forces in case a biological warfare agent is used in conflict.
- An intention, i.e. the intent to be able to deploy a Biological Weapon (BW) in order to start, maintain or influence a conflict and cause casualties that would decisively alter the paradigm of such conflict. This intent could be driven by an aspiration to have and be able to use a weapon of mass destruction that is less visible in development than a chemical or nuclear weapon would be, and possibly as a response to a perceived adversary’s capabilities.

The development of a biological weapons programme would in the early stages be impossible to distinguish from medical (including veterinary medical) and environmental research; indeed, much of the relevant and necessary information could be derived from completely open and legal microbiological, infectious disease and environmental research. Only in the later stages, where agent survivability and large-scale dissemination needs to be tested, will accidents or observations give indications of the activity. This might be in the shape of a non-transparent pathogen testing programme, unusual vaccine or biological countermeasures development, the establishment of “false-flag” bio-containment or BSL-3, BSL-3Ag+ or preferably -4 facilities, or the appearance of small outbreaks or clusters of unusual disease.

1.5 THE NON-STATE ACTOR

The non-state actor would typically be a belligerent group driven by radical ideas, by definition independent of any government, willing to use violence or death as a tool and willing to extend the use of this tool to include deliberate introduction of dangerous pathogens into society. Most such groups would be considered terrorists, but could be deniably supported by some states. If such a group obtains a safe haven in any country for the purposes of establishing headquarters and, in this case, an R&D base, they would after about a year move into a third category, the state-supported actors with capabilities that will lie between the non-state actors and states with a bio-weapons programme. This third category will not be discussed further in this document, but will be classed in the state category.

In our context, a non-state actor has restricted overt access to technology and scientific institutions. The ability to communicate without being compromised is also limited, as is the ability to travel. The actor will have limited
scientific and technological capabilities quantitatively or qualitatively, and will also be constrained in time when they are a known threat: counter-terrorism has a high priority. This again restricts the freedom of choice in targeting, means and scope of an attack.

There are many definitions of terrorism\(^1\), but the most comprehensive and easily understandable one [2] we have found defines five necessary elements:

- Terror is a method and not an ideology in itself\(^2\). Terror is in other words a tool.
- Terror contains the threat of systematic and directed violence.
- Terror is usually directed against civil society, it is apparently indiscriminate and its targets often of symbolic value.
- The fear derived as a consequence of the first three elements, and which makes tactical success possible, is the precondition for the fifth element: a strategic goal, which is political.

From this one may also draw the conclusion that a non-state actor using a BTA need not cause overly many casualties, but must give the impression that they can and will. The non-state actor will have an intention to cause terror by the deliberate use of biological agents causing disease and death against their targets.

### 1.6 DISSEMINATION

Based on the requirements for the precise and predictable effect of weapons use from state actors and the need for spectacular attributability from the non-state actor, a requirement will usually be that the effects of an attack occur as simultaneously and within as defined a time-frame as possible. Besides demonstrating that this is an intended event and maximising injury, it will also minimise the ability of health services to respond sufficiently. A comparison of some possible dissemination routes is given below with regard to timing and efficiency. Terminology has been garnered from Sackett et al. for the phasing [3].

<table>
<thead>
<tr>
<th>Dissemination via</th>
<th>Period of Infection</th>
<th>Prodromal Phase</th>
<th>Acute Disease</th>
<th>End-State</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfaces</td>
<td>Days</td>
<td>1 – 10 days</td>
<td>6 – 20 days</td>
<td>Uncertain</td>
</tr>
<tr>
<td>Foods</td>
<td>Days – Weeks</td>
<td>Days – Weeks</td>
<td>Days – Weeks</td>
<td>Uncertain</td>
</tr>
<tr>
<td>Water</td>
<td>Days</td>
<td>1 – 10 days</td>
<td>6 – 20 days</td>
<td>Uncertain</td>
</tr>
<tr>
<td>Aerosol</td>
<td>Minutes</td>
<td>1 – 5 days</td>
<td>3 – 8 days</td>
<td>4 – 14 days</td>
</tr>
</tbody>
</table>

---

1. Terrorism is defined by the U.S. Department of Defense as “the unlawful use of – or threatened use of – force or violence against individuals or property to coerce or intimidate governments or societies, often to achieve political, religious, or ideological objectives.” [www.pbs.org/wgbh/pages/frontline/teach/alqaeda/glossary.html](http://www.pbs.org/wgbh/pages/frontline/teach/alqaeda/glossary.html) downloaded 11 September 2007. The main challenge faced when trying to define terrorism, and which tends to defy lawmakers, is that acts of terror encompass several different criminal acts that are part of the phenomenon, and are difficult to use to formulate an exclusive, formal definition. Terror may be explained analytically, or definitely defined.

2. Rote Arme Fraktion is an example where violence (Urban guerrilla warfare) was a goal in itself as it countered (Max Webers observation on) “... das Gewaltmonopol des Staates...”. (Max Weber: “Politics as a Vocation”, closely followed by Thomas Hobbes in “The Leviathan” where he describes the necessity of an instrument of violence to retain power).
Notes:

- Contaminating surfaces will certainly cause infection, but within an undefined time-frame and dependent on agent survivability. Contagion will thus be distributed over days depending on contact time and amount, received dose will vary, and disease will occur over a longer time axis that does not give a clear indication of an attack. Gastrointestinal and cutaneous infection is achieved, with an uncertain outcome with respect to the number of dead and sick.

- Foodstuffs may be infected intentionally, and will give a long-lasting, low-dosage exposure dependant on when and how the relevant food is consumed. It will complicate identification, but also weaken the claim and effects of a biological attack. Gastrointestinal infections will result, but with even less certain outcome than after surface contamination.

- Contaminating water-supplies, while doable may give the same effects as contaminating surfaces. Tap water is difficult to contaminate due to disinfectants and dilution, but local sources or piping areas may be efficiently utilised. The impact will be very variable, depending on water use in the target.

- Infection from inhaled air containing an aerosol gives an immediate dose and an acceptable probability of massive, simultaneous contagion in the target. Disease progression will be similar in all affected, and the probability of the desired effect being reached high.

1.7 THE RISK POSED BY THE AGENTS

When evaluating the risk posed by the different agents in these actor-scenario settings, absolute numbers are very complex to find or even estimate. What will be covered is the relative risk represented within the group of agents chosen, based on an evaluation of the different factors related to the agents. Thus, an easily identifiable and accessible, suitably pathogenic agent that is stable in culture and has a good shelf-life may have a higher relative risk than one which is more difficult to culture and identify, even though the latter may have higher pathogenicity. As these factors are to a large extent decided by access to knowledge, technical expertise and equipment they will be weighted differently between the non-state and state dummy when estimating the technical probability of use.

![The path from intent to effector](image-url)

**Figure 1-1: Overview of the Process of Creating a Biological Weapon.**
1.8 THE ACTOR

We are facing two potential groups of actors: the state-supported, or national who bases their effects on political and military power, and the non-state actor basing their effects on terrorism.

In both cases the four factors – intent, knowledge, ability and skills – are decisive, as are the differences in constraints: few constraints need bother the state actor provided there is an intent, while the non-state actor, operating outside the law and norms of society, is at least constrained in time, knowledge, ability, skills and, not least, security.

We have developed a matrix to describe some of these constraints as they influence agent production, and to all intents and purposes the level we should be considering when assessing the dummy non-state actor is 2, the state actor somewhere between 3 and 4.

It is important to bear in mind that the principal quantitative difference between these two groups is caused by the unequal constraints of resources, supplies, containment and storage facilities, delivery vehicles, time, knowledge and skills.

### Table 1-2: Competence and Facility Requirements.

<table>
<thead>
<tr>
<th>Level</th>
<th>Agents</th>
<th>Competence</th>
<th>Facilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Contamination with fecal matter or similar</td>
<td>No scientific background necessary</td>
<td>None necessary</td>
</tr>
<tr>
<td>1</td>
<td>Agents in impure culture</td>
<td>Basic microbiology</td>
<td>Simple lab</td>
</tr>
<tr>
<td>2</td>
<td>Pure, identified agents</td>
<td>+Technical skills and diagnostic knowledge</td>
<td>Containment corresponding to « almost BSL-3 »</td>
</tr>
</tbody>
</table>

3 Intent is described earlier, and is the basis for any assessment or evaluation of threat. An actor must have the intent of using biological agents to cause harm. Knowledge cover the whole aspect of tacit and intangible knowledge necessary to run the process of producing a biological agent in such a form as to be usable to fulfil an intent. Ability covers accessibility to facilities and equipment that makes the process achievable, skills are those necessary to exploit these abilities.
Level 0 needs resources that a transnational group might have; these would then be considered outside the non-state actor group, more similar to states.

At Level 0, the actor is seen capable of using pollutants directly to contaminate the environment. There is no demand on scientific competence or need for facilities to accomplish this; fecal matter polluting a well is a sufficient example.

At Level 1, agents, only probably identifiable, would be cultivated in impure cultures and with questionable, if any, effectiveness. At Level 2, isolated and identified strains would be developed and used, at Level 3 we would find stabilized and confirmed agents and at Level 4 the biological warfare agents.

1.9 IDENTIFYING AND CHOOSING AGENTS

Identifying and choosing biological agents for development into effectors is crucial to any success, and is the first test of the capability of the actor.

The steps (2) – Choosing agent

A state actor needs a weapon with defined and predictable effects in order to use it as part of a campaign plan and be able to exploit the results. It needs an agent that gives an operational or strategic advantage; the effect must be large enough to achieve this and at the same time be a deterrent. Based on the knowledge base represented by the state’s collective R&D resources, a state may choose a suitable effector, with a focus on properties that work towards an operational or strategic advantage.
A non-state actor does not need the same quantitative EFFECTS; they need to create fear or a state of terror. The agent must be recognisable as DANGEROUS, but as mentioned earlier attributability is crucial, it must be apparent that the event is intentional.

Our group, RTG/HFM-186, has gone through the first stage one would see in any kind of programme, be it defensive or offensive, involving biological agents in a threat scenario. This involves the use of skills, abilities and knowledge to identify and evaluate agents as threats. Any choice of agents will always be debatable, some will have a preference for one while excluding another, others would like to include many more, some would even deny the possibility of any agent having a belligerent potential. The mix of expertise in different fields associated with defence against biological weapons has, however, led us to a consensus list containing 15 agents, of which three are toxins. A state would probably be able to ID and choose any 14 of our 15 agents, variola major (smallpox) being a special case, as knowledge, skills and abilities are not serious constraints. Non-state actors would be restricted in choices due to these constraints, and would probably choose agents based on prior knowledge of effects and high accessibility. This knowledge would be acquired by studying former BW programmes, high-impact diseases and the morbidity and mortality potential of accessible agents.

Most of the agents listed will be familiar to anyone interested in this aspect of microbiology, and based on an evaluation of virulence, pathogenicity, stability, availability, ease of production and dissemination, possibilities of modification, and knowledge of the potential the different agents have to cause harm when used belligerently, we ended up with this choice:

1) Variola major (smallpox);
2) *Bacillus anthracis* (including MDR) (anthrax);
3) *Yersinia pestis* (plague);
4) *Francisella tularensis* (tularemia);
5) Filovirus Ebola;
6) Filovirus Marburg (viral hemorrhagic fevers);
7) *Clostridium botulinum* toxin (botulism);
8) Alphaviruses (VEE, EEE, WEE) (viral encephalitis);
9) *Brucella* species (brucellosis);
10) *Burkholderia mallei* (glanders);
11) *Burkholderia pseudomallei* (melioidosis);
12) *Coxiella burnetii* (Q-fever);
13) Staphylococcal enterotoxins;
14) *Rickettsia prowazekii* (typhus fever); and
15) Ricin toxin.

Some of these will be familiar from former, discarded weapons programmes; the reason for this being, of course, that behind the selection of agents chosen for development in the former arsenals of some Nations lay a great deal of science and knowledge of effects that is difficult to bypass. The others may be seen as what we regard as the most potential of the representatives of new or emerging disease-causing agents. With the exception of Variola major, they are all zoonotic with animals as their main host.
Further challenges are provided by the continuous emergence of these kinds of agents, and as science advances, so does the ability to use and change these agents into new agents of war or terrorism. A special concern is the synthesis of highly pathogenic microorganisms, either unintentionally as seen with mouse ectromelia virus [4] in 001 or intentionally. These concerns must be covered elsewhere.

1.10 PROCURING SEED STOCK

Finding the right pathogen sub-strain to work with is a crucial success criterion in any kind of offensive programme.

![Image of Procuring Seed Stock]

This is the “do, die or don’t” of a programme. Again, non-state actors are hampered by lack of the access states have to diagnostic laboratories, research labs and culture collections in their own or friendly Nations. They are reduced to stealing from laboratories or collecting from outbreaks, a complicating uncertainty factor as regards the effectiveness of the agent. States will succeed if they put their minds to it; non-states may have to go for the tried and tested agents. This is an important limiter for the non-states, and is where they and states part ways with regards to technical probability, and thus risk.

1.11 CHARACTERISATION

The actor further needs to characterise agents with regard to virulence and pathogenicity and stability of properties during further work and development. This may be one of the points where the Aum Shinrikyo group failed; they produced an ample load of anthrax spores only to experience the embarrassment of them being a vaccine strain.
Where the state actor will work towards optimization of the chosen strain, the non-state actors efforts may be hampered by lack of access to really virulent strains, and the lack of ability, time and resources to modify the available strains. In our spore dissemination experiments at the FFI we have assumed an inhalational LD 90 of around 25000 spores, which may be in the region expected from modestly virulent wild-types of *B anthracis*. This gives a 40 cu m effective aerosol using COTS equipment [5]. Finding a better sub-strain, say with an LD 90 of 5000 would increase the effective cloud by a factor of 5 – 8.

However, 25000 is assessed to be good enough for a non-state actor if the intent is to produce mass fear rather than mass casualties.

### 1.12 ISOLATE, CULTURE AND CONFIRM PROPERTIES

At this step the actor needs to find the actual individual colonies they are using for their effector, the actual seed-stock, and properties like stability of virulence and viability must be re-determined. At this stage the qualitative and quantitative aspects of the final effector are to a very great degree determined. We have assessed the 15 agents the RTG/HFM-186 has chosen, and each stage and step is assigned a numeric value describing how dangerous or suitable an agent is at that step. The numbers from 1 through 9 are negotiable but based on what we could find from textbooks, practical and clinical experience and discussions with colleagues and others. The number 4 denotes neutral, less is difficult or unsuitable, more is easier or suited.
The steps (5) – Isolate, culture and confirm properties

- Isolate agent, culture and reconfirm properties
- At this step the actual seed-stock for BTA or BW must be found, and properties like stability of virulence and viability re-determined

Figure 1-6: Determining Agent Properties.
### Table 1-3: Evaluation of Agent Properties as Biological Threat Agents (Aerosol Release)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Availability</th>
<th>ID</th>
<th>Culture</th>
<th>Isolation from Culture</th>
<th>Virulence</th>
<th>Stability of Properties</th>
<th>EoP</th>
<th>EoH</th>
<th>Morbidity</th>
<th>Mortality</th>
<th>TTE</th>
<th>RoE</th>
<th>Modifiable</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em></td>
<td>9</td>
<td>7</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>7/3*</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Botulinum toxin</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>9</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>5</td>
<td>Y</td>
</tr>
<tr>
<td>Variola major</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>– (?)</td>
</tr>
<tr>
<td><em>F. tularensis</em></td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>7/3*</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>?</td>
</tr>
<tr>
<td>Ebola VHF</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Marburg VHF</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><em>B. mallei</em></td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>5</td>
<td>?</td>
</tr>
<tr>
<td><em>B. pseudomallei</em></td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>?</td>
</tr>
<tr>
<td><em>C. burnetii</em></td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>?</td>
</tr>
<tr>
<td>Staph enterotoxin</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>9</td>
<td>9</td>
<td>Y</td>
</tr>
<tr>
<td>R. prowazekii</td>
<td>5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>8</td>
<td>N/A</td>
<td>2</td>
<td>5</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>?</td>
</tr>
<tr>
<td>Alphaviruses**</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>3</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>Y</td>
</tr>
<tr>
<td>Ricin toxin</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>?</td>
</tr>
<tr>
<td><em>Brucella spp</em></td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>?</td>
</tr>
</tbody>
</table>

* As wet or (dry) agent formulation.

** VEE, WEE, EEE / Viral encephalitis.

**Explanatory notes:** Effect/suitability is described by Figure 1-1 to Figure 1-9, where 1 denotes very difficult/inefficient or unsuited, 9 denotes very easy/highly effective or suited. 4 is neutral. The reference point here is an experienced laboratory-trained microbiologist, an un-trained person would probably not manage to handle anything rated 4 or less; **Availability** denotes how easily obtainable the agent is from environmental or laboratory samples or actual cases; **ID** describes how easily the agent is definitively identified; **culture** is a description of skills and conditions necessary to culture or produce the agent; **isolation** is how easily the agent is isolated from a mixed culture; **virulence** describes the ability of the agent to cause disease when the optimal sub-strain is chosen; **stability** expresses how well the agent retains its properties with regards to virulence, viability, etc., when cultured in vitro; **EoP** is “ease of production” throughout the production process resulting in usable quantities of weapons grade agent; **EoH** or “Ease of Handling” describes the ease with which the agent can be safely handled without loss of properties from production to dissemination; **morbidity** denotes relative morbidity; **mortality** denotes relative mortality in the absence of countermeasures; **TTE** is “Time To Effect” and denotes probability of significant effect within a given time-frame; **RoE** is “Reliability of Effect” and denotes the probability of a release causing the desired disease-effects, given the constraints imposed by EoH. Finally, modifiable denotes possibility of modifying agent.

The perceived, public effect is presumed high for all these agents. All values given are estimates based on textbooks, reference texts, practical and clinical experience and discussions with colleagues and others, and are negotiable.

**Special note on estimated virulence of VHF:** It has a low value because of very complicated infectious routes both from clinical cases and the environment that are difficult to exploit when used as a biological threat weapon.
1.13 HARVEST AND STORE

At this step in the process most of the focus is on viability, and how long the effector retains its properties during production and storage. This needs to be tested and determined. Storage usually degrades the agent unless special precautions are taken, both by autolytic and extraneous influences, but for our generic non-state actor this would be a very short phase, and would represent yet another constraint in that the time from production to use would have to be short. This has an important influence on planning and targeting.

![Diagram of Harvest and Store process]

Figure 1-7: Storage Constraints.

1.14 WEAPONIZATION

The next step is the weaponization process. For a state this is a complex process, and continued testing and QA is mandatory, especially with regard to how well the effector retains desired properties in relation to shelf-life and dissemination. This decides the operational efficiency and impact of the effector. During this process protection of the agent from environmental contamination, and also protecting the handlers, is important, and influences the operational impact (RoE). For a non-state actor it is another, crucial constraint, even though the weaponization process itself can be simplified.
1.15 DISSEMINATION AND EFFECT

Given targeting within operational constraints, the effect will then be decided by the dissemination method and the quantity and qualities of the agent.
1.16 TECHNICAL PROBABILITY – NON-STATE

A non-state actor will experience constraints in time, technology and security that are assessed to lead them to emphasise availability and factors related to finding the effector they want (ID, culturability, isolation, etc.). EoP (Ease of Production) and mortality will also be deciding criteria, and using the table these have been summed to express a technical probability factor. Any agent with a score lower than 4, i.e. neutral, for at least one of these parameters has been relegated to “lower risk”. This is based on the thesis that obtaining a result sufficient to provoke attributable fear is the deciding factor in effector production.

The first figure is the sum of Availability+ID+culture+isolation, the added figure is EoP+Mort:

- **B. anthracis**: 31 + (7+8)
- **Staph Etox**: 27 + (5+5)
- **R. prowazekii**: ? but several parameters assessed as below 4; relegated
- **Ricin**: 26 + (7+8)
- **Alphaviruses**: 20 + (6+6)
- **Botulinum toxin**: Several parameters below 4, incl EoP; relegated
- **Variola major**: Assessed as very low probability; relegated
- **F. tularensis**: 23 + (5+5)
- **Y. pestis**: 25 + (5+8)
- **B. mallei**: 3 on availability and culture; relegated
- **B. pseudom**: 0 + (4+7)
- **Marburg VHF**: Several parameters below 4; relegated
- **Brucella spp**: EoP is assessed as 3; relegated
- **C. burnettii**: 21 + (4+5)
- **Ebola VHF**: Several parameters below 4; relegated

This leaves eight agents, ranked as follows, followed by estimated effect/consequence (0 – 9) based on probable effector efficiency after successful release (average of TTE (Time To Effect) and RoE (Reliability of Effect):

- **B. anthracis**: 31 + (7+8) est effect: 7.5 HIGH TP (Technical probability)
- **Ricin**: 26 + (7+8) do 6 HIGH TP
- **Y. pestis**: 25 + (5+8) do 7 HIGH TP
- **Staph Etox**: 27 + (5+5) do 9 HIGH TP
- **F. tularensis**: 23 + (5+5) do 5.5 MEDIUM TP
- **Alphaviruses**: 20 + (6+6) do 6.5 MEDIUM TP
- **B. pseudom**: 20 + (4+7) do 4.5 MEDIUM TP
- **C. burnettii**: 21 + (4+5) do 4.5 MEDIUM TP

All these agents have sufficient morbidity or mortality, and though differing in assessed consequence will all satisfy a non-state actor’s requirement for an effector.
1.17 TECHNICAL PROBABILITY – STATE

As mentioned, a state actor will focus on the efficiency and reliability of their effectors in a weapons system. This technical probability, given the intent to use, represents the probability of use defined at the start of the document.

Based on the previously shown table, and working out the sums of factors TTE, RoE, morbidity, mortality, and adjusting for availability, we get a list ranking the agents as follows:

<table>
<thead>
<tr>
<th>Agent</th>
<th>Score (Availability)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em></td>
<td>40 (9)</td>
</tr>
<tr>
<td>Staph Etox</td>
<td>39 (7)</td>
</tr>
<tr>
<td><em>R. prowazekii</em></td>
<td>36 (5)?</td>
</tr>
<tr>
<td>Ricin</td>
<td>34 (9)</td>
</tr>
<tr>
<td>Alphaviruses</td>
<td>34 (6)</td>
</tr>
<tr>
<td>Botulinum toxin</td>
<td>34 (5)</td>
</tr>
<tr>
<td>Variola major</td>
<td>34 (1) availability restricts choice until GT is efficient</td>
</tr>
<tr>
<td><em>F. tularensis</em></td>
<td>32 (7)</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>32 (5)</td>
</tr>
<tr>
<td><em>B. mallei</em></td>
<td>32 (3)</td>
</tr>
<tr>
<td><em>B. pseudom</em></td>
<td>31 (6)</td>
</tr>
<tr>
<td>Marburg VHF</td>
<td>29 (2)</td>
</tr>
<tr>
<td><em>Brucella spp</em></td>
<td>28 (8)</td>
</tr>
<tr>
<td><em>C. burnetii</em></td>
<td>27 (7)</td>
</tr>
<tr>
<td>Ebola VHF</td>
<td>26 (3)</td>
</tr>
</tbody>
</table>

Given this ranking we get three obvious sets of agents:

- *B. anthracis, R. prowazekii, Y. pestis* and *F. tularensis* emerge in order as the higher risk bacterial agents; Variola major, Alphaviruses and Marburg/Ebola VHF as the high risk viral agents. However, seeing the problems related to availability, ID and production of Variola and the VHFs, Alphaviruses should be considered higher risk in this group. With advances in gene technology this may change.

- Staphylococcal Enterotoxin, Ricin and Botulinum toxin all come out as high risk in that order.

The remaining agents are assessed to have an availability that precludes them having a higher relative risk than the others.

Comparing the ranking of bacterial, viral and toxin agents with one another is difficult, but generally the technology for mass-producing bacterial agents is more widely known, which may give bacteria and bacterial toxins an advantage.

The possibility of modifying strains to give higher virulence, or transferring plasmids or other genetic elements to change the basic properties of some agents complicates the picture, and must be borne in mind in any evaluation or assessment of biological agent use. The progress in biotechnology will certainly have an impact on the availability of some agents, some of which may be usable in attacks. The fact that some research groups have managed to produce or modify some agents does not, however, mean that these technologies are widely
available for immediate use: the work done to achieve these steps is time and resource demanding and is not assessed to be widely available to biological agent producers for some years.

1.18 INTERPLAY BETWEEN TARGETING, AGENT CHOICE AND INTENT

“Why would Saddam Hussein want to produce aflatoxins as bioweapons? They don’t give effects until several years after use, and then as liver cancers…” ANON

Ideally an actor would focus on targeting in order to obtain an objective:

- What is to be achieved?
- When must it be achieved?
- What effect is necessary in the target (effect constraints, both upper and lower)?
- Which effector will be suitable to achieve this?

In a conventional setting this is done using the familiar and tested resources available to the warfighter, within overall political and technical constraints imposed on the operation. A terrorist will use guns, flammables and explosives, states will utilise military might (or at least threaten to use it). At a strategic level, the same reasoning is used when developing new weapon systems and types. This would also be valid when states develop unconventional weapons such as those based on a biological effector. In a conflict where biological weapons or terrorism agents are first used, the actual effects will be largely unexplored and add to the complexities in planning and execution. Targeting in the case of non-state actors may visibly be dependent on agent choice which again will influence and modify the objectives that can be reached.

This will impact states less than it will non-state actors due to the difference in resource and time availability. In the interplay between targeting, agent choice and intent the results may appear very uncertain and difficult to envisage both for attacker and defender. In the case of a non-state actor this may be assumed to reinforce their leaning towards agents that are “tried and tested”, and restricts their choice. This will limit their arsenal to threat agents that are seen as easier to produce and procure, and that may not need much testing due to good documentation. This will also simplify defensive preparations in society, with the possibility of giving priority to the most probable threats.

The state actor poses a significantly more complex threat with regard to detection and countermeasures, given a less limited choice of agents with the possibility of modifying or altering them. Technical considerations will, however, probably play an important part here as well, and will, combined with effective and reliable intelligence, make countermeasure prioritization possible.

1.19 A FINAL WORD ON CONSEQUENCES

The Health Services in most countries are very capable, but operate at a level that handles day-to-day situations more or less easily, with (possibly) some extra capacity available during the influenza season and the like. Additionally, the health services can usually be mobilised through emergency re-prioritization to provide an emergency capacity in addition to this.

A non-state actor will rarely, based on the studies we have done at the FFI, be able to cause significant NUMBERS of disease casualties (enough to overwhelm the emergency services) when using BTAs, though the
capacity of the primary health services may be overwhelmed due to high numbers of worriers. The primary effect will be intentionally caused disease striking at will and making individuals targets of a population.

A state attack using biological weapons might leave a very high number of diseased victims, enough to overwhelm the treatment and logistics capacities of any health systems. In practice this will leave a sizeable part of the affected population without necessary access to medical assistance, and leave to the aggressor, through their choice of agent, to decide who lives and dies. The goal of all countermeasures work should be to prevent this from happening.

Figure 1-10: Casualties vs. Treatment Capacity.

_HScap: Health services medical emergency treatment capacity in a non-epidemic situation._

_Augm: Capacity following re-prioritization._

_The coloured oval denotes the group of casualties outside any possible treatment capacity when you have a time-compressed, enormous number of acutely ill, infectious-disease victims, the kind of consequences a BW attack might entail._

### 1.20 REFERENCES


Chapter 2 – GENOME-BASED “OMICS” TOOLS TO DEVELOP VACCINES AGAINST BIOThREAT AGENTS

Gustavo Palacios and Mariano Sanchez-Lockhart
Department of Defense (DoD) and Defense Technical Information Center (DTIC)
UNITED STATES

Corresponding Author (Palacios): gustavo.f.palacios.ctr@mail.mil

2.1 OVERVIEW

A Scientific Technical Information (STI) relevance and gap analysis was conducted to identify and assess current state-of-the-art of the nucleic acid based “omics” technologies and their association with vaccinology, in particular for selected Biological Warfare Agents (BWAs) and their associated diseases. Information was collected for each “omics” field and their respective disease progression and associated biomarkers. This STI includes a description of the state-of-the-art status of each associated “omics” technology, analyzing its current and immediate future capacity to support the design, production and evaluation of vaccines against selected biological warfare agents.

A comprehensive search was conducted for each area, including Bacterial and Viral Genomics, Bacterial and Viral Population Genomics, Host and Pathogen Transcriptomics, and Immunomics and Dissection of the Immune Response. The list was down-selected to a number of reviews and publications from the last few years that best reflect the current state-of-the-art and the innovations that will likely have an impact in each field in the next few years. Several sections of this report are mainly based on the work of:


2.1.1 Literature, Database, and Web Search

A comprehensive literature search was conducted for each field. Databases searched included:
GENOME-BASED “OMICS” TOOLS TO DEVELOP VACCINES AGAINST BIOTHREAT AGENTS

- The Defense Technical Information Center (DTIC) Online Multi-search;
- The Chemical, Biological, Radiological, and Nuclear Defense Information Analysis Center (CBRNIAC) database (HighWire);
- ScienceDirect;
- BIOSIS; and
- Medline/PubMed.

Other open-source references were sought using standard internet search engines. Keywords included the following:

- Biomarkers;
- Genome/genomics;
- cDNA;
- mRNA;
- Transcriptome/transcriptomics;
- Comparative genomics;
- Immunomics;
- Antibody repertoire;
- TCR repertoire;
- Correlates of immunity; and
- Correlates of protection.

Initial search results were entered into EndNote®, a commercial reference management software package, used to manage bibliographies, abstracts and references. Using the above search criteria, several hundred citations and abstracts were retrieved for each field. USAMRIID Subject-Matter Experts (SMEs) reviewed all abstracts and identified specific articles judged as most promising to extract information and data pertinent to this study.

2.2 GENOME-BASED “OMICS” TOOLS FOR VACCINE DEVELOPMENT

2.2.1 Introduction

Vaccines are the most effective tools to prevent infectious diseases, which still are the most important threats to health worldwide. Effective vaccines have lowered morbidity and mortality everywhere they have been used. As a result, vaccines are considered among the greatest successes of modern medicine [8], [9]. Nonetheless, the threat of pandemics and emerging infectious diseases accents the need to develop new and more effective methods of vaccine discovery and production.

From the current crop of successful vaccines, we have learned that the fundamental determinants of a successful design are a clear understanding of the mechanism of microbial pathogenesis, prior identification of the main virulence factors, and a solid characterization of the immune response after infection and vaccination [10]. However, despite the common origins of vaccinology and immunology, both disciplines have occupied parallel universes. Model antigens were used to understand the fundamentals of modern immunology, and, until recently,
little attention was paid to the study of vaccine biology in humans. Therefore, most vaccines had been developed without enough attention to the mechanisms by which they confer protective immunity.

As elegantly described by Pulendran [13], the classical approach to vaccine development utilized an empirical model (e.g. the “isolate → inactivate/attenuate → inject approach”) [11] that, although successful in numerous occasions, had several flaws:

a) It utilized only whole pathogen (live or inactivated) antigens;

b) Ignored immunogenetics (individual differences in immune responses to the same antigen(s)) and immunogenomics (population-level variations in immune responses);

c) It is a failed paradigm for vaccines against hyper-variable viruses and complex pathogens (i.e. parasites, fungi);

d) It required an intact cold chain for vaccine viability;

e) It resulted in expensive vaccines which leads to under-utilization and poor vaccine coverage; and

f) It was unsuccessful for the development of vaccines against incurable diseases, including HIV/AIDS, malaria and others [12].

It is now clear that pathogen and host variability, as well as the interactions between them, must be considered in vaccine design [13]. There is a sentiment in the scientific community that all “easy” vaccines have been discovered, and a detailed understanding of cellular and molecular mechanisms will be necessary to optimize or develop new vaccines for the remaining biological threats [12], [14].

Molecular biology first, and the genomics era later, has revolutionized pathogen vaccine design. From a vaccinology point of view, the availability of the complete genome sequence of a pathogen represents a large reservoir of genes encoding for potential antigens that can be selected, screened and tested as vaccine candidates. Potential surface-exposed proteins can be identified, starting from the genome sequence rather than from the microorganism. This novel approach has been termed “reverse vaccinology” [15]-[17] and it would be the focus of the first part of this report. Systems vaccinology and the unified immune response network theory are efforts to study and apply tools from systems biology to vaccine studies, which have started to bear fruit in the past few years [18]-[21] and are the focus of the second part.

2.2.2 The Effect of “Omics” Methods in Vaccine Generation and Production

2.2.2.1 Bacterial Vaccine Production

Traditional vaccinology consisted of the biochemical evaluation of whole pathogens in order to identify surface exposed antigens that could be potentially be utilized for vaccine development. “Reverse vaccinology” is a reversal of that traditional workflow in which the first step is obtaining the genome sequence of the microorganism of interest followed by bioinformatics analysis of the genome to identify potential vaccine antigens. The approach was first proposed by Pizza et al. [22] and allows fast identification of candidate antigens for use in vaccination. As such, it has been predicted to provide solutions for those vaccines that have been difficult or impossible to develop [16], [23]. The first application of reverse vaccinology was the development of the *N. meningitides* serogroup B (MenB) vaccine [24], [25], [22]. Briefly, the process can be summarized as follows:

1) The genome sequence of a virulent strain of MenB was obtained [26];
2) Surface-exposed antigens were determined using bioinformatic algorithms that selected Open Reading Frames (ORFs) coding for putative surface-exposed proteins or secreted proteins; those ORFs were successfully expressed, purified and used as immunogens;

3) The corresponding antisera were used to evaluate the surface localization of the proteins;

4) Selected ORFs were then prioritized based on their ability to induce broad strain coverage in passive protection studies;

5) Five antigens selected by this approach were combined into one multi-component vaccine, in order to increase the breadth of the vaccine coverage and avoid selection of escape mutants; and

6) A very large collection of clinical isolates representing the MenB global population diversity was assembled and genetically characterized; and the more divergent strains were used to properly test protection.

The vaccine formulated with aluminum hydroxide induced bactericidal antibodies in mice against 78% of the 85 genetically diverse strains. Coverage was increased to 90% using different adjuvants [15].

The Genomics Revolution initiated with the Human Genome Race not only allowed the characterization of multiple eukaryotic genomes but also provided a huge increase in the characterization and availability of complete annotated bacterial sequences from diverse origins. While the genome sequence of a single strain reveals many aspects of the biology of a species, it fails to address how genetic variability drives pathogenesis within a bacterial species. The availability of genome sequences for different isolates of a single species enables quantitative analyses of their genomic diversity. The higher the number of isolates and the broader the selection of strains, the better the estimate of heterogeneity [27], [28]. Novel bioinformatic analysis tools are now available that allow the comparison of microorganism genomes at different levels (strains, species or genus) and allow us to compare differences to identify phenotypic markers, host range, and molecular evolution of virulence. In a similar form, several curated databases are now being developed to provide comprehensive information about experimentally validated antigens, e.g. Protegen [29], IEDB [30], AntigenDB [31], at different taxonomic levels.

The Pan-genome can be defined as the global gene repertoire pertaining to a species. In general, it can be divided into three parts: the core-genome, which includes the set of genes invariably present and conserved in all the isolates; the “dispensable genome”, comprising genes present in some, but not all the strains; and the strain-specific genes, which are present only in one single isolate. Maione et al., focusing on core genes, achieved the discovery of universal vaccine candidates against S. agalactiae, the Group B Streptococcus (GBS) [32].

Another interesting application of the concept was the re-development of the pneumococcal vaccine. Streptococcus pneumoniae is one of the most important human pathogens, accounting for considerable morbidity and mortality rates worldwide. S. pneumoniae is the most common etiologic agent of community-acquired pneumonia, as well as bacterial meningitis, otitis media and sepsis. Conjugated polysaccharide-based vaccines are available and extremely effective against the serotypes included in the formulation [33]. However, a few years after the introduction of the “Prevenar”™ glycoconjugated vaccine, the emergence of serotypes not covered by the vaccine has been reported, posing some concern on the long-term effectiveness of these types of vaccines [34]. In order to fill a gap, approaches including reverse vaccinology and immunoscreening [35] with patient sera were used to identify all possible surface-exposed and immunogenic molecules encoded by S. pneumoniae. Interestingly, both methodologies resulted in the identification of a genomic locus (rlrA islet) in the pathogenic strain TIGR4 that possesses all the typical features of a Pilus-encoding Island (PI). Subsequently, immunization studies in mice confirmed that three pilus sub-units are able to confer highly significant protection.
against lethal challenge [36]. However, the islet was not widespread among all strains. Analysis of a global collection of currently circulating \textit{S. pneumoniae} strains reported the frequency to be \( \sim 30\% \) overall (although it was \( 50\% \) among antibiotic-resistant strains) [37]-[39]. Thus, a global genomic approach was initiated which successfully identified a second pilus island [40]. Analysis of 305 clinical \textit{S. pneumoniae} isolates has shown that the second pili variant (PI-2) is present in approximately 16\% of analyzed strains, and is mostly associated with non-Prevenar-7 serotypes lacking the PI-1 islet, including the ones considered to be emerging serotypes both in industrialized as well as developing countries. The combination of the two pili antigens could then cover about 49 – 66 \% of currently circulating pneumococcal isolates. Thus, in this case, the identification of virulence factors by comparative genomics allowed effective design of a complementary vaccine.

An alternative pan-genome approach is the comparison between pathogenic and non-pathogenic strains of the same species. Elegant examples of comparative genome analysis revealed that an extensive genome reduction had occurred in non-pathogenic strains of \textit{E. coli}, \textit{Listeria}, \textit{Yersinia} and \textit{Neisseria} with loss of genes, particularly those involved in virulence, host interaction and metabolic pathways [41]-[44]. From a vaccine point of view, genes encoding for antigens conserved both in pathogenic and non-pathogenic strains can be discarded during the selection, which will reduce the number of candidates to express and test in the animal model, consequently reducing the time for the delivery of a vaccine.

2.2.2.2 Synthetic Biology and Viral Vaccines

The relatively small genome sizes of viruses allows for the rapid and accurate complete chemical synthesis of most virus genomes. It has become common for the genome sequences of emerging viruses to be published before widespread access to the actual virus becomes available. For example, the genome sequences of the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in 2012 and influenza A (H7N9) in 2013 in China were available weeks to months before the actual viruses were available to most researchers. The exchange of genome sequence data followed by chemical synthesis of the genome and reverse genetics to recover replicating viruses can allow far-flung researchers to begin development of countermeasures quickly after an outbreak is detected.

2.2.3 The Effect of “Omics” Methods in Vaccine and Immune Response: Vaccinomics

2.2.3.1 The Unified Immune Response Network

The “immune network hypothesis” was first proposed by Nels Jerne in the 1970s. It theorized that the adaptive immune system worked as an idiotypic network to explain the regulation of clonal immune responses [45]. This work was expanded by the “symmetrical network theory” by Geoffrey Hoffman [46] and others over the following 30 years, but none of the changes were aimed at defining the network as applied to systems-level vaccine-induced immune responses. Thus, factors outside the immediate immune system itself were not included (e.g. gene polymorphisms, epigenetics, or the influence of the host microbiome). The proposal of the immune response network theory [47], was to focus on systems-level vaccine-induced immune responses including host genetics and non-host factors, as the basis for the construction of a mathematical model and predictive equation that lead to pre-determined immune responses [19], [20], [48]-[52]. Among the host factors, individual components (immune response genes, epigenetic phenomena and contributory SNPs) and group components (gene pathways, gene networks) of the immunogenetic system are considered. Gender, age and microbiome composition are usually included as non-host factors, at both individual and population levels [47], [53], [54]. Biostatistics and bioinformatics tools are used to find correlations between the individual components and the immune response phenotypes. These mathematical models can be used to explain, and eventually predict, the immune response to a vaccine [53]. Vaccinomics, as the field was named, uses this and other “omics”
information to engineer novel vaccine candidates that overcome genetic barriers to developing protective immunity [48], [55]-[62], [11], [63]. The new field has multiple practical and beneficial applications that go beyond the vaccine design stage (e.g. vaccine monitoring and the study of vaccine-induced adverse events) that will be discussed later in this document.

Oyston and Robinson [64] recently summarized some of the key issues and barriers currently faced in vaccine development. One identified challenge area is vaccine delivery, and includes not only socio-economic concerns regarding vaccine availability and manufacturing, but also the mode of administration of the vaccine and appropriate dosing. It is here that the systems approaches from personalized medicine and pharmacogenomics can lead to “individualized” vaccine delivery. Better understanding of the genetic and non-genetic drivers of the immune response to vaccine antigens at a network level can be used to inform the rational design of new and improved personalized vaccine candidates. As the importance of personalized medicine increases, it might be desirable that vaccines are developed with a data-driven appreciation of the genetic differences between individuals and sub-groups within a population instead of a “one size fits all” approach. In particular, this approach might be particularly important for the immunosenescent population by defining and understanding, at the systems level, perturbations in the immune system, immunogenome, and microbiomes that prevent the development of protective immune responses. Likewise, it could be possible to engineer around a particular genetic restriction (the failure of a viral vaccine antigen to interact with its intended receptor due to a genetic polymorphism, for example) to improve vaccine effectiveness. In general, this type of approach would allow a “discover–validate–characterize–apply” paradigm of vaccine discovery and development. This paradigm would start with the discovery of a key genetic restriction, move to replication and validation of the effect in relevant sub-groups, progress to functional studies to uncover the mechanism(s) and finally engineering of a new candidate vaccine.

2.2.3.2 Population Genetic Considerations for Targeting Vaccine Development

Genetic research on infectious diseases requires studying genetic variation in the genomes of both the host and the pathogen. It is clear that the identification of genetic variants that affect susceptibility or progression of infection will provide crucial information for the development of therapeutics and vaccines. Classical studies to understand the role of genetics in regulating immunity were performed by comparing responses in monozygotic and dizygotic twins, which have resulted in the observation of varying degrees of heritability [65]-[68]. While there are examples of host genetic variation impacting susceptibility to infection (e.g. A beta-globin polymorphism that protects against malaria infection [69]; deletion in the Chemokine Receptor 5 (CCR5) gene that provides resistance to HIV infection [70]), susceptibility appears to be the result of the combination of the small effects across multiple genes, in addition to the environment and the pathogen. Thus, methods used to study the genetics of other common, complex disorders have been adapted to the study of infectious disease [71].

Genome Wide Association Studies (GWAS) have been used for the analysis of susceptibility to infectious diseases, and a number of variants have been identified. The identified variants seem to concentrate in Human Leukocyte Antigen (HLA) regions [72]-[77]. GWAS have been used successfully to identify genetic variants important to smallpox vaccination [78]-[81]. The methods theoretically provide a way to detect novel genetic factors associated with infection, susceptibility and disease progression, without the need of a priori knowledge about the genes being tested. Although the method has been successful in some instances [77], [82], it has also been plagued by the same problems seen in other complex diseases: the associations discovered have small effect sizes and not all associations have been replicated [71]. Given that until now the majority of GWAS studies have focused on main effects (primarily via an additive model of common variants), it is possible that only by examining the contribution of rare variants [83] or by using network models that can do a secondary analysis to identify epistatic interactions and aggregate-level characteristics (e.g. pathway, network), it will be
able to completely realize the potential of GWAS studies [84]. Nevertheless, as a successful example, the results of this approach prompted the development of a new anti-retroviral therapy based on the finding of a CCR5 mutation (CCR5Δ32) in HIV-resistant individuals [85].

Network models are more useful to study complex states where the disease is not the consequence of a single molecular abnormality, but the result of the interactions of numerous small abnormalities. These arrays of affected genes, called modules when they are functionally related, might provide further insight into disease mechanisms and outcomes. The generalized use of novel methods of network analysis, like differential network analysis (modeling the change in the network across time or across multiple conditions) [86] or cross-species interaction networks (modeling the interaction of multiple hosts with the same pathogen) [87], might be needed before the proper impact of population genomics methods can be assessed.

2.2.3.3 Immunogenetics (Summarized from Poland et al.)

The field of immunogenetics combines the approaches and methods of genetics with the ones of systems biology to correlate genotype with immunological phenotypes [88]. The applications of immunogenetics to the field of vaccines has focused on understanding the genetic restrictions of the immune system in order to identify vaccine antigens, and the genetic polymorphisms associated with its ability to respond to vaccination.

Briefly, the first approach consists of three steps:

a) Identification of immunogenic pathogen-derived peptides detected in the HLA binding groove;

b) Comparing and contrasting peptide sets among hyper- and non-responders; and

c) Using the information to develop HLA supertype vaccines.

The rationale for this approach is that some of the peptides identified from the grooves of HLA molecules will bind to a wide spectrum of HLA alleles. These wide-spectrum peptides may be useful for a peptide-based vaccine approach among individuals with the same HLA supertype [89], [90]. Adding peptide sets from multiple HLA supertypes, may allow coverage of greater than 90% of a given population [91]-[93]. Poland et al., used mass spectrometry to isolate and identify naturally presented and processed pathogen-derived peptides from the HLA peptide binding groove of hyper- or hypo-responders to the smallpox vaccine [23], [89], [94] demonstrating the feasibility of the approach. An alternative strategy would be to use a similar approach to identify a “universal” immunodominant T-cell epitope that could be used to mount a protective immune response in all individuals, overcoming genetic restrictions [95].

Several studies have demonstrated associations between genetic polymorphisms in the host and the levels of protection induced by different viral vaccines [96]-[109]. Homozygous HLA alleles are associated with failure to develop antibodies and cytotoxic T-cells during HIV infection [96], [110]. Additionally, polymorphisms in several genes have been shown to have an effect in susceptibility to HIV-1 infection [96], [111]-[116]. These observations are not restricted to viruses. A study analyzing vaccine-induced antibody responses to Bacillus anthracis Protective Antigen (PA) vaccine suggested a genetic basis for individual variations in immune responses following vaccination. DRB1-DQA1-DQB1 haplotypes were associated with lower production of PA-specific antibodies and lower PA-specific T lymphocyte proliferation levels. The future of personalized vaccines will depend on our understanding of the balance of the immune response they elicit and host genetic diversity.

Several studies have demonstrated gender-based differences in the immune responses to vaccine antigens [98], [117]-[122]. These observations are not restricted to the humoral aspects of immunity; differences in cellular
immune responses [123], and circulating NK T-cell levels and CD4+ NK T-cell sub-sets have been observed associated with gender and age-based differences in vaccine responses [124]. These seminal studies might represent the start of the development of prophylactic vaccines customized to gender.

Vaccinomics has also been applied to investigate why historically successful vaccines are effective [125]-[127], to elucidate mechanisms behind vaccine escape variants [128], and to develop vaccines that target conserved regions of circulating strains of highly variable pathogens [129]. An extremely intriguing new approach is the study of the effect of the diversities of B and T lymphocytes receptor repertoires on the outcome of an immune response. Receptor repertoires can be shaped by prior antigen exposure as well as by intrinsic factors such as aging. Wiley et al. [130] found that a Toll-like receptor agonist adjuvant increased the diversity of antibodies, which led to improved antigen neutralization ability and a better protective response.

2.2.3.4 Pathogen Evolution

The other side of the coin when considering evolutionary changes affecting vaccine response is the study of pathogen genetic variation. Our ability to predict pathogen evolutionary paths could significantly improve vaccine development. Not surprisingly, the same processes that affect the ability of pathogens to develop antibiotic resistance will also affect their ability to escape the human immune system. For example, the study of epistasis (i.e. the effects of different genetic backgrounds on the development of drug resistance and immune escape) is already considerably influencing the field. Recent research on the genetic diversity of Mycobacterium Tuberculosis Complex (MTBC) suggests that different lineages of MTBC may have adapted to specific human populations in different geographical regions. This will drive treatment management since particular strains of TB (e.g. the “Beijing” family) may be more likely to develop drug resistance mutations [131]. This case of “positive epistasis”, where the genetic background might lower the fitness cost of drug resistance conferring mutations would be obviously mirrored by cases of “negative epistasis” where the cost is increased. Identifying genetic backgrounds that show “negative epistasis” for the development of immune escape mutants would be a worthy goal for vaccine development. Given that this discipline is completely novel, most tools to identify, model and predict the effects of genetic interactions within host and pathogen genomes, as well as interactions between the two genomes, are still in development.

2.2.3.5 Microbiome and Vaccine Immunity

In the last few years, the characterization of the microbiome has become one of the most intriguing and critical ways to study the relationship between the host immune system and the microbiota. The microbiome, comprised of bacterial, fungal and viral species that naturally inhabit the human body, has become quickly characterized by using high-throughput sequencing [132], [133] and new informatics tools [134]-[136]. Genomic characterization of microbiomes allows the identification of specific changes in microbial communities that correlate with particular conditions, such as protection, response or disease outcome. Falling sequencing costs and increasing analysis throughput facilitates comparison of longitudinal and experimental group-samples, which are starting to provide a clearer picture of the interaction between the immune system and the microbial flora. Several recent studies have demonstrated the power of this approach. Compositional comparisons of bacterial communities have demonstrated that the colonization of the gut promotes regulatory (IL-10 and CD4+ regulatory T-cells) or pro-inflammatory pathways (Th17) in the host [137]-[144]. In fact, depletion of microbiota with antibiotics revealed critical changes in the status of readiness of the gut immune systems [137], [139], [140], [143]-[150]. Some reports using mice have suggested that, under the absence of the microbial flora, the capacity of the immune system to control infection is substantially diminished and, consequently, susceptibility to infection is augmented. This line of work suggests that the composition of the microbiota has major implications for public health and vaccines.
2.2.4 The Effect of “Omics” Methods on Vaccine Evaluation: Correlates of Protection and Immunity

2.2.4.1 Immunomics (Summarized from Six et al.)

Despite the successful development of vaccines, mechanistic events leading to protective immune responses are often poorly understood. Biological correlates of immune-mediated protection are currently based on antibody titers and ELISPOT assays. However, the peak of vaccine-induced antibody titers (i.e. antibody response quantity) does not solely explain antibody-mediated protection. The quality of such antibody responses (e.g. avidity, target epitopes, glycosylation, diversity), as well as that of the associated T-cell responses, have been identified as determining factors of high-affinity antibody responses and efficient immune memory. Different types of memory T-cells (central-memory and effector-memory) have been identified based on their functional and migratory properties, and therefore contribute differently to long-lasting cellular immunity [151]. To develop successful vaccines for the remaining “difficult” pathogens, novel ‘readout systems’ or ‘correlates’ of immune-mediated protection that reliably predict immune responses to novel vaccines in vivo are needed.

To develop a successful immune response following vaccination, both the innate and the adaptive immune systems must synergize. Antigen-Presenting Cells (APC) take up antigens and traffic to the draining lymph nodes. There, processed antigens are presented to naïve CD4+ and CD8+ T Lymphocytes (CTL). These are stimulated to proliferate and differentiate, resulting in effector and memory T-cells. The CD4+ T-cells help B cells to mount antibody responses and naïve CD8+ T-cells to differentiate into CTLs. The innate immune response determines the quality and quantity of the adaptive immune response. Initial pathogen recognition by innate immune cell receptors (such as toll-like receptors) upon ligation of different classes of pathogen-associated molecular patterns, is a key event in the overall immune response since it determines the initial direction of the process [152], [153]. While most antigens and vaccines trigger both B- and T-cell responses, the nature of the vaccine and its delivery (e.g. nature of antigen, route of administration, quality of antigen presentation, adjuvants, timing between challenges, immunocompetence status of the subject) exerts a direct influence on the type of immune effectors that are predominantly elicited [154], [155].

In summary, an effective vaccine should be able to:

i) Successfully activate APCs to initiate antigen processing and presentation to T-cells;

ii) Activate T and B cells broadly to give a high yield of memory cells;

iii) Generate memory B cells which produce neutralizing antibodies or antibodies that target the invading pathogen for destruction by either complement or antibody-dependent cellular cytotoxicity;

iv) Generate memory CTL that limit the spread of the pathogen by recognizing and killing infected cells or secreting-specific cytokines;

v) Generate memory CD4+ T-cells that reduce, control or clear pathogens by producing cytokines that support activation and differentiation of B cells and CTL;

vi) Generate memory T-cells to several distinct epitopes, to overcome MHC variations across the population and limit immune response escape of the pathogen;

vii) Promote the correct balance of regulatory T lymphocytes (T_reg) concomitant to the induction of vaccine-specific immune responses; and

viii) Confer long-term immune memory.
New methods of analysis that can incorporate the growing number of vaccine-associated immune parameters need to be developed. These methods should:

1) Assess vaccine efficacy not only at the population level, but also at the individual immune cell level including mucosal response, local antibody production, timely B- and T-cell responses, and appropriate effector or regulatory biological pathways.

2) Consider multi-scale and multi-parametric variables including the immunocompetence of the host (age-, disease- or treatment-related immunodeficiency) and the antigenicity of the preparation.

3) Investigate the dynamics and repertoire modifications following immunization and challenge, to better understand the mechanisms of cell activation, and to derive models of efficient vaccine-induced immune responses.

4) Assess pathogen prevalence levels, pathogen diversity, population changes, host factor deficiencies or HLA polymorphisms.

In this line, a wealth of complementary immunomonitoring technologies is available:

i) Transcriptomics and ribosome profiling for evaluation of the innate immune response on single-type and single-cell target cells, e.g. dendritic cell activation [156], inflammatory response, and complement activation;

ii) Dissection of the adaptive antibody responses, e.g. antibody and B-cell immune responses [157], neutralizing antibodies [158], non-neutralizing antibodies, and antibody-dependent cell cytotoxicity;

iii) Dissection of adaptive T-cell immune responses, e.g. CTL activity, T-cell specificity using tetramer/peptides, and cytokine production [159]; and

iv) Characterization of the lymphocyte repertoire diversity, e.g. Immunoscope/CDR3 spectratyping, TCR/BCR rearrangement quantification, and TCR/BCR deep sequencing [160], [161].

Given the large diversity of pathogenic microbes, a better understanding of immune responses will benefit from global approaches aimed not only at studying the individual components involved, but also at studying and modelling the complex interactions between these components and most importantly their spatial and temporal aspects. To tackle this type of complexity, the analysis of large data sets with non-supervised methods and/or through modelling, to extract/generate statistically significant results, irrespective of preconceived hypotheses, is an ideal approach [162], [163]. This systems biology framework should provide novel analyses of immune responses, identifying response-specific signatures and assessing their predictive value. The principle of this approach is to integrate high-throughput data, e.g. any -omics, and produce a model of the immune response triggered. Future advances for vaccine development will be based on taking a systems biology approach to the immune system, leading to the creation of a virtual or *in silico* immune system capable of complex simulations [163]-[165].

Predictions generated by the simulations can be used for guiding vaccine development and refinement, for predicting vaccine efficacy in different settings, and for guiding vaccination policies and regulatory decisions. To this end, the expanding knowledge on the molecular mechanisms of immune responses, the availability of high throughput genomic and proteomic technologies and the development of integrative systems biology offer new approaches for modelling vaccine-induced immune responses and open the possibility to establish predictive signatures of effective responses.

A proof of concept study was performed by Pulendran and colleagues who applied a systems biology approach to study the immune response induced by the yellow fever vaccine, one of the most successful vaccines ever
developed [166]. Their strategy involved immunology, genomics and bioinformatics in order to gain a global picture of the nearly 30,000 genes, the resulting proteins and the cells participating in immune responses to vaccination. Using this approach, the investigators identified gene expression signatures in the blood a few days after vaccination that could predict, with up to 90% accuracy, the strength of the immune response to the yellow fever vaccine. Sékaly and colleagues made similar observations using functional genomics coupled to polychromatic flow cytometry, showing a strong and coordinated initial response that determines the ensuing efficient poly-functional and lasting adaptive response [167]. Both studies underline a strong correlation between the early innate immunity-related events and protective vaccine response. The consistency of these predictive signatures across several trials, for both CD8+ T-cell and antibody responses to the yellow fever vaccine, raises the possibility that these rules or their components might have broad applicability for different types of immunogens designed to protect against diverse pathogens.

Several detailed studies of the innate and adaptive immune responses to vaccination have been published in recent years [19], [20], [166]-[168]. A good example of a successful vaccine, the yellow fever vaccine YF-17D, is based on a live attenuated virus that confers long lasting protection with only one shot. It achieves this by inducing neutralizing antibodies, CTL and Th1/Th2 cells and by signaling dendritic cells through Toll-like receptors 2, 7, 8 and 9 [19]. Two independent studies by Querec et al. [166] and Gaucher et al. [167] revealed that genes of the antiviral Type I interferon pathway, complement pathway and inflammasome were induced 3 – 7 days after vaccination, preceding the development of the adaptive immune response. Moreover, it also showed that several regulators for innate sensing and signaling were also observed. Interestingly, using this early gene expression signature, it was possible to predict with high accuracy the number of CD8+ T-cells and neutralizing antibody titers [166], [169], [170]. These studies on yellow fever vaccine were quickly followed with similar success by Pulendran et al. studying the innate and adaptive immune responses in healthy adults receiving trivalent inactivated or live attenuated influenza vaccines [50]. Interestingly, several interferon-related genes were found to be up-regulated by both attenuated live viral vaccines. On the other hand, the inactivated vaccine response was mostly observed from plasma B cells. In both studies, TNFRSF17, the receptor gene for B cell growth factor BLyS, was found to be among the top predictors of the antibody response.

The finding of common response genes across vaccines for different pathogens suggests that some signatures could be predictive across vaccine set-ups while others might be vaccine-specific. Nevertheless, in addition to identifying the key gene expression signatures of early successful innate immune activation, the systems biology approach generated new hypotheses to determine the factors that regulate control memory T-cells and antibody production, which, in some cases, might not be completely related to inflammatory immune responses. The creation of a reference immunome database has been proposed to facilitate the comparison of the vaccine-induced responses [171].

The objective of these systems biology approaches is the identification of molecular biomarkers associated with the successful immune response after vaccination. Multiple molecular measurements and gene expression data can be utilized to identify novel biomarkers predictive of vaccine response.

In addition to simple correlation analysis, machine learning algorithms can be utilized to identify patterns of biomarkers indicative of a successful vaccine response. The authors of the above-mentioned studies used the ‘Discriminant Analysis via Mixed Integer Programming’ (DAMIP) algorithm [50], [166], [172], an optimization-based predictive modeling framework that combines a discrete support vector machine coupled with a robust feature selection module [50], [166]. This approach can guide future trials in model systems [12], [21], [173]. An obvious consideration is the need for validation of the signature biomarkers after the bioinformatics analysis. The development of nanoliter-volume multiplexed real-time PCR allows simultaneous, high resolution, temporal quantification of expression signatures. This is particularly useful for detailed analysis of a candidate signature
over many conditions (e.g. multiple time points or stimuli) and at different levels of resolution (single cells, rare cell types and flow sorted/deconvoluted sub-sets).

2.2.5 Conclusions

The revolution initiated by “omics” technologies in all fields of science has important correlates on the field of vaccine design. This effect will probably be even more sustained in the field of biothreats countermeasure design, test and evaluation. Almost all “desired” vaccines for biothreat agents would need to be evaluated under the animal rule. Given the smaller studies and datasets possible under that paradigm, it is expected that the level of scrutiny of regulatory agencies will be higher, ensuring that all available technologies are applied to each “in vivo” study. Nevertheless, the effect of “omics” technologies will be even more drastic on the areas of vaccine design and biomarker discovery. Almost by definition, the amount of data available for biothreat agents had been extremely limited. The genomics revolution is changing that by providing huge amounts of data on genetic diversity, host expression profiling, bacterial expression profiling, and microbial population genomics. All of those factors have potentially the ability to change the field by facilitating the design of vaccines and the identification of druggable targets.

2.3 REFERENCES


DISTRIBUTION STATEMENT

“This report is a work prepared for the United States Government by USAMRIID. In no event shall either the United States Government or USAMRIID have any responsibility or liability for any consequences of any use, misuse, inability to use, or reliance upon the information contained herein, nor does either warrant or otherwise represent in any way the accuracy, adequacy, efficacy, or applicability of the contents hereof.”
Chapter 3 – CHALLENGES AND TECHNOLOGICAL ADVANCES IN VACCINE DEVELOPMENT FOR BIODEFENSE

Paula Bryant\textsuperscript{1, 2}, Daniel N. Wolfe\textsuperscript{1} and William Florence\textsuperscript{1}

\textsuperscript{1}: Defense Threat Reduction Agency, J9-Chemical and Biological Technologies Department
Translational Medical Division, Fort Belvoir, VA; \textsuperscript{2}: National Institutes of Health,
National Institutes of Allergy and Infectious Disease, Division of Microbiology
and Infectious Diseases, the Office for Biodefense, Research Resources,
and Translational Research, Rockville, MD

UNITED STATES

Corresponding Author (Bryant): paula.bryant@nih.gov

3.1 INTRODUCTION

Vaccines are among the greatest successes in the history of public health, and are considered one of the most
cost-effective interventions in modern medicine for combatting infectious diseases. Ever since Edward Jenner
used cowpox-infected materials to immunize against smallpox in 1796, the use of vaccines became
indispensable to eradication of disease. In the second half of the 20\textsuperscript{th} century, advances in bacterial attenuation,
virus propagation and attenuation, and inactivation of microorganisms led to the golden age of vaccine
development, yielding vaccines against polio, measles, mumps and rubella [1]. In the latter half of the
20\textsuperscript{th} century, the reliance on attenuated or killed/inactivated vaccines to directly mimic natural infection was
replaced with the emergence of newer technologies, including protein conjugation to capsular polysaccharides
and methods to engineer recombinant DNA, leading to the development of vaccines for prevention of bacterial
pneumonia and meningitis, hepatitis B and the recent development of the Human Papillomavirus (HPV) vaccine
[1]. Recent technological advances in molecular genetics, molecular and cellular immunology, structural
biology, bioinformatics, computational biology, nanotechnology, formulation technologies, and systems biology
have heralded in a new era of reverse vaccinology and/or ‘vaccinomics’ [1]-[4]. Vaccinomics replaces empirical
methodologies commonly referred to as the ‘isolate-inactivate-inject” paradigm, and thus should facilitate
rationale vaccine design against the ‘challenge pathogens’, Emerging Infectious Diseases (EIDs), and biothreat
agents outlined above [1]-[4].

Despite significant progress, vaccines still do not exist for the majority of priority global diseases (e.g. HIV,
Tuberculosis, Malaria, Dengue, a Universal Influenza vaccine) [5], and the timeline for vaccine development
continues to lengthen with a concomitant increase in cost [6]. For many of these pathogens it has been difficult
to show preventive immunity due to their complex pathogenic mechanisms, extensive strain and/or antigenic
variability, and/or the evolvement of immune evasion mechanisms by the microbe. In addition, for pathogens
such as CMV, herpes simplex, \textit{Mycobacterium tuberculosis}, and \textit{Burkholderia} sp., a carrier state is established
with reactivation occurring in situations of immunosuppression. Thus, new vaccine discovery and novel
immunization paradigms will likely be required for successful vaccine development against these challenging
pathogens. Moreover, in addition to these ‘traditional’ pathogens, new threats to human health are posed by
re-emergent- and antibiotic-resistant agents along with the appearance of novel and unanticipated forms of
infectious diseases [7]. The picture is significantly more complicated when considering the risk of any of these
microbes or their products being improperly used as biological weapons. There are currently only three FDA-
licensed vaccines for protection against biological threats: the original Edward Jenner live-attenuated Vaccinia
virus vaccine (Dryvax) against smallpox; a second-generation smallpox vaccine ACAM2000, which contains
live Vaccinia virus cloned from the same strain used in Dryvax but cultured in Vero cells rather than in the skin
of calves; and a cell-free culture filtrate vaccine (AVA) effective against Anthrax. Second- and third-generation vaccines for both anthrax and smallpox are in various stages of clinical development as well as first-in class recombinant vaccines against inhalational plague and BoNT A/B, but the path to licensure for all has been extremely slow.

The failure to translate technological advancements into products is in large part due to the most significant bottleneck in vaccine development, i.e. the lack of understanding of key vaccinology principles in humans. This includes the need for greater understanding of disease-specific mechanisms of protective immunity, immune evasion mechanisms, and strategies to drive the immune system toward preferred responses by immunization [1]. There persists a continued reliance on testing potential vaccine antigens in animal models. Using animal models as a primary screen to identify potential vaccine antigens is costly and labor intensive, and not always predictive of the response in humans. Moreover, for many pathogens, appropriate and relevant animal models have not been developed. Indeed, vaccine discovery research waits far too long to consider the human response – not only in determining the potential safety and efficacy of vaccine candidates, but also in identifying critical biomarkers and correlates of protection that can support the candidate in the clinic as it traverses the regulatory path.

The gap in understanding the human response to a given pathogen and corresponding vaccine candidate is intensified for biodefense agents. For the majority of Category A and B biothreat agents, human cases and outbreaks are rare and/or occur in hard to access areas of the world. Unlike traditional infectious diseases, human efficacy trials are not feasible for biodefense vaccines. Instead, biodefense vaccines currently in development will be subject to the Food and Drug Administration’s (FDA) “Animal Rule”, issued in 2002, and intended to expedite the development of new drugs and biologic products as MCMs to chemical, biological, radiological, and nuclear threats. The Animal Rule applies only to new drug or biologic products for which definitive human efficacy studies are not ethical or feasible to be conducted, and thus covers the majority of CBRN agents [8]. The Animal Rule was born of the controversy over administration of investigational drugs and biologics to soldiers under military orders without informed consent, under an administrative rule for waiver of informed consent in certain military exigencies [8]. Since its’ issuance, only four drug products for humans have been approved by the FDA on the basis of efficacy studies in animals. To date, no vaccine has been licensed/approved via the animal rule. Success will require instilling confidence (of the regulatory agency) that the animal model is predictive of the human response. Since human data for exposure to biothreat agents is at best limited, innovative ex vivo human mimetics require further development and exploitation in order to better model and predict the human response and help advance reliable animal models for licensure of biodefense vaccines.

The potential for exposure of military personnel to not only known, but also new or emerging biological threats also demands that our investment strategy for vaccines embraces agility and adaptability. This requires an approach that exploits innovative platforms that can be easily modified to ‘rapidly respond’ and create vaccines as new threats are identified. Rapid threat identification, antigen/target selection, and lead candidate development must be strategically integrated. Moreover, a paradigm shift is required to enable the rapid, reliable and cost-effective sustainable access to biodefense vaccines and other countermeasures in sufficient quantities to treat the military and affected populations. Past models involving the construction and operation of facilities dedicated to the production of a single vaccine product utilizing traditional expression technologies, such as prokaryotic and egg-based platforms, are not sustainable for biodefense (or next-generation vaccines for EIDs and unmet public health needs), and thus an alternative approach is needed.

The need to create flexible and adaptive countermeasure strategies requires development of new regulatory paradigms that go beyond the “Animal Rule”. Investments must be made in regulatory sciences and technologies to increase the likelihood of success and facilitate the advancement of vaccines for biodefense along the
regulatory path. These investments include reliable animal models for threat agent diseases, identification and validation of biomarkers (correlates of protection), ex vivo technologies to predict/determine human safety and efficacy of vaccines in humans prior to clinical studies, flexible and agile expression and manufacturing platforms, as well as downstream processing methods and analytical tools to provide novel methods of assessment. The need to create rapid, flexible and adaptive countermeasure strategies along with new regulatory paradigms is not restricted to the development of biodefense vaccines, but is also required to meet the ever increasing public health demand for new vaccines to combat antibiotic-resistant microorganisms and emerging/re-emerging infectious disease outbreaks.

3.2 RATIONALE VACCINE DESIGN: ANTIGEN DISCOVERY TECHNOLOGIES

3.2.1 Reverse Vaccinology

Selecting the optimal antigen represents the cornerstone in vaccine design [2]. When the genome of the first living organism was sequenced in 1995 [9], it was realized that genomic technologies, by determining the whole proteomic potential of the infectious organism, could boost the chances of identifying the protein, or mixture of proteins, that could be used to develop an efficacious vaccine [4]. The method, coined ‘Reverse Vaccinology (RV)’, replaced the traditional process of selecting candidate antigens and testing them one by one. The latter relied heavily on identifying antigens by analyzing sera from infected individuals that were protected from reinfection to identify molecules characterized by their presence and conservation in the infectious agent, their visibility to the host immune system, and their ability to elicit a protective immune response [4]. Although this approach identifies a restricted set of candidates that dominate the host immune response, it fails to identify those components that are not highly immunogenic, but are nonetheless able to confer protective immunity. In RV, the coding potential of a pathogen’s genome is exploited by in silico selection, high throughput screenings, and genomic/proteomic profiling technologies to define promising antigens in relation to in vivo expressed genes and clonal variation [2]. The RV approach allows identification of a much broader spectrum of candidates, to include proteins previously masked by other immunodominant antigens. In addition, it enables the identification of potential vaccine antigens in organisms that are difficult to cultivate in the laboratory [4].

The first pathogen for which RV was attempted was serogroup B Neisseria Meningococcus (MenB). The entire genome of the virulent MenB strain MC58 was sequenced, and bioinformatic analysis predicted that over 600 potential encoded proteins were exposed on the surface or secreted. Of these, 350 were cloned in E. coli, expressed in soluble form, purified, and used to immunize mice [4]. The sera of immunized mice were then screened in a serum bactericidal assay that is known to correlate with protection. The process led to the identification of five previously unknown vaccine candidates that subsequently have completed clinical trials in a vaccine combination known as 4CMenB, and approved by the European Medicines Agency (EMA) with the commercial name of Bexsero® [4]. In each of the vaccine projects based on RV, the original design had to be modified to adapt to the peculiarities of the target species. To use RV successfully required a more detailed understanding of bacterial population biology and of host-pathogen interactions, and how both impact the development of a vaccine. Besides RV, genomic-based antigen discovery has been enhanced by new technologies enabling interrogation of the comprehensive antigen repertoire using libraries of genetically expressed antigens and screening for immunogenicity of the proteins during infection, termed “antigenome analysis” [10]. In addition, advances in mass spectrometry have enabled direct testing of the presence and quantity of antigens on the surface of bacteria [11].

The testing of identified vaccine candidates in animal models still produces the major bottleneck of the entire RV process. The bottleneck is not only because screening in animals is labor intensive (and costly), but also
because mice are not always predictive of the human response. As recently demonstrated by a Phase III clinical trial of an experimental *Staphylococcus aureus* vaccine [12], RV and anti-genome technologies remain limited in their capacity to predict which antigens are protective. Thus, a large effort has been devoted to defining a set of screening procedures that could integrate the original bioinformatics selection to significantly reduce the number of candidates that need to be tested in animal models [4]. Integrating new technologies such as microarrays, RNA-Seq, proteomics, algorithms, and novel *ex vivo* human mimetics into the RV process could not only serve to streamline the original ‘brute-force’ approach, but enable better prediction of the right antigens to be incorporated into vaccines.

### 3.2.2 Structural Vaccinology

A second approach in the rationale design of vaccines is termed ‘structural vaccinology’, in which immunogens are rationally engineered using available structural information. Recent advances over the past decade in X-ray crystallography and NMR spectroscopy have increased the throughput of protein structure determination so that we can now determine the structure of most vaccine antigens and their epitopes [13], [14]. Structure vaccinology has been used in the design of cross-protective candidate immunogens for pathogens that exhibit high antigenic diversity and strain variation, such as HIV, influenza, RSV, hepatitis C, and MenB. This approach exploits the discovery of broadly Neutralizing Antibodies (bNAbs) and knowledge of structural information about the bNAb epitope to guide the design of immunogens to induce responses similar in structural recognition, breadth, and potency [14]. This strategy has been termed ‘reverse vaccine engineering’, or more specifically as ‘antibody- or epitope-guided’ vaccine design. The first proof of concept for reverse engineering of vaccines was achieved for RSV, where computationally designed immunogens mimicking the binding site for an RSV-neutralizing mAb (Palivizumab; currently licensed for prophylaxis against RSV in infants) successfully elicited RSV-specific bNAb in NHPs [13], [14].

There is a complexity to ‘antibody- or epitope-guided’ reverse vaccine engineering that is not sufficiently captured in the title of the approach. First, lessons learned in carrying out this approach include the fact that the epitopes recognized by antibodies are larger than the ‘patches’ of amino acids that can be identified using conventional epitope mapping, and that the individual amino acids identified as being essential for an epitope can be less important than preserving the overall epitope surface [14]. In addition, many bNAbs are atypical, with an unusually high degree of somatic mutation or long CDRH3 regions, and thus are not easily elicited. Moreover, a successful vaccine candidate must be designed to bind the germline antibody precursor, select for the appropriate primary recombination events, and direct its somatic mutations toward the appropriate mature form [5]. Such is the case for HIV, in which reverse engineering of vaccine approaches have had to exploit recent technological advances in isolating monoclonal antibodies from memory B cells and plasma cells from infected patients, deep sequencing, and bioinformatics [1], [5], [14], [15]. Thus, one must use knowledge of both structural biology and antibody evolution to design vaccines that will elicit antibodies of known specificity [5]. The steps to reverse vaccine engineering include:

1. Identification of bNAbs from subjects by single-cell memory B cell techniques with or without antigen selection and cloning the H- and L-chains into immunoglobulin G vectors;
2. Determination of the structure of the binding sites using crystallographic methods; and
3. Mimicking the epitope binding sites of such bNAbs on carrier protein scaffolds or vectors to serve as the basis for immunogens to elicit such bNABs [1].

The latter step is facilitated and enhanced by computational and protein design tools that enable the engineering of more stable, homogenous, and efficiently produced vaccine protein antigens [14].
In order to focus responses on protective epitopes, one approach (termed ‘antigen minimization’) is to generate stable domains of larger viral proteins that contain the epitopes of interest but lack other non-protective or potentially undesirable epitopes [14]. This approach utilizes knowledge of the relevant protein structure to guide selection of stable domains and/or to guide design of stabilizing features that compensate for domain isolation. The antigen immunization approach was utilized in immunogen design for both HIV and Flu, as well as several other agents [14]. Alternatively, the crystal structure of a peptide or protein in complex with an Ab can serve as the starting point for design of epitope-scaffold immunogens, in which epitopes are transplanted to unrelated scaffold proteins for conformational stabilization [16]-[18]. Studies have demonstrated that epitope-scaffolds bound antibody with significantly higher affinity than the corresponding epitope peptide alone [17], [18]. The greatest challenge in scaffolding is to transplant and stabilize discontinuous epitopes involving two or more segments of protein backbone. A hybrid computational-experimental approach for scaffolding discontinuous epitopes was developed and succeeded to transplant a two-segment portion of the epitope of the anti-HIV bNAb b12 [14], [19]. Finally, the ultimate goal of rationale vaccine design is to generate a vaccine that provides broad-coverage against multiple strain variants of a given pathogen, for example such as a universal Flu vaccine that would avoid the need to generate new seasonal vaccines every year. Such an accomplishment was achieved for MenB by combining both reverse vaccinology and structural vaccinology approaches described above. Specifically, a single immunogen was engineered that contained immunodominant regions from all three MenB sequence groups, and which was able to elicit protective antibodies against all the tested variants of the MenB serotype [20].

3.3 VACCINE DELIVERY AND ADJUVANTS

Practically every type of vaccine platform/delivery system has been exploited in generating candidate vaccines against the viral, bacterial, and toxin bio-threat agents; and these approaches are described in detail in the chapters dedicated to a given pathogen and corresponding MCM. These include whole cell inactivated or attenuated approaches, sub-unit vaccines, viral- or bacterial-vectored expression platforms, gene-based vectors or replicons, Virus-Like Particles (VLPs), and ‘Naked’ DNA-based vaccines delivered by electroporation, etc. The goal of exploiting any one of these platforms is to elicit a potent humoral and/or cell-mediated immune response against the expressed antigen, and each have their own pros and cons for inclusion in vaccine design. Vectored delivery systems have been designed to stimulate mucosal and system immunity [1]. Viral-vectored and gene-based delivery of vaccine antigens results in the synthesis of the antigen within antigen presenting cells for endogenous presentation on major histocompatibility complex Class I and II molecules. Replicating vectors closely mimic efficacious live attenuated vaccines. DNA-expression vectors, replication-defective viruses, or prime-boost combinations of the two are effective in eliciting bNAb’s [5]. Prime-boost vaccine regimens that use DNA and viral vectors have increased both humoral immunity and memory CD8 T-cell responses [5].

Despite the availability of these platform technologies, and the significant advances in both reverse- and structural-vaccinology described above, the resulting vaccine formulation may still be in need of an adjuvant. Adjuvants may be required for vaccine candidates comprised of poorly immunogenic antigens (i.e. sub-unit and recombinant protein-based vaccines such as Anthrax and Ricin), vaccine targets which rely heavily on T-cell responses (e.g. F. tularensis, Burkholderia sp.), and vaccines targeting antigenically-diverse pathogens to broaden protection (i.e. Filoviruses, Alphaviruses). Moreover, in the case of vaccines for biothreat agents, adjuvants may be required to ensure dose-sparing (to lessen the stockpile burden), achieve single-dose immunization schedules, and to achieve rapid onset of protective immunity. In the past decade, there have been considerable advances in identification of signaling pathways and receptors of the innate immune system and a greater appreciation of the importance of innate immunity in influencing adaptive immune responses. As a result, the literature is rich in newly identified ‘adjuvants’ – or rather immune cell stimulators that may or may not be effective as vaccine adjuvants. We have reached a point where it is now possible to design adjuvants to
potentiate and direct a desired, very specific immune response. However, adjuvants are not approved alone but rather as part of the final formulation of a vaccine, with safety and efficacy of the combination product considered as a whole [21]. When an adverse event occurs in a clinical trial involving an adjuvanted vaccine, it is often the safety of the adjuvant that is called into question [21], [22]. Indeed, the lack of new human adjuvants is not from a lack of potent adjuvant compounds but instead from a lack of adjuvants with robust safety and tolerability [22]. Adjuvant discovery programs to date are based on screening compounds for their ability to induce inflammatory cytokines; however such activity leads to high reactogenicity [22]. Thus, new paradigms in adjuvant discovery and development are needed that exploit ‘non-inflammatory’ pathways [22]. In the case of facilitating the advancement of biodefense vaccines along the regulatory path to licensure, it is perhaps best to utilize already FDA-approved adjuvants when possible, as the additional regulatory hurdles of including a novel adjuvant may be insurmountable when coupled to the inherent hurdles associated with biodefense (i.e. lack of human data and thus understanding of correlates of protection and clinical endpoints, animal rule). When Alum just won’t cut it, adjuvants with ‘regulatory experience’, i.e. those currently in clinical development by major vaccine pharmaceutical companies, should be exploited.

3.4 **EX VIVO HUMAN MIMETICS**

As discussed, successful translation of the technological advances outlined above into a licensed vaccine is dependent on the ability to predict the safety and efficacy of the candidate in humans prior to entry into clinical trials. Human clinical trials to determine medical countermeasure efficacy against the majority of high priority biothreats cannot be conducted because there are too few cases, the occurrence is too sporadic, and the severity of the disease is too great. Instead, licensure in the United States for these MCMs will only be possible using the “Animal Rule” of the FDA, which allows for a demonstration of efficacy in one or more well-characterized animal models which are considered relevant to the disease in humans. Thus, U.S. government agencies have ongoing large investments in the development of animal models for many of the top biothreats. The challenge lies in developing models that truly recapitulate human disease. Again, insufficient understanding of biothreat disease pathogenesis in humans, protective human immune responses, and identification of immune correlates of protection make identifying the appropriate animal model difficult.

To close this gap, over the past decade the U.S. Department of Defense invested in the development of Vaxdesign’s Modular Immunv in vitro Construct (MIMIC™) Technology [23], [24]. The MIMIC™ is an in vitro mimetic comprised of individual engineered tissue constructs seeded with isolated primary human immune cells, that accurately simulates the human immune response to candidate vaccines [23]-[26]. The tissue constructs are designed to stimulate the environment and cellular communication necessary for proper induction of immune responses under physiologic conditions. The MIMIC™ platform consists of three different modules: the Peripheral Tissue Equivalent, the Lymphoid Tissue Equivalent, and a Functional Assay or Disease Module [23], [24]. The ability of the MIMIC™ to accurately predict the innate, cellular and humoral immune responses was demonstrated for a large panel of vaccines (FDA approved and experimental) and adjuvants [23]-[33]. The technology can be used to dissect the mechanisms of actions of both vaccines and adjuvants, define both cellular and humoral correlates of protection, assess vaccine immunogenicity and efficacy between various formulations, determine the effects of human genetic and diversity on vaccine effectiveness, and characterize the mechanisms of antibody-mediated pathogen neutralization [23]-[33]. In addition, Vaxdesign has developed a number of highly sensitive functional MIMIC™ assays, to include a sensitive multiplexed ELISA-like assay, termed Antibody Forensics, to examine antibody quantity and quality [23]. Moreover, the MIMIC™ was designed with all the attributes required for an enabling technology that industry could exploit to truly facilitate vaccine development: It is modular; utilizes a reliable, consistent, and well-characterized cell source (GRP-compliant donor program); and is fully automated to increase assay-throughput and reproducibility [23].
The MIMIC™ technology has been validated by seeking opportunities to correlate MIMIC™ data to that obtained in human clinical trials with the same vaccine. Both successful vaccines (FDA approved) and failures (candidates that didn’t make it past first in human trials) were examined, and the MIMIC™ successfully reproduced the clinical data for each, leading to the purchase of Vaxdesign by Sanofi Pasteur in 2010. The MIMIC™ and its’ associated assays are being designed and validated so the resulting data can be utilized to support IND package submissions as well as aide in the analysis of clinical trial samples, and thus could serve to advance regulatory sciences and eliminate further bottlenecks in vaccine development. Thus, technologies like the MIMIC™ provide significant and critical assets across the vaccine development spectrum that can serve to streamline efforts, provide additional validation of results obtained in animal models, and increase the probability of clinical success and licensure.

Additional ex vivo mimetics are needed to better understand human mucosal immunology, as the respiratory mucosa is a critical exposure portal for natural and bioengineered pathogens and an attractive drug delivery route. For example, an improved in vitro three-dimensional (3-D) Fluidic-Enhanced Airway Model is being pursued that reproduces not only epithelial function, but also encompasses the integrated epithelial-interstitial-microvasculature structure of the human respiratory air-blood barrier. This model will employ primary human lung cells in a self-contained engineered fluidic chamber enabling independent control and access to the three cell types (i.e. bronchial epithelial cells, fibroblasts, and microvascular endothelial cells). These respiratory models offer a tool to study the impact of biological threats, and vaccines against them, at a likely site of exposure.

In vitro platforms of human organ constructs that could accurately predict human safety, efficacy, and pharmacokinetics of candidate medical countermeasures are a long-term prospect and goal of U.S. government agencies involved in MCM development. The ultimate goal of this research would be to reduce the overall burden of in vivo testing in the development of products for human use. A platform comprised of in vitro human organ constructs in communication with each other (i.e. liver, lung, heart, kidney, vasculature, and blood brain barrier with neuronal component) could accurately assess efficacy, toxicity, and pharmacokinetics of drugs in a way that is relevant to humans. Development of a platform that is both reliable and accessible to the scientific community at a reasonable cost would greatly facilitate medical countermeasure development.

3.5 FUTURE OF BIODEFENSE VACCINES

3.5.1 Challenge of Evolving Biological Threats

The future of biological defence vaccines is inevitably tied to the evolving threat. The biological threat has changed dramatically since the passing of the Biological and Toxins Weapons Convention went into force in 1975 as a result of asymmetrical warfare, emerging infectious diseases, and advances in biotechnology. The adversary has changed; the focus has shifted in large part from state-run biological weapons programs to biological terrorism. This in itself impacts the breadth of biological agents for which we need medical countermeasures.

An increasing awareness of emerging infectious diseases also continues to shape our picture of the biological threat. For example, since the discovery of Ebola virus in 1976, four other Ebola species have been identified [34], which has impacted the experimental design when seeking a “pan-Ebola virus” vaccine. As biosurveillance efforts continue to expand globally and encompass both clinical and agricultural studies, newly emerging threats will inevitably be found. The recent discovery of a novel rhabdovirus, Bas-Congo virus, as the etiological agent of a 2009 viral hemorrhagic fever outbreak in the Democratic Republic of the Congo [35] is a prime example of
how the combination of increasing surveillance with technological advances will likely increase the rate at which new pathogens are discovered.

While technological advances increase our ability to detect and diagnose new pathogens, they also bring the potential for new threats. Genetic engineering is common practice in microbiology labs world-wide to tease out biological functions. Combined with an increasing understanding of how given biological functions and networks impact a system as a whole, the creation of synthetic viruses and bacteria has become a reality [36], [37]. The 2010 Presidential Commission for the Study of Bioethical Issues concluded that synthetic biology was not a threat at the time [38]. However, the field of synthetic biology will need to be monitored for advances that could lead to new threats, or perhaps more importantly, new opportunities for biological defence capabilities.

3.5.2 Streamlining Vaccine Development

Defence organizations need to rapidly develop and field safe and effective MCMs against biological threats to warfighters. To this end, and in response to key strategic direction from The White House through Homeland Security Presidential Directives, Executive Orders, and other correspondences, the U.S. Department of Defense initiated the Medical Countermeasure Initiative (MCMI) [39]. The goal of the MCMI is to provide the innovative S&T base and critical flexible and agile biomanufacturing capability required to ensure a rapid response to multiple and/or changing threats for both the military and civilian populations. New investments are required in vaccine prototypes that leverage adaptable antigen expression platforms and flexible and agile biomanufacturing technologies that utilize scalable, single-use/disposable systems. Borrowing from enabling investments in flexible manufacturing, the Department of Defense is seeking to enhance the CBDP’s ability to adaptively manufacture countermeasures through new expression vehicles such as plants, cells, and de novo synthetic platforms. Methods to demonstrate and develop such platforms toward new capabilities that can integrate into a multi-vaccine product advanced development facility and large-scale production are being explored [39].

The development of vaccines against traditional biological threats for which the CBDP needs vaccines remains a challenge. The evolving threat landscape further complicates this scenario, providing a moving target that necessitates the development/utilization of adaptable, ‘plug-and-play’ vaccine platforms exploiting flexible and agile manufacturing technologies. Negotiating the critical path for biodefense vaccine development in a timely manner requires the implementation of translational medicine principals aimed at product delivery. Advancements in regulatory sciences and technologies are needed to increase the likelihood of success and facilitate the progression of MCMs for biodefense along the regulatory path. Novel in vitro assays and ex vivo human mimetics (as well as in silico approaches) for the rapid screening of vaccine candidates should enable the ability to better predict the efficacy, potential toxicity, and immune response in humans prior to entry into clinical trials. Investments aimed at understanding the molecular mechanisms of pathogenesis and protective immunity in humans for both traditional and emerging threats will continue to be required to facilitate not only vaccine candidate discovery but subsequent advanced development.

3.6 REFERENCES


Disclaimer: This manuscript represents the opinion of the authors and not necessarily that of the Defense Threat Reduction Agency, the Joint Science and Technology Office, or the National Institutes of Health (OBRTR/DMID/NIAID/NIH).
Chapter 4 – USE OF ADJUVANTS FOR ENHANCEMENT OF VACCINE POTENCY

Garry Morefield
VaxForm, Bethlehem, PA
UNITED STATES

Corresponding Author (Morefield): garry.morefield@vaxform.com

4.1 INTRODUCTION

Historically, the first vaccines discovered utilized bacteria or viruses that had been attenuated or killed prior to administration [1]. Because these vaccines utilized whole organisms as the antigen they retained immune modulators, such as toll-like receptor agonists and exotoxins, intrinsic to the pathogen. Due to the presence of these intrinsic immunomodulators these early vaccine formulations had issues with high rates of reactogenicity. As technology improved allowing for the purification of sub-unit antigens, which removed the intrinsic immunomodulators, the safety profile of vaccines improved, however the potency of vaccine antigens declined. To enhance the potency of these highly purified antigens, adjuvants must be incorporated into vaccine formulations.

Currently, worldwide the most commonly utilized adjuvants in marketed products are based on aluminum salts and squalene emulsions. The only FDA-approved biodefense vaccine, BioThrax® for protection against anthrax, incorporates an aluminum salt adjuvant [2]. Other biodefense vaccines in development targeting pathogens such as plague, botulinum neurotoxins, and staphylococcal enterotoxins incorporate aluminum salts as well [3]-[5]. In addition to aluminum salts and squalene emulsions many other classes of adjuvants are in development including Toll-Like Receptor (TLR) agonists, saponins, and cytokines [6]-[9]. These next generation adjuvants enhance potency and target the appropriate immune response to efficiently protect patients from pathogens.

4.2 ALUMINUM SALT ADJUVANTS

Aluminum-containing adjuvants are currently the most widely used in marketed vaccines. These adjuvants were first discovered by Glenny et al. in 1926 and have a long history of safe use in vaccine formulations. Glenny was investigating diphtheria and observed an increase in the immune response of diphtheria toxoid when precipitated with potassium alum versus a solution of the toxoid [10]. Insights into the mechanism of action and properties of aluminum adjuvants have been determined in recent years.

The mechanism of action of aluminum-containing adjuvants has typically been divided into three functions including recruitment of Antigen-Presenting Cells (APCs), enhancing the uptake of antigen, and influencing the differentiation of APCs and T-cells. Once a vaccine incorporating an aluminum-containing adjuvant is administered an inflammatory response is initiated and immune cells such as monocytes, neutrophils, and eosinophils are recruited to the administration site [11]-[14]. Once APCs are recruited to the site of administration aluminum adjuvants facilitate antigen uptake by making the antigen particulate in nature [15]. Particulate antigens are more efficiently internalized by resident and infiltrating APCs [16]. Once internalize by APCs aluminum salt adjuvants influence the activation and differentiation of immune cells through the Nalp3 inflammasome [17]-[21]. This interaction with cells of the immune system typically results in a Th2 response with enhanced levels of antibodies produced following vaccination [22].
When developing vaccines incorporating aluminum salt adjuvants it is important to understand both the nature of the surface of the adjuvant and how the antigen interacts with that surface. Surfaces of aluminum salt adjuvants are dynamic in nature with a continuum of surfaces with slightly different properties, with aluminum hydroxide adjuvant on one end of the continuum, and aluminum phosphate adjuvant on the other. Aluminum hydroxide adjuvant is chemically crystalline Aluminum Oxyhydroxide $\text{Al(OH)}_3$ [23]. The surface of aluminum hydroxide adjuvant is able to carry an electrical charge as it is composed of hydroxyls that are able to accept and donate protons [24]. Because the adjuvant surface can have a positive or negative charge depending on the pH the adjuvant has a Point of Zero Charge (PZC). The PZC of aluminum adjuvants is analogous to the isoelectric point for proteins. It is important to understand what the PZC of the adjuvant is, as this gives an indication of whether the surface charge at a given pH will be positive or negative which in turn impacts antigen adsorption. The PZC of aluminum hydroxide adjuvant is 11.4 so at physiological pH the surface has a positive charge. Through treatment with excipients such as phosphate anion the PZC of aluminum hydroxide adjuvant can be decreased to an acidic value [25]. The decrease in PZC is proportional to the level of phosphate exposure as the greater amount of phosphate exposure results in the lower the PZC value. Aluminum phosphate adjuvant is chemically amorphous aluminum hydroxyphosphate, $\text{Al(OH)}_3(\text{PO}_4)y$, and is not a stoichiometric compound. The PZC of aluminum phosphate adjuvant decreases with increasing phosphate incorporation and commercially available material generally has a PZC between 5.0 and 5.5 [26]. Therefore, at physiological pH aluminum phosphate adjuvant generally has a negative surface charge.

Once the physical properties of the aluminum adjuvant being incorporated into a formulation are understood, how the target antigen interacts with the adjuvant needs to be determined. There are many mechanisms by which an antigen can adsorb to an aluminum-containing adjuvant such as electrostatic attractive forces, hydrophobic interactions, ligand exchange, hydrogen bonding and van der Waals forces [27]. The predominate adsorption mechanisms observed in vaccine formulations are electrostatic attractive forces, ligand exchange and hydrophobic interactions. Electrostatic attractive forces act in formulations where the antigen and adjuvant have opposite electrical charges. At physiological pH aluminum hydroxide adjuvant has a positive charge and will have electrostatic attraction with antigens having an acidic isoelectric point. Aluminum phosphate adjuvant has a negative charge at physiological pH and will have electrostatic attraction with antigens that have a basic isoelectric point. Model antigens, such as albumin which has an acidic pI and adsorbs preferentially to aluminum hydroxide adjuvant and lysozyme which has a basic pI adsorbs preferentially to aluminum phosphate adjuvant have been utilize to investigate electrostatic attractive forces [28]. A method to determine the extent to which electrostatic attractive forces are involved in adsorption of an antigen is to treat the formulation with increasing concentrations of sodium chloride [29]. The ions from the salt shield the charges on the adjuvant surface and antigen, therefore as the ionic strength increases the adsorption level of the antigen will decrease if electrostatic attractive forces are the predominate adsorption mechanism.

The strongest adsorption force is ligand exchange which occurs when a phosphate group on an antigen exchanges with a hydroxyl on the surface of the aluminum adjuvant. Due to the strength of ligand exchange binding the interaction can occur even in the presence of electrostatic repulsive forces. This was demonstrated by preparing ovalbumin with varying amounts of phosphorylation and determining the level of adsorption with aluminum phosphate adjuvant [30]. In these formulations there were electrostatic repulsive forces present between the antigen and the adjuvant. Ovalbumin with low phosphate content exhibited no adsorption to the adjuvant as electrostatic repulsion was the dominant force. Hyperphosphorylated ovalbumin could overcome the electrostatic repulsive force with ligand exchange binding and was 99% adsorbed to aluminum phosphate adjuvant. However, caution must be used when ligand exchange is the primary mode of antigen adsorption. The high strength of binding associated with this interaction can have a deleterious impact on antigen stability and potency. Hepatitis B surface antigen as well as alpha-casein, a model antigen, have shown that as the strength of binding increases the resulting potency in mice decreased [31], [32]. The observed decrease in
vaccine potency resulted from interference in antigen processing and presentation. Therefore, antigen adsorption strength must be considered, in addition to the level of adsorption when developing a stable, immunogenic vaccine formulation with aluminum salts.

Historically it was hypothesized that the vaccine antigen must be adsorbed to the surface of the adjuvant to observe an enhancement of immunogenicity. However, recently it was shown that antigen adsorption is not an absolute requirement for enhancement of potency for vaccines incorporating aluminum salt adjuvants [33]. The necessity of adsorption is antigen dependent. Some antigens do require a high level of adsorption to maintain optimal potency, while for others equivalent immunogenicity is achieved for adsorbed and non-adsorbed formulations, and others exhibit optimal immunogenicity in non-adsorbed formulations. The determining factor often is changes in the physical state of the antigen between adsorbed and non-adsorbed formulations. The correlation of potency and adsorption is generally investigated by evaluating the immunogenicity of the target antigen in adsorbed and non-adsorbed formulations. Manipulation of the surface charge of the adjuvant through addition of phosphate anion is utilized to obtain adsorbed and non-adsorbed formulations. This has been performed for anthrax antigens. It was determined that recombinant Protective Antigen (rPA) had a higher affinity for aluminum hydroxide adjuvant versus aluminum phosphate adjuvant [34]. Additionally, it was shown that as the level of phosphate treatment of aluminum hydroxide adjuvant increased the adsorption of rPA decreased. This treatment is able to decrease the binding strength of rPA to the adjuvant thereby increasing the potency and stability of the antigen [35]. Once the importance of adsorption is determined the focus of subsequent development efforts can be targeted towards optimizing the stability of the adsorbed or non-adsorbed formulation.

When optimizing formulations incorporating aluminum adjuvants the impact of the micro-environment pH must be evaluated if adsorption is critical for antigen potency. Since vaccine formulations having aluminum salt adjuvants are suspensions of charged particles in an aqueous solution the charged adjuvant surface attracts ions of opposite charge from the surrounding water. This includes attraction of protons by a negatively charged adjuvant surfaces and attraction of hydroxyls by a positively charged adjuvant surfaces. The result of attracting protons or hydroxyls to the adjuvant surface induces a micro-environment pH which can be up to 2 pH units different than what can be measured in the bulk of the formulation [36]. The difference in pH between the bulk and micro-environment can have a dramatic impact on the stability of adsorbed antigens as antigen stability depends on pH. By understanding the impact of pH on antigen stability the formulation pH can be manipulated to ensure the pH of the microenvironment is in a stabilizing range for the antigen. Conditions that balance adsorption level, adsorption strength, and micro-environment pH are generally found to be the optimal formulation conditions for vaccines.

4.3 EMULSION ADJUVANTS

An emulsion is a dispersion of at least two immiscible liquid phases stabilized with an emulsifying agent [37]. The most common emulsion types utilized in vaccine formulations are water-in-oil and oil-in-water emulsions. Emulsions were first evaluated as vaccine adjuvants by Freund in the 1940s [38]-[40]. He found that enhanced immunogenicity could be obtained by formulating antigens utilizing a water-in-paraffin oil emulsion either incorporating Mycobacterium tuberculosis, Complete Freund’s Adjuvant, or with-out the mycobacteria, Incomplete Freund’s Adjuvant (IFA). Salk and colleagues evaluated IFA with whole virus influenza vaccine in humans and non-human primates and found that formulation with the adjuvant induced a faster and longer-lasting immune response than formulation with saline alone [41]-[43]. The success of water-in-oil emulsion vaccine formulations in clinical studies led to the licensure of a seasonal influenza vaccine incorporating IFA in the United Kingdom [44]. In the initial years of licensure approximately 1 million doses of the emulsion
adjuvanted influenza vaccine were administered, but the vaccine exhibited high reactogenicity and their use was discontinued over time due to those safety issues [45].

Squalene-based oil-in-water emulsions are the current focus of vaccine adjuvant development. Squalene is a biodegradable oil which is a precursor of cholesterol. The primary source of squalene is shark liver oil, however methods for obtaining squalene from renewable sources such as olives are being developed [46]. Typically non-ionic surfactant emulsifiers such as Tween 80 and Span 85 are used to prepare stable emulsions of squalene. To manufacture the emulsion generally the two phases are prepared separately then mixed together. The squalene is mixed with the low Hydrophilic-Lipophilic Balance (HLB) emulsifier as a low HLB indicates a preference for the oil phase, and the high HLB emulsifier is mixed with the aqueous phase. The oil phase is then dispersed into the water phase. Finally, a homogeneous preparation having a sub-micron particle size can be obtained through processes such as microfluidization or temperature-induced phase inversion. This provides a preparation that can be sterilized by terminal filtration and stored ready to use.

The mechanism of action of oil-in-water emulsions incorporates multiple mechanisms to induce the adjuvant effects [47]. The emulsion stimulates the release of chemokines which attract monocytes and granulocytes to the site of injection. Once recruited to the site of injection the emulsion adjuvant enhances antigen uptake followed by stimulation of monocytes to mature into dendritic cells. In addition to these direct effects on cells of the immune system it has also been shown that squalene emulsions can interact with skeletal muscle to promote the immune response [48]. Recently, it was determined that that the emulsion as a whole is required to stimulate enhancement of vaccine potency [49].

Squalene-based emulsion adjuvants in development include by the major vaccine manufacturers include MF59 (GlaxoSmithKline), AS03 (Novartis Vaccines and Diagnostics), and AF03 (Sanofi Pasteur). These emulsion adjuvants have been generally utilized in pandemic and seasonal inactivated influenza vaccines. All three of these squalene emulsion adjuvants have been found to boost the immune response to both seasonal and pandemic strains of influenza [50]-[55]. Emulsion adjuvants have been used to enhance the potency of biodefense vaccines such as botulinum neurotoxin [56]. Because of the ability to induce Th1 responses, the dose sparing potential and relative safety demonstrated by the squalene-based emulsion adjuvants they will continue to play an important role in the future of vaccine development.

4.4 ALTERNATIVE ADJUVANT SYSTEMS

With the increasing purity and complexity of antigens under development for novel vaccines aluminum salt and squalene emulsion systems do not achieve the desired potency enhancement or stimulate the appropriate immune response. Therefore, many alternate adjuvant systems have been investigated including TLR agonists, saponins, and cytokines. There is one marketed vaccine by GSK, Ceverix, which contains a TLR agonist, MPL, combined with an aluminum salt. In addition to this successful introduction of an alternative adjuvant system to protect against HPV infection there are many examples of studies with biodefense antigens during development.

Toll-like receptors allow cells to recognize conserved molecular patterns of pathogens and provide a link between the innate and adaptive immune system. Because of this link between innate and adaptive immunity, agonists to TLRs have been the focus of many adjuvant development studies for vaccines. A recent study utilizing recombinant Protective Antigen (rPA) targeting anthrax infection compared immune responses to a variety of alternative adjuvant systems including TLR agonists, CpG DNA, LPS, IL-1α, Pam3CSK4, and resiquimod [57]. This study found that all adjuvant systems were able to enhance potency of rPA following intranasal administration; however IgG sub-class and T-cell cytokine profiles were altered depending on the
adjuvant used. This demonstrates the ability of using various TLR agonists to target stimulation of the appropriate immune response to provide the greatest protection against disease.

Saponins are typically plant-derived products composed of amphipathic glycosides. There are multiple commercial saponin adjuvant systems available such as ISOMATRIX™, Matrix-M™, and Advax™, however saponin adjuvant systems are not in any FDA-approved product to date. While there is little data available for biodefense antigens formulated with saponin adjuvant systems, there has been success using these types of adjuvants with influenza, rabies, and hepatitis [58]-[60]. Saponin adjuvant systems are able to stimulate both CD4+ and CD8+ T-cell responses following vaccination. The broad immune activation induced by saponin-based adjuvant systems suggests their potential to enhance the efficacy of vaccines targeting biodefense antigens.

Cytokines are a diverse group of proteins that regulate immune responses to infection as well as vaccination. The use of cytokines as adjuvants has been investigated for use in vaccines targeting biodefense threats such as anthrax, plague, and SEB. The cytokine IL-1α has recently been investigated for potency enhancement of nasally administered anthrax vaccine [61]. It was found that IL-1α acts on both stromal and hematopoietic cells following nasal administration to stimulate the immune response. This cell stimulation caused increased production of lethal factor-specific antibodies demonstrating the potential for use of IL-1α as an adjuvant in novel anthrax vaccine formulations.

Interleukin-12 has been investigated as a potential adjuvant for use in a vaccine against plague [61]. IL-12 was combined with inactivated whole cell *yersinia pestis* and administered intranasally to mice. It was found that the adjuvanted vaccine increased the immunogenicity of the whole cell vaccine allowing protective immune responses to be achieved with lower doses. The higher serum IgG as well as mucosal IgA antibodies induced with IL-12 correlated with protection against pulmonary challenge. IL-12 has also been used in DNA vaccination against plague demonstrating the versatility in the cytokines ability to act as an adjuvant [62].

Finally, interleukin-15 has been shown to increase the potency of an SEB vaccine to protect against toxic shock [63]. When IL-15 was administered with a genetically attenuated SEB antigen it was found to stimulate the maturation of spleenocytes into dendritic cells. This activation increased antibody production and enhanced protection against challenge with the wild-type toxin. The study also demonstrated that IL-15 could be co-administered with aluminum salt adjuvants which target the cytokine to phagocytic monocytes increasing the efficiency of dendritic cell maturation.

Scientists working to develop vaccines targeting biodefense pathogens have a broad array of adjuvant systems from which to choose to produce a safe and effective vaccine product. Traditional aluminum salt and squalene emulsion adjuvant systems will continue to play a large role in vaccine development due to their established safety and potency profiles. However, as the need for more potent adjuvant systems that target activation of specific immune responses are required as new antigen targets are discovered the need for novel adjuvant systems will grow. TLR agonists, saponins, and cytokines are a few of the novel classes of adjuvant systems that show promise to be effective adjuvants for these developing vaccines. As the safety profiles and mechanisms of action of these adjuvants become better understood their use in vaccines will continue to grow.

### 4.5 REFERENCES


USE OF ADJUVANTS FOR ENHANCEMENT OF VACCINE POTENCY


[58] Distefano D, Antonello JM, Bett AJ, Medi MB, Casimiro DR and Ter Meulen J. Immunogenicity of a reduced-dose whole killed rabies vaccine is significantly enhanced by ISCOMATRIX™ adjuvant, Merck amorphous aluminum hydroxyphosphate sulfate (MAA) or a synthetic TLR9 agonist in rhesus macaques. Vaccine. 2013 1 Oct;31(42):4888-93.


Chapter 5 – STATE-OF-THE-ART THERAPEUTIC MEDICAL COUNTERMEASURES FOR BIOLOGICAL THREAT AGENTS

Melissa V. Stundick¹, Aruna Sampath², Matthew Metz¹ and Joseph C. Larsen¹
¹: U.S. Department of Health and Human Services, Office of the Assistant Secretary for Preparedness and Response, Washington, DC; ²: Science Applied International Corporation (SAIC) Contractor to the U.S. Department of Health and Human Services, Office of the Assistant Secretary for Preparedness and Response, Washington, DC

UNITED STATES

Corresponding Author (Larsen): joseph.larsen@hhs.gov

5.1 INTRODUCTION

The threat of an attack with biological warfare agents has resulted in significant public sector investment towards the development of Medical Countermeasures (MCMs: vaccines, therapeutics, and diagnostics). Prior to the 2001 terrorist attacks within the U.S., much of this work was sponsored by the U.S. Defense Department and Ministries of Defence from other Partner Nations. Primarily, this work focused on pre-event mitigation through vaccination of military personnel. While this strategy is appropriate for personnel deploying to combat areas that are, or may be, contaminated with an agent, it does not focus on the risks to vulnerable civilian populations.

In light of the attacks of September 11, 2001, a large-scale release of biological agent(s) on a civilian population appears more plausible. The mass vaccination of civilian populations is prohibitive, as the risk of vaccination of the general population outweighs the benefit when framed in the context of probability of exposure. Therefore, there is significant interest in developing prophylactics and therapeutics that can prevent morbidity and mortality from these diseases after individuals have been exposed or have become symptomatic, respectively. The enormous expense of drug development, estimated at $1.2 billion (U.S.) to bring a drug from discovery to approval [46], necessitates that only promising strategies and/or products are supported.

Further, many of the candidate therapies for biodefense-related agents must pursue a non-traditional regulatory pathway, via the U.S. Food and Drug Administration’s (FDA) Animal Rule. This rule allows animal efficacy data to be used to support approval when human clinical evaluation of efficacy is not feasible or ethical. There are, however, a number of substantive requirements for seeking approval under this rule [246]. To date, approval through this pathway does not appear promising, as there have been only two products approved since its establishment in 2002, despite a robust investment in the development of therapeutic MCMs. Given this, it is likely that drug development costs and timelines for these agents will likely exceed industry averages. Thus, it is critical that a greater number of technologies and candidate therapeutics enter the developmental pipeline in order to counter the high rate of attrition in this mission space.

Treatments are generally defined as those interventions that are administered once it becomes evident that a victim has been infected. The trigger for administering treatment is the onset of symptom(s) indicative of disease. Administration of drugs as Post-Exposure Prophylactics (PEP) occurs when they are given to individuals exposed to an agent and at risk of illness, but not yet showing symptoms. In this chapter, the term therapeutic is used broadly as a drug that might be useful as a treatment and/or a PEP. If used for treatment, a therapeutic must be more potent, as it must defeat a pathogen that has already established a symptom-causing infection. Yet, use for treatment generally encounters fewer safety and tolerability requirements because administration will only occur in cases of illness (life-threatening in this context). On the other hand, PEP use
requires relatively more safety and tolerability because many more individuals, some who are exposed, but might never become ill, would receive the therapeutic.

This chapter provides an overview of state-of-the-art MCMs for biological agent exposure or infection. Five categories of emerging therapeutics are described:

- Pathogen-targeted small molecule therapeutics;
- Host-targeted therapeutics;
- Immunotherapies;
- Antimicrobial peptides; and
- Nucleic acid based therapies.

The benefits and technical challenges associated with these emerging countermeasures are discussed.

5.2 PATHOGEN-TARGETED SMALL MOLECULE THERAPEUTICS

Small molecule therapeutics that target a pathogen are typified by anti-microbial compounds which disrupt the metabolism or lifecycle of the pathogen, either crippling or killing it. The earliest of these were naturally occurring compounds evident in biological competition among microbial rivals. Subsequent pharmaceutical innovations have provided both modifications of natural products, and entirely novel chemistries. The concept of using small molecules to inhibit an essential component of a pathogen’s life cycle is not novel, yet the historical investment in this type of MCM against biodefense agents has lagged behind other measures, such as vaccines. The application of traditional and state-of-the-art drug development methodologies for these agents has been initiated only in the last few years. Regardless, significant progress has been made in advancing therapeutics for several agents. For the purposes of this discussion, small molecule therapeutics will be grouped into anti-viral, anti-bacterial (often called antibiotic), and antitoxin compounds.

5.2.1 Anti-Virals

5.2.1.1 Poxviruses

Two candidate anti-viral compounds have emerged for the treatment of orthopoxvirus infection in recent years, ST-246 and CMX-001. ST-246 (Tecovirimat) is an inhibitor of viral egress from host cells that has demonstrated significant protective efficacy in ectromelia virus and rabbitpox challenge models [269], [200], [165]. Further, ST-246 has demonstrated 100% protection in a lethal monkeypox challenge model in Non-Human Primates (NHPs) when administered up to three days post-infection (a time point at which lesions become evident), suggesting the drug may hold promise as a therapeutic anti-viral treatment for smallpox [90]. ST-246 has undergone clinical trials to establish human safety, tolerability, and pharmacokinetics (Clinicaltrials.gov identifier NCT00907803 – https://clinicaltrials.gov/ct2/show/NCT00907803).

Cidofovir, a cytidine analog targeting viral DNA replication, has demonstrated PEP efficacy against a lethal monkeypox challenge in cynomolgus macaques [229]. In addition, cidofovir is FDA-approved for the treatment of Cytomegalovirus (CMV) retinitis in Human Immunodeficiency Virus (HIV) patients. However, the nephrotoxicity of cidofovir precludes its widespread use in a public health emergency. Because of these safety concerns, CMX-001, a pro-drug derivative of cidofovir, is being developed as an oral formulation. CMX-001 possesses broad spectrum anti-viral activity against a number of dsDNA viruses such as BK, adenovirus, and herpesviruses and is also being developed for its utility as a smallpox anti-viral [48], [238].
CMX-001 demonstrated 100% protection in a lethal Rabbitpox (RPX) virus challenge when administered one day post-infection [205]. In a parallel study in the same model when CMX-001 was administered at lesion onset, a dose dependent improvement in survival was observed between the treatment groups [206]. A Phase I clinical study (Clinicaltrials.gov identifier NCT00780182 – https://clinicaltrials.gov/ct2/show/NCT00780182) has been conducted for CMX-001, and other clinical trials are in preparation.

ST-246 and CMX-001 act synergistically when evaluated in combination against vaccinia or Cowpox Virus (CV) in vitro [200]. Effects of the combination therapy were also evaluated in mice infected with CV. Treatment with the combination of ST-246 and CMX-001 at individually sub-therapeutic doses of 1 and 3 mg/kg/day (respectively) was effective in reducing mortality even when the drugs were administered six days after lethal CV challenge. No evidence of toxicity was observed with the combination either in vitro or in vivo. The drugs have different anti-viral mechanisms; therefore, the combination may provide an additional benefit in decreasing resistance development.

5.2.1.2 Filoviruses

An alykylated porphyrin chlorphyllide selected from a cell-based, High Throughput Screen (HTS) for Hepatitis B Virus (HBV) inhibition showed broad spectrum activity against filoviruses and other enveloped viruses including flaviviruses and arenaviruses [74]. One of the chemophores of chlorphyllide, chlorin E6, inhibited Marburg Virus (MARV) in Vero cells at low micromolar concentrations. As yet undetermined pharmacological properties and mechanism of action for this prospective anti-viral will determine whether a useful drug can be developed.

High content imaging technology has enabled cell-based HTS for inhibitors of viruses. Panchal et al., [189] reported an image-based method to screen 580 compounds against MARV and Ebola Virus (EBOV) using a viral replicon construct fused with the Green Fluorescent Protein (GFP) reporter gene. One compound, NSC 188491, a nucleoside analog, showed 70% inhibition of EBOV at 1.5 µM. Clearly, more potent analogs of this compound will need to be developed and yield acceptable pharmacology for a useful drug to result.

Virus pseudotypes have been used to conduct HTS for compounds capable of inhibiting viral entry [265]. A compound, designated LJ001, was identified in a screen of approximately 80,000 compounds for inhibitors of Nipah virus entry into host cells. When tested against other viruses, LJ001 possessed the ability to inhibit the entry of a number of negative sense, enveloped RNA viruses. The compound was capable of inhibiting EBOV entry in vitro and protected 80% of mice when administered at the time of infection. However, once daily injections of LJ001 initiated immediately post-infection and then redosed every 24 hours for 7 days did not protect mice from a lethal challenge with EBOV. This shortcoming was likely due to insufficient steady state plasma concentrations. Given that the compound is a viral entry inhibitor, blocking establishment of infection, its utility in the treatment of symptomatic EBOV infection is likely low.

5.2.1.3 Venezuelan Equine Encephalitis Virus (VEEV)

A review of the existing literature conducted by the U.S. Department of Defense’s Defense Threat Reduction Agency identified significant gaps in the data, assays, and animal models required for successful development of drugs to combat Equine Encephalitis Viruses (EEVs) [203]. Specifically, the development of HTS assays, the resolution of the crystal structure of the non-structural proteins, and the development of animal models to support therapeutic evaluation were cited as missing prerequisites to a successful target-based drug development program.
Carbocyclic cytosine (carbodine) has been shown to inhibit an attenuated vaccine strain of VEEV (TC-83) in Vero cells. Inhibitory activity is conformation dependent where the (–) enantiomer had EC_{50} concentrations of 0.2 µg/ml in a mouse model infected with TC-83 [103]. Further, pre-treatment with 200 mg/kg of the (–) enantiomer significantly improved survival when administered up to four days post-infection. Carbodine is limited by its toxicity, hence it is necessary to develop and characterize its analogs to improve the therapeutic index of this compound.

Historically, and particularly in the biodefense space, development of anti-viral therapeutics has lagged behind those against bacteria. The candidates for anti-viral therapies have been most frequently identified through in vitro screens of chemicals for their ability to inhibit the virus life cycle. The proportion of candidates that then appear to have safe and effective pharmaceutical properties has been very low.

It is clear that multiple robust, coordinated drug discovery and development campaigns will be required to identify sufficient numbers of candidate compounds to overcome the rate of attrition of anti-viral therapeutic development. A definitive concept of use for candidate viral MCMs (treatment and PEP) would help steer research and development efforts by clearly defining the requirements for efficacy, safety, and route of administration. Lastly, a strategic shift from supporting individual, disparate academically focused drug discovery efforts to supporting larger product focused programs will be more likely to extend the development of promising candidates beyond the proof-of-concept stage.

### 5.2.2 Anti-Bacterials

#### 5.2.2.1 Conventional Anti-Bacterials

Doxycycline and ciprofloxacin are the most well-known and widely accepted therapeutics against bacterial threats. CDC recommended treatments exist for the most common bacterial biodefense agents (Table 5-1), although animal model data to support efficacy is limited. The recommendations are based primarily on small numbers of clinical experiences with off-label use. Moreover, despite off-label use as a treatment or prophylaxis for biodefense agents, the FDA has not approved label expansion for these antibiotics to indicate their use against biodefense agents.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Antibacterials Currently Recommended as Therapeutics by the CDC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em></td>
<td>Ciprofloxacin, doxycycline, penicillin</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>Streptomycin, gentamicin, the tetracyclines, chloramphenicol</td>
</tr>
<tr>
<td><em>F. tularensis</em></td>
<td>Doxycycline, ciprofloxacin, streptomycin or gentamicin</td>
</tr>
<tr>
<td><em>B. mallei</em></td>
<td>Tetracyclines, ciprofloxacin, streptomycin, novobiocin, gentamicin, imipenem, ceftriaxime, the sulfonamides</td>
</tr>
<tr>
<td><em>R. prowazekii</em></td>
<td>Doxycycline, chloramphenicol, azithromycin, and fluoroquinolones; rifampin may also be considered</td>
</tr>
<tr>
<td><em>C. burnetii</em></td>
<td>Doxycycline; quinolones can also be considered</td>
</tr>
<tr>
<td><em>Brucella spp.</em></td>
<td>Combination of doxycycline and rifampin</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>Azithromycin, erythromycin, doxycycline</td>
</tr>
</tbody>
</table>
A relatively large body of published data obtained from animal model studies supports the use of levofloxacin, a fluoroquinolone, for PEP and/or treatment of *B. anthracis*, *Y. pestis*, or *F. tularensis* infections. Levofloxacin is an effective treatment in rat models of *B. anthracis* infection. After determination of antigenemia, intravenous levofloxacin treatment for five days at a total daily dosage of 25 or 12.5 mg/kg, conferred 90% and 70% survival, respectively [270]. Levofloxacin given to mice following infection with *F. tularensis* SCHU S4 was able to protect the animals, even when administration was delayed 72 hours post-infection [113]. Mice that were successfully treated with levofloxacin were later shown to have developed adaptive immunity to *F. tularensis* SCHU S4 administered via the intranasal route. In a second *F. tularensis* efficacy study using a marmoset model, animals were given levofloxacin twice daily for ten days beginning 24 hours post-challenge; this PEP regimen conferred 100% survival [168].

One study compared the effects of levofloxacin versus ciprofloxacin administration against *Y. pestis*, *F. tularensis*, or *B. anthracis* infections in three animal models. Levofloxacin alone was effective against infection with *Y. pestis* or *F. tularensis* in rabbit, mice and guinea pig models. Ciprofloxacin and levofloxacin had comparable activity against anthrax infection in the same animal models, yet the doses of ciprofloxacin and levofloxacin required to protect against inhalation anthrax were approximately 18-fold higher than the doses of levofloxacin required to protect against pneumonic plague and tularemia [194]. The critical period for drug administration following exposure to *B. anthracis* or *Y. pestis* was 24 hours while mice challenged with *F. tularensis* could effectively be protected even if drug administration was delayed for 72 hours post-challenge. Prolonged therapy was necessary for anthrax, whereas a shorter-term therapy for plague and tularemia was often successful. Overall, these results suggest the use of levofloxacin as an effective alternative to ciprofloxacin as a therapeutic against anthrax, plague, or tularemia.

Other fluoroquinolones, such as moxifloxacin and gatifloxacin, have been assessed for efficacy against biodefense pathogens. Moxifloxacin is approved by the FDA to treat acute bacterial sinusitis, acute exacerbation of chronic bronchitis, community acquired bacterial pneumonia, uncomplicated and complicated skin and skin structure infections, and complicated intra-abdominal infections. Recent studies demonstrate that moxifloxacin has activity against *Y. pestis in vitro* and in animal infection models. Based on PK/PD studies, Louie et al. [137] predict that a regimen of 400 mg/day of moxifloxacin could be highly effective for the treatment of plague in humans. This is the same dose that has been FDA-approved for the indications listed above. Gatifloxacin, FDA-approved as a 0.5% ophthalmic solution for the treatment of pink eye, has also been evaluated for treatment efficacy in a murine *B. anthracis* inhalation infection model. PK/PD studies suggested that a daily dose of 400 mg for adults or 10 mg/kg of body weight for children gives a 100% probability of attaining the PK/PD target [10]. Additional animal model studies are needed to corroborate the potential applications of moxifloxacin and gatifloxacin.

While many studies have been focused on the activity of fluoroquinolones against biodefense pathogens, other antibiotic classes may also be effective. Azithromycin, a macrolide antibiotic, demonstrates strain/species-specific effectiveness against *Francisella* spp [5]. Experimental data suggests linezolid, an oxazolidinone, is effective for PEP against *B. anthracis*. Survival rates of mice administered linezolid 24 hours after challenge were comparable to ciprofloxacin through 90 days of evaluation [79]. Weiss et al. [261] determined that, in a PEP model, doxycycline, ofloxacin, imipenem, and gentamicin given individually 24 hours after infection each conferred protection against *B. anthracis* infection, yet upon termination of treatment after 30 days, animals died from respiratory anthrax. The rate of mortality varied depending on the drug used. The group further tested doxycycline and ciprofloxacin as treatments administered after animals were determined to be bacteremic. Doxycycline was able to cure animals with bacteremia levels up to about $10^5$ CFU/ml, whereas ciprofloxacin could cure animals with bacteremia levels up to $9 \times 10^4$ CFU/ml. When either antibiotic was administered in combination with anti-PA antibodies, the therapy (both PEP and treatment) was more effective. This study
supports the therapeutic potential of doxycycline and ciprofloxacin (especially in combination with anti-PA antibodies) while indicating that ofloxacin, imipenem, and gentamicin are unlikely to be viable alternatives for anthrax therapy.

Daptomycin, a cyclic lipopeptide with activity against Gram-positive bacteria, was also evaluated as a therapeutic for *B. anthracis* infection [82]. Daptomycin’s mode of action involves inserting into, and disrupting, the bacterial membrane. In a murine model of *B. anthracis* infection, twice daily daptomycin given 24 hours after challenge for either 14 or 21 days produced survival rates comparable to ciprofloxacin administered using the same regimen. Culture results from tissues removed at the termination of the experiment at 43 days were negative. Additional studies in NHP models will be needed to advance this drug as a potential anthrax therapeutic.

Drugs already FDA-approved for other indications have also been tested against biodefense pathogens. Lovastatin, a statin and cholesterol lowering drug, was tested for its activity against *Y. pestis*. Although it had no *in vitro* effect on *Y. pestis*, when tested as a pre-exposure prophylaxis, lovastatin did show some protective effect. Seventy-three percent of control animals died after *Y. pestis* inoculation compared to a 20% mortality rate in the animals that had received lovastatin administered IP at a dose of 20 mg/kg/day [13]. Lovastatin is available for human use in 10, 20 or 40 mg tablets with a maximum recommended dose of 80 mg/day. Identifying possible alternative therapeutics that are already FDA-approved may accelerate approval for biodefense indications. However, it is critical that the drug levels that are administered remain at or below the FDA-approved dosing for the existing indication. If the dosing levels exceed the FDA-approved dose, additional toxicology and safety studies will be required. Further, the strategy of expanding a label of an FDA-approved drug will only be successful if there is a willingness on the part of the original innovator to serve as a drug sponsor for regulatory submission.

### 5.2.2.2 Modifications of Conventional Antibiotics

Most ongoing anti-bacterial innovation is focused on chemical modifications to conventional or precededent antibiotic classes. A representative list of compounds that fit into this category and are currently in development is presented in Table 5-2. This list is not comprehensive and is subject to continual change as drug development is discontinued or more candidates emerge from the discovery phase. This section will focus on those five compounds which have publicly available test data against biodefense pathogens: BAL30072, dalbavancin, oritavancin, cethromycin, and TR-701 (highlighted below). We will then briefly discuss select broad spectrum compounds in late stage clinical development that merit testing against biodefense pathogens.

<table>
<thead>
<tr>
<th>Class</th>
<th>Class Mechanism of Action</th>
<th>Compound Name</th>
<th>Developer</th>
<th>Stage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycoside</td>
<td>Disrupt protein synthesis; Some disrupt integrity of the bacterial cell membrane</td>
<td>ACHN-490</td>
<td>Achaogen</td>
<td>Ph II</td>
<td>[4], [191], [204], [121]</td>
</tr>
<tr>
<td>β-lactamase inhibitor</td>
<td>Inhibit β-lactamase</td>
<td>NXL 104</td>
<td>Novexel</td>
<td>Ph II</td>
<td>[41]; <a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MK-7655</td>
<td>Merck</td>
<td>Ph I</td>
<td>[35]</td>
</tr>
<tr>
<td>Class</td>
<td>Class Mechanism of Action</td>
<td>Compound Name</td>
<td>Developer</td>
<td>Stage</td>
<td>References</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------------------------------------------</td>
<td>---------------</td>
<td>--------------------------</td>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>β-lactam antibiotic</td>
<td>Inhibit cell wall synthesis</td>
<td>BAL30072</td>
<td>Basilea Pharma</td>
<td><em>In vitro</em></td>
<td>[185], [161], [211]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MC1</td>
<td>Pfizer</td>
<td><em>In vivo</em></td>
<td>[59], [118], [89], [56]</td>
</tr>
<tr>
<td>Carbapenem</td>
<td>Inhibit cell wall synthesis</td>
<td>Razupenem</td>
<td>Protez</td>
<td><em>In vivo</em></td>
<td>[120]</td>
</tr>
<tr>
<td>Cephalosporin</td>
<td>Disrupt synthesis of peptidoglycan layer within cell wall</td>
<td>CXA-101</td>
<td>Cubist</td>
<td>Ph II</td>
<td>[141], [134], [156]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TD-1792</td>
<td>Theravance</td>
<td>Ph II</td>
<td>[195], [130]; <a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a></td>
</tr>
<tr>
<td>Depsipeptides</td>
<td>Variable</td>
<td>TripropeptinC</td>
<td>Inst. Micro. Chem.</td>
<td><em>In vitro</em></td>
<td>[78], [77]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WAP-8294A2</td>
<td>aRigen Pharmaceuticals</td>
<td>Ph I</td>
<td>[76]; <a href="http://www.arigen.jp">www.arigen.jp</a></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS-2041835</td>
<td>Astellas Pharma</td>
<td><em>In vivo</em></td>
<td>[256]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ramoplanin</td>
<td>Nanotherapeutics</td>
<td>Ph III</td>
<td>[63], [247], [218]</td>
</tr>
<tr>
<td>Fluorocycline</td>
<td>Interfere with protein synthesis</td>
<td>TP-434</td>
<td>Tetraphase</td>
<td>Ph II</td>
<td>[235], [236], [92], [208], [227], [49], [159]; <a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TP-271</td>
<td>Tetraphase</td>
<td><em>In vivo</em></td>
<td>[227], [72], [49]</td>
</tr>
<tr>
<td>Glycopeptide</td>
<td>Interfere with cell wall synthesis</td>
<td>Dalbavancin</td>
<td>Pfizer</td>
<td>Ph III</td>
<td>[81], [262]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oritavancin</td>
<td>Targanta Therapeutics</td>
<td>Ph III</td>
<td>[80], [25]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT-00786</td>
<td>Bioman</td>
<td><em>In vitro</em></td>
<td>[38]</td>
</tr>
<tr>
<td>Lipopeptide</td>
<td>Variable</td>
<td>CB-183,315</td>
<td>Cubist</td>
<td>Ph II</td>
<td>[155]; <a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a></td>
</tr>
<tr>
<td>Macrolides/Ketolides</td>
<td>Inhibit protein synthesis</td>
<td>Solithromycin (CEM-101)</td>
<td>Cempra</td>
<td>Ph II</td>
<td>[158], [222], [207], [179], [57], [228]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cethromycin</td>
<td>Advanced Life Sciences</td>
<td>Ph III</td>
<td>[149], [62]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bicyclides</td>
<td>Enanta</td>
<td>Pre-clinical</td>
<td>[96], [20], [99]</td>
</tr>
<tr>
<td>Oxazolidinone</td>
<td>Interfere with protein synthesis</td>
<td>PNU-100480</td>
<td>Pfizer</td>
<td>Phase I</td>
<td>[252]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciprofloxacin Derivatives</td>
<td>Univ NB</td>
<td><em>In vitro</em></td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Radezolid</td>
<td>RibX</td>
<td>Ph II</td>
<td>[142], [126], [127]; <a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TR-701</td>
<td>Trius Therapeutics</td>
<td>Ph III</td>
<td>[152], [135], [139], [196], [28]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LCB01-0371</td>
<td>Handong Univ, LegoChem Bio</td>
<td>Pre-clinical</td>
<td>[98]</td>
</tr>
</tbody>
</table>
The β-lactam antibiotic, BAL30072, showed superior *in vitro* activity against *B. pseudomallei* when compared with ceftazidime, meropenem, or imipenem [54]. BAL30072 also has strong *in vitro* activity against Multi-Drug-Resistant (MDR) *Pseudomonas aeruginosa* and *Acinetobacter sp.* isolates [185] suggesting that it may be an effective broad spectrum agent. However, the compound is still very early in development and its suitability in animal models remains to be tested.

Dalbavancin and oritavancin are novel, semisynthetic lipoglycopeptides being developed for the treatment of complicated skin and skin structure infections. A limited body of data suggests that they may have activity against other Gram-positive species as well. Both compounds have a long half-life in humans, which supports less frequent dosing. Dalbavancin demonstrated potent *in vitro* activity against *B. anthracis* [81]. Dalbavancin was further tested in mouse models of inhalation anthrax. Delayed treatment beginning 36 or 48 hours post-challenge resulted in 70 – 100 % survival, depending upon the dose used. In a PEP model, all control mice died within four days, yet 80 – 100 % of the dalbavancin treated mice survived at all dose regimes tested. Though dalbavancin is administered intravenously, one advantage of dalbavancin over ciprofloxacin is that its long half-life in humans supports just once weekly dosing, suggesting it could have enhanced utility in a mass public health emergency.

Oritavancin causes bacterial cell death through depolarization and permeabilization of the cell membrane [19]. It accumulates significantly in macrophages and demonstrates potent *in vitro* activity against *B. anthracis*. In PEP studies, a single intravenous oritavancin dose of 5, 15, or 50 mg/kg 4 hours post-challenge with *B. anthracis* provided 40, 70, and 100 % survival, respectively after 30 days. By comparison, untreated animals died within 4 days and ciprofloxacin (administered twice a day for 14 days) treated animals had 90% survival. Oritavancin was also effective if administered after symptom development. A single intravenous dose of 50 mg/kg 42 hours post-challenge provided 56% survival. Thus, oritavancin shows great promise as a *B. anthracis* MCM.

Restanza™ (cethromycin), a novel ketolide used for once-a-day oral treatment of community acquired pneumonia, also seems to have *in vitro* activity against biodefense pathogens [62], [149]. Cethromycin has marginal *in vitro* efficacy against *B. pseudomallei* with MIC values ranging from 4 to 64 mg/L [149]. Resistance in *B. pseudomallei* has been noted, especially in clinically isolated strains. High-level resistance is likely mediated by constitutive AmrAB-OprA efflux pump overexpression. Cethromycin also exhibits *in vitro* activity

---

1. [https://clinicaltrials.gov/ct2/show/NCT00865280]
against *B. anthracis*. When Frean *et al.* [62] tested 11 anti-microbial agents on 26 isolates of *B. anthracis*, cethromycin was one of the most active compounds. These data will need to be validated in animal models.

TR-701 is the phosphate pro-drug of the anti-bacterial oxazolidinone TR-700. TR-700 is active against all clinically relevant Gram-positive and some Gram-negative pathogens [152], yet is primarily being developed for the treatment of Methicillin-Resistant *S. aureus* (MRSA) as well as complicated skin and skin structure infections. TR-700 activity extends to linezolid-resistant strains of staphylococci and enterococci; in all cases, MIC values for TR-700 were much lower than for linezolid [135]. Effectiveness of TR-701 as a PEP against *B. anthracis* infection has been compared to ciprofloxacin and levofloxacin in a mouse inhalation model [139]. After 14 days, all control mice died, yet survival rates for those administered identical oral doses of ciprofloxacin, levofloxacin, or TR-701 were 50%, 50%, and 90%, respectively. These results support further testing for TR-701 biodefense indications.

Data on the activity of the remaining compounds in Table 5-2 against relevant biodefense pathogens have not been published. However, several have shown activity against a broad spectrum of Gram-negative pathogens (including drug-resistant pathogens), demonstrate acceptable human safety and PK profiles and are currently in clinical trials for indications such as acute respiratory infections, complicated skin and skin structure infections or community acquired pneumonia. Compounds exhibiting these qualities merit testing against biodefense pathogens. These compounds include NXL-104, ACHN-490, and TP-434.

NXL 104 is a new β-lactamase inhibitor in Phase II clinical trials for use in patients suffering from urinary tract infections (Clinicaltrials.gov identifier NCT01281462 – https://clinicaltrials.gov/ct2/show/NCT01281462). *In vitro* testing demonstrated that NXL-104 is effective against a range of bacteria [162], [163], [52], [41]. On several occasions, NXL-104 was tested in combination with ceftaroline or ceftazidime. The latter compounds are susceptible to inactivation by common β-lactamases. The addition of NXL-104 as a β-lactamase inhibitor protects the cephalosporins from inactivation and lowers the resulting MIC values, thus expanding their spectrum of activity [41], [162], [163].

ACHN-490, a sisomicin analog, neoglycoside/next generation aminoglycoside, is being developed to combat drug-resistant bacteria in a health care setting. ACHN-490 is effective against *P. aeruginosa* and acted synergistically with four other antibiotics against that same pathogen [191]. In separate studies, ACHN-490 was effective against *Enterobacteriaceae* family members as well as *P. aeruginosa* and *S. aureus* in mouse models of infection and against clinical isolates of *A. baumannii* [204], [121]. ACHN-490 inhibited the growth of aminoglycoside-resistant *Enterobacteriaceae* and was also shown to be effective against aminoglycoside-resistant staphylococci [4]. Overall, these results suggest that ACHN-490 could be a useful broad spectrum antibiotic, and it is currently in a Phase II clinical trial to determine efficacy against complicated urinary tract infections (Clinicaltrials.gov identifier NCT01096849 – https://clinicaltrials.gov/ct2/show/NCT01096849). Achaogen holds a contract with the Biomedical Advanced Research and Development Authority to develop this compound as a therapeutic for biodefense agents (Contract # HHSO100201000046C).

TP-434 is a synthetic tetracycline that inhibits protein synthesis. TP-434 has demonstrated *in vivo* activity against MRSA, *S. pneumonia*, *S. pyogenes* and multi-drug-resistant Gram-negative bacteria. Phase II assessments of the ability of TP-434 to treat community-acquired complicated intra-abdominal infections (Clinicaltrials.gov identifier NCT01265784 – https://clinicaltrials.gov/ct2/show/NCT01265784) as well as further studies on the oral formulation of TP-434 are planned. So far, the data indicate that this compound could have efficacy against a broad spectrum of pathogens.
### 5.2.2.3 Novel Anti-Bacterial and Anti-Virulence Chemistries and Targets

The need for novel anti-microbials that have unique mechanisms of killing bacteria or preventing infection has been widely agreed upon. As such, numerous efforts are under way to identify and/or design drugs targeting critical bacterial pathways with new chemistries and modes of action. A representative list of novel compounds currently in development can be found in Table 5-3. The focus of this section will be on the sub-set of compounds under development that have been tested against biodefense pathogens. In addition, we will discuss several bacterial pathways or proteins that are being targeted and could be exploited while developing drugs for biodefense use.

**Table 5-3: Representative List of Novel Anti-Bacterial and Anti-Virulence Compounds in Development.**

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Developer</th>
<th>Stage</th>
<th>Notes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3SS inhibitors</td>
<td>Univ. of MA</td>
<td><em>In vitro</em></td>
<td>Targets T3SS</td>
<td>[186]</td>
</tr>
<tr>
<td>LcrF inhibitors</td>
<td>Paratek Pharma.</td>
<td><em>In vivo</em></td>
<td>Targets T3SS (vs. <em>Y. pseudotuberulosis</em>)</td>
<td>[109], [64]</td>
</tr>
<tr>
<td>Salicylidene Acylhydrazides</td>
<td>Kaolinska Inst.</td>
<td><em>In vitro</em></td>
<td>Targets T3SS (vs. <em>Salmonella enterica</em>)</td>
<td>[167]</td>
</tr>
<tr>
<td>Salicylaldehyde Acylated Hydrazones</td>
<td>Umea U. &amp; Innate Pharmaceuticals, Sweden; Institute Pasteur</td>
<td><em>In vitro</em></td>
<td>Targets T3SS (vs. <em>Y. pseudotuberculosis</em> and <em>Chlamydia spp.</em> )</td>
<td>[172], [160]</td>
</tr>
<tr>
<td>LED209</td>
<td>U. Texas</td>
<td><em>In vivo</em></td>
<td>inhibits QseC- activation of virulence gene expression (vs. <em>F. tularensis</em> and others)</td>
<td>[202]</td>
</tr>
<tr>
<td>Oligo G</td>
<td>Algipharma</td>
<td>Ph I</td>
<td>Alginate sub-unit; disrupts biofilms</td>
<td>[108]; NCT00970346</td>
</tr>
<tr>
<td>NVC-422</td>
<td>NovaBay Pharma</td>
<td>Ph II</td>
<td>Derivative of a natural anti-microbial; Inhibits biofilm formation</td>
<td>[201]; NCT00781339; NCT01243125</td>
</tr>
<tr>
<td>Bis-indole analogs</td>
<td>Microbiotix</td>
<td><em>In vitro</em></td>
<td>Some had activity against <em>B. anthracis</em></td>
<td>[33], [170], [188]</td>
</tr>
<tr>
<td>Pyridazinone and Pyrazolethione analogs</td>
<td>UCLA</td>
<td><em>In vitro</em></td>
<td>Sortase A inhibitor</td>
<td>[234]</td>
</tr>
<tr>
<td>RNPA1000</td>
<td>Univ. Rochester</td>
<td><em>In vitro</em></td>
<td>Inhibits enzyme involved in rRNA and mRNA degradation</td>
<td>[180]</td>
</tr>
<tr>
<td>Pyocins</td>
<td>AvidBiotics</td>
<td><em>In vitro</em></td>
<td>Narrow spectrum anti-microbial</td>
<td>[264]</td>
</tr>
<tr>
<td>AVS group</td>
<td>Hanyang Univ.</td>
<td><em>In vitro</em></td>
<td><em>B. anthracis</em> AHAS inhibitor</td>
<td>[65]</td>
</tr>
<tr>
<td>NI01</td>
<td>U. Manchester; Wigan and Leigh</td>
<td><em>In vitro</em></td>
<td>Membrane depolarizing agent; Prevents biofilm formation</td>
<td>[212]</td>
</tr>
<tr>
<td>Compound Name</td>
<td>Developer</td>
<td>Stage</td>
<td>Notes</td>
<td>References</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>-------</td>
<td>-------</td>
<td>------------</td>
</tr>
<tr>
<td>NI02</td>
<td>U. Manchester; Wigan and Leigh</td>
<td><em>In vitro</em></td>
<td>Mechanism unknown</td>
<td>[213]</td>
</tr>
<tr>
<td>NAP and other chemistries</td>
<td>U. of Guelph; U. of Toronto; Carlsberg Laboratory, Denmark</td>
<td><em>In vitro</em></td>
<td>Mono-ADP-Ribosyltransferase Toxins (<em>P. aeruginosa</em> and <em>V. cholerae</em>)</td>
<td>[244]</td>
</tr>
<tr>
<td>Coelomycin</td>
<td>Merck</td>
<td><em>In vitro</em></td>
<td>Fungal metabolite – highly subst. 2,6-dioxo-pyrazine (vs. <em>S. aureus</em>)</td>
<td>[69]</td>
</tr>
<tr>
<td>Aurodox</td>
<td>Kitasato U, Japan</td>
<td><em>In vivo</em></td>
<td>linear polyketide compound (vs. <em>Citrobacter rodentium</em>)</td>
<td>[110]</td>
</tr>
<tr>
<td>CHIR-090</td>
<td>Novartis</td>
<td><em>In vitro</em></td>
<td>LpxC inhibitor</td>
<td>[268], [39], [22]</td>
</tr>
<tr>
<td>NVB302</td>
<td>Univ of Leeds; Novacta Therapeutics</td>
<td>Ph I</td>
<td>Type B lantibiotic</td>
<td>[101], [93]; <a href="http://www.novactabio.com">www.novactabio.com</a></td>
</tr>
<tr>
<td>Walkmycins</td>
<td>Inst Micro Chem; Kinki Univ.</td>
<td><em>In vitro</em></td>
<td>Inhibitors of histidine kinase involved in sensing signals from the two-component signal transduction system</td>
<td>[101], [178], [50], [94]</td>
</tr>
<tr>
<td>AFN-1252</td>
<td>Affinium</td>
<td>Ph I</td>
<td>Fab I inhibitor</td>
<td>[260], [106], <a href="http://www.afnm.com">www.afnm.com</a>; [105]</td>
</tr>
<tr>
<td>MUT056399</td>
<td>FAB Pharm; Mutabilis</td>
<td>Ph I</td>
<td>Fab I inhibitor</td>
<td>[58], [226], [29]</td>
</tr>
<tr>
<td>SBPT04</td>
<td>Colorado State Univ.</td>
<td><em>In vivo</em></td>
<td>FabI inhibitor</td>
<td>[53]</td>
</tr>
<tr>
<td>GSK1322322</td>
<td>GSK</td>
<td>Ph II</td>
<td>Peptide deformylase Inhibitor</td>
<td>[131], [132], [24], [164], [199]</td>
</tr>
<tr>
<td>Phenylthiazolylurea-sulfonamides</td>
<td>Bayer</td>
<td><em>In vivo</em></td>
<td>Phenylalanyl t-RNA synthetase inhibitor (vs. <em>S. aureus</em> and <em>S. pneumoniae</em>)</td>
<td>[21]</td>
</tr>
<tr>
<td>GSK299423</td>
<td>GSK</td>
<td>Pre-clinical</td>
<td>Topoisomerase inhibitor</td>
<td>[18]</td>
</tr>
</tbody>
</table>

*Y. pestis* uses the plasmid-encoded Type III Secretion System (T3SS) to overcome host defences. This system injects a set of proteins known as Yops into the cytosol of host cells that come in contact with the bacteria. This system is critical to virulence, and is broadly employed by Gram-negative bacterial pathogens. A screen for small molecules that inhibit Type III secretion in *Y. pestis* yielded a number of prospective inhibitors, eight of which were selected for further study [186]. Four of the eight showed promise as leads for future structure-activity relationship studies. All had chemical structures distinct from each other and from previously described Type III secretion inhibitors.
In a more targeted approach, a series of N-hydroxybenzimidazoles were synthesized and tested to determine if they act as inhibitors of LcrF, a multiple adaptational response transcriptional factor, which regulates expression of the Type III secretion system. While some were capable of inhibiting LcrF-DNA binding, none showed \textit{in vitro} anti-bacterial activity against \textit{Y. pseudotuberculosis}, \textit{S. aureus}, or \textit{E. coli}, indicating that they do not target bacterial cell growth [109]. The developers of these compounds published results suggesting that while not anti-bacterial, the compounds can disrupt virulence [64]. Mice given either of two compounds 18 hours prior to \textit{Y. pseudotuberculosis} infection and at four additional time points after infection had a 75% survival rate in a single experiment. The compounds did not completely eliminate virulence – the LcrF deletion mutant was completely non-lethal. The partial effectiveness at protection, and the requirement for pre- and multiple post-infection dosing suggest that anti-virulence treatments alone may not be suitable therapeutics, but may be useful in combination with other therapies to enhance protection. These compounds are still very early in development and will need optimization and further \textit{in vivo} testing. If, after optimization, they are still only useful as combination therapies, one will need to question whether further pursuit of this approach is worthwhile.

Historically, most clinically relevant anti-microbials target DNA, RNA, protein and glycans of the cell wall. Currently, however, an alternative target for novel anti-bacterial drug discovery is the Type II Fatty Acid biosynthesis (FASII) pathway. In particular, FabI, the FASII enoyl-ACP reductase, was identified as a possible broad-spectrum target for chemotherapeutic intervention [53]. SBPT04, an inhibitor of the enoyl-ACP reductase enzyme ftaFabI, was assessed for its efficacy against \textit{F. tularensis} infection in a mouse model. SBPT04 delivered intraperitoneally cleared infection by day four of treatment; when delivered orally, it delayed dissemination [53]. No evidence of disease relapse was noted. SBPT04 treatment resulted in an overall reduction in the gene transcripts encoding products involved in cell wall and lipid synthesis, intermediary metabolism and respiration, and RNA and protein synthesis [53]. The use of FabI as a drug target has run into technical challenges, however. Since the enoyl-ACP reductase function is performed in several pathogens by enzymes unrelated to FabI [27], this target is only suitable for distinct pathogens. Moreover, studies revealed that low-GC Gram-positive pathogens are resistant to fatty acid biosynthesis inhibitors \textit{in vitro} when their culture broth is supplemented with exogenous fatty acids in a concentration that would be present in human serum [27], [26]. Therefore, although the compound works well \textit{in vitro}, there is data to suggest the contrary in the context of an infected host. FabI inhibitors may have limited potential as therapeutics.

Three enzymes have recently been the targets of biodefense anti-microbial drug development: Sortase A, Acetohydroxyacid Synthase (AHAS), and LpxC. The Sortase A enzyme of \textit{S. aureus} and \textit{B. anthracis} helps attach surface proteins to the cell wall that play a key role in the infection process [234]. During a HTS, Suree \textit{et al.} [234] identified several pyridazinone and pyrazolethione analogs that inhibit Sortase A in \textit{S. aureus}. Interestingly, these compounds were able to reduce Sortase A activity in \textit{B. anthracis} as well, suggesting broad-spectrum Sortase A inhibition. AHAS, which catalyzes the first common step in branched-chain amino acid synthesis, is the second enzyme of interest. Screening of a chemical library for inhibitors of \textit{B. anthracis} AHAS resulted in four chemicals: AVS2087, AVS2093, AVS2387, AVS2236 [65]. Similarly, the LpxC enzyme has been investigated as anti-microbial target. LpxC is a deacetylase involved in lipopolysaccharide biosynthesis. CHIR-090 tightly binds to LpxC derived from several bacterial species including \textit{Yersinia enterocolitica} (which has an ortholog in \textit{Y. pestis}) and has demonstrated promising \textit{in vitro} activity against a range of pathogens [39], [146], [14], [22], [15]. While the Sortase A, AHAS, and LpxC inhibitors are still very early in development, this research does highlight three alternative drug targets.

RNA processing and degradation are required for bacterial survival and thus provide potential drug targets. RNPA1000, a small molecule inhibitor of RnpA (an enzyme which catalyzes rRNA and mRNA degradation) exhibited \textit{in vitro} anti-microbial activity against MRSA, vancomycin intermediate susceptible \textit{S. aureus}, vancomycin resistant \textit{S. aureus}, biofilm-associated \textit{S. aureus} and other Gram-positive bacterial pathogens [180].
RNPA1000 was able to ameliorate disease in a mouse *S. aureus* infection model [180]. The compound does not affect other drugs used to treat MRSA infections, including vancomycin, daptomycin, or rifampicin; however, it does potentiate oxacillin, making it more potent. Unfortunately, RNPA1000 is somewhat toxic to human cells in *in vitro* assays. Nonetheless, it may be possible to use RNPA1000 as a starting point to synthesize analogs with similar function, but less toxicity.

Peptide Deformylase Inhibitors (PDIs) are another class of anti-bacterials that has garnered interest recently [102], [97], [34], [73]. The enzyme is encoded by the *def* gene which is found in all pathogenic bacteria [73]. The number of PDIs that have undergone early development is too numerous to mention here, but at least three have entered clinical trials: LBM415, BB-83698, and GSK1322322. Development of the first two was terminated shortly after Phase I trials. Development of GSK1322322 appears to be continuing and a Phase II study for the treatment of acute bacterial skin and skin structure infections was recently completed (Clinicaltrials.gov identifier NCT01209078 – [https://clinicaltrials.gov/ct2/show/NCT01209078](https://clinicaltrials.gov/ct2/show/NCT01209078)). Unfortunately, there are drawbacks to PDIs including *in vitro* activity limited to Gram-positive anaerobes, bacteriostatic activity, and relatively high resistance frequency [73]. Thus, it is not surprising that development of these compounds has had significant technical challenges.

In addition to GSK1322322, GlaxoSmithKline (GSK) has two other anti-microbials in the pipeline which have unique chemistries. GSK2251052, a boron-based inhibitor of leucyl t-RNA synthetase with a reported broad range of activity against Gram-negative pathogens has entered Phase I safety trials (Clinicaltrials.gov identifier NCT01262885 – [https://clinicaltrials.gov/ct2/show/NCT01262885](https://clinicaltrials.gov/ct2/show/NCT01262885)). The second is a Novel Bacterial Topoisomerase Inhibitor (NBTI) that is structurally and mechanistically distinct from fluoroquinolones, GSK299423. Crystal structures reveal that the compound forms a complex with DNA gyrase and DNA; it appears to stabilize a pre-cleavage enzyme-DNA complex and inhibit DNA strand separation [18]. As a result, double-stranded DNA cleavage, as well as a subsequent enzymatic conformational change, are both inhibited. GSK299423 was approximately 70 times more potent against *S. aureus* DNA gyrase than reported for NXL101, an NBTI class compound that progressed to human clinical trials [18]. It was also over 2,000 times more potent than ciprofloxacin for inhibition of *S. aureus* DNA gyrase supercoiling. GSK299423 had anti-bacterial activity against both Gram-negative and Gram-positive bacteria, including clinical isolates with fluoroquinolone resistance mediated by DNA gyrase and topo IV mutations. To date, there are no reports of testing the two GSK compounds on biodefense pathogens.

Currently, there is considerable interest in inhibitors of biofilm formation. One way to inhibit biofilm formation is by disrupting quorum sensing. Bacteria use this process to regulate gene expression in response to local cell population densities by sensing the concentrations of chemicals known as autoinducers. A wide range of bacterial gene expression is controlled by quorum sensing including symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation [263], [12], [36]. Quorum signals can also interfere with the host immune response [221].

Several laboratories have identified candidate compounds capable of inhibiting quorum sensing *in vitro* [157], [253], [248], [84], [140], [171], [111], [75], [231], [138]. These compounds represent different chemical families yet, to date, only one, LED-290, has been tested in animal model of biodefense pathogen infection to confirm the *in vitro* activities observed. Rasko *et al.*, [202] tested the effects of LED-290 on *F. tularensis* infection. Three hours after *F. tularensis* infection, mice were orally administered one dose of LED-290. Eighty percent of the LED-290 treated mice lived for nine days, yet only 10% of the untreated mice survived this long. *In vitro* experiments showed that LED-290 decreased the expression of *F. tularensis* virulence genes and was able to significantly reduce the number of *F. tularensis* recovered from macrophages [202]. While this approach does not eliminate infection, its utility may lie in enabling the host immune system and/or anti-bacterial therapy to
take effect while pathogenesis is disrupted. This could reduce morbidity and mortality. Quorum sensing inhibitors are currently a very active area of research (e.g. [37], [271], [116], [100]). It is important to note that quorum sensing inhibitors will not kill bacteria, thereby possessing the added benefit that they significantly reduce the risk of provoking bacterial resistance through mutations and selective pressure, and reduce impact on helpful microbiota such as gut microflora.

Another class of alternative anti-bacterials is efflux pump inhibitors. Efflux pumps are membrane proteins which transport toxins, including antibiotics, from within the bacterial cell to the outside environment and have been associated with multi-drug resistance. Two methods have been pursued by researchers in an attempt to overcome the effect of efflux pumps – the first is to develop efflux pump inhibitors that would be administered in conjunction with antibiotics; the second is to keep the role of efflux pumps in mind as new antibiotics, or analogs of existing antibiotics, are designed [259]. Structural modifications to the antibiotic that reduce the ability of the pump to extrude it from the cell, yet do not compromise its activity should be considered [259].

Additional unique compounds active against biodefense pathogens are being designed or identified through small molecule screens; pyocins and indoles are two examples. Pyocins resemble bacteriophage tail structures. They are naturally made by some P. aeruginosa strains, and possess bactericidal activity against other strains of the same species [264]. The bacterial specificity of a pyocin is conferred by its tail fiber. By altering the tail fibers of P. aeruginosa pyocins and replacing them with tail fibers from bacteriophage that infect either E. coli or Y. pestis, Williams et al., [264] created pyocins which have an altered killing profile more specific to the species that from which the tail was derived. Although pyocins would have very narrow applications, this approach may allow for targeting of a specific biodefense pathogen of interest. The relatively large, proteinacious nature of these molecules may preclude them from having suitable pharmacological properties. Finally, Panchal et al. [188] have identified four bis-(imidazolinylindole) compounds with in vitro activity against biodefense pathogens as measured by the protection of macrophages and by inhibition of bacterial growth in vitro. These four compounds are designated MBX 1066, MBX 1090, MBX 1113, and MBX 1128. In vivo studies in mouse models revealed protection by MBX 1066 and MBX 1090 against infection with B. anthracis, Y. pestis, or S. aureus. During all B. anthracis and Y. pestis therapeutic studies, the MBX compounds were delivered by intraperitoneal injection; additional studies will be required to determine if the products are effective when delivered as oral or IV formulations, as would likely be required in a public health emergency.

Molecular modeling approaches are also used in combination with genomic analysis to suggest prospective new targets for anti-bacterial drugs. Availability of genome sequences and functional analyses of essential genes in bacterial genomes facilitates the search for targets. The lysine biosynthesis pathway in B. anthracis was selected as a potential target for anti-bacterial intervention using genomic analysis and structural prediction of the active site of a key enzyme, dihydrodipicolinate synthase [249]. This particular target has not yet had a novel anti-bacterial drug created to work against it. However, the capability to predict targets, discern their molecular structure, and then pursue structure-based design of inhibitors, offers a new tool that can complement existing approaches to drug candidate identification.

5.2.3 Anti-Toxins

5.2.3.1 Botulinum Neurotoxin

Botulinum Neurotoxin (BoNT) is one of the most deadly substances in nature; only nanogram amounts of toxin are required for lethal intoxication. The seven serotypes of BoNT (A-G) differ significantly at the amino acid level, making the identification of broadly effective therapy for all serotypes technically challenging. Current
treatments include a heptavalent equine anti-toxin and BabyBIG®, a human polyclonal antibody product for the treatment of infant botulism [11]. With U.S. government sponsorship, significant effort has been put into developing effective next generation MCMs against BoNT, much of which has been focused on the development of small molecules capable of inhibiting intoxication (reviewed by Larsen [124]). The developmental goal of these MCMs is to provide greater administration flexibility in a mass casualty scenario.

There are, however, significant concerns regarding the technical feasibility of a small molecule therapy for botulism. These include the long half-life and retention of BoNT inside neuronal cells, coupled with the limited in vivo activity reported to date for many of these compounds. Much of the recent work has focused on the development of small molecules which inhibit the catalytic activity of the toxin [133], [273], [30], [31], [32], [23], [117], [55]. The large enzyme:substrate interface, coupled with the reported flexibility in the active site of the enzyme, has made the design of effective candidate small molecule inhibitors technically challenging [119], [274], [223], [224]. Despite this, extensive structural and bioinformatic modeling, coupled with strong medicinal chemistry campaigns have generated several promising leads. Many of these show strong inhibition of BoNT in in vitro assays with Ki in the low micromolar to high nanomolar range. To date, however, few compounds have advanced to evaluation in in vivo models.

Of particular note are compounds described by Pang et al. [190]. Using a computer-aided design and optimization scheme, BoNT inhibitors were reported with approximately 700 – 800 nM Ki. One particular inhibitor possessed a half-life of 6.5 hours and did not possess toxicity in the preliminary experiments conducted. In murine challenge experiments, the compound provided 100% and 70% protection when administered prior to challenge at periods equivalent to two and four half-lives of the drug, respectively. This is in comparison to 40% and 0% survival among untreated mice. Given their in vivo activity, these compounds are some of the most potent BoNT inhibitors that have been reported to date. In order to offer significant therapeutic utility, it will be imperative to determine if these compounds provide protection against intoxication when administered at times following signs of intoxication.

Hydroximate-based metalloprotease inhibitors of BoNT have been developed and evaluated for their ability to inhibit BoNT activity in in vitro, ex vivo, and in vivo assays [238]. These compounds possess low micromolar Ki for the inhibition of BoNT/A. Treatment of nerve muscle preparations with BoNT/A diminished electrophysiological parameters associated with normal neurotransmission, such as quantal content and nerve evoked muscle twitches. The candidate inhibitors were capable of reversing these effects, partially restoring muscle twitches following a 40 – 90 minute delay. However, when these compounds were evaluated in a mouse hind limb model of localized paralysis, they provided no evident reversal of paralysis. These data suggest that while the in vitro and ex vivo assays for the evaluation of BoNT inhibitors are useful experimental tools to screen for candidate compounds, recapitulation of results from these assays to in vivo systems is not assured.

5.2.3.2 Ricin

While not derived from a microbial pathogen, ricin (from castor beans) has the potential to be counteracted with therapeutics as do microbial toxins. Cell-based HTS assays have been used to identify candidate ricin inhibitors [250]. Four compounds were identified which possessed dose dependent inhibitory activity with effective concentrations in the low micromolar range. Structural modeling indicated that candidate inhibitors targeted the active site of the ricin A-chain. Although significant chemical modification of these compounds is needed prior to any successful determination of in vivo efficacy, these data suggest that cellular screens for ricin inhibitors can yield hits that possess inhibitory activity.
5.2.3.3  *B. anthracis* Toxins

*B. anthracis* Lethal Factor (LF) and Edema Factor (EF) are toxins critical to the pathology of inhalational anthrax. Most MCMs under development focus on antibody-based neutralization of toxin action, yet there has been little attention paid to the development of small molecule inhibitors of anthrax toxins. Such compounds include positively charged β-cyclodextrin derivatives which are capable of blocking the channel formed by the Protective Antigen (PA) *in vitro*, and protecting LF mediated cell death in macrophages [104]. These compounds were also evaluated in *in vivo* models of *B. anthracis* challenge [151]. In rats, complete protection was observed when the compounds were administered prior to, or at the time of, challenge. In a murine infection model, one compound provided significant protection alone and in combination with ciprofloxacin. However, these studies were conducted with the attenuated Sterne strain of *B. anthracis* and compound administration was initiated at day one following challenge, leaving their potential PEP or treatment efficacy as yet undetermined.

Soluble receptor decoys have been developed as a means to prevent toxin entry into cells. The ANTXR2 domain of the *B. anthracis* toxin receptor was assessed as a recombinant protein for its ability to prevent intoxication by wild-type LF+PA and mutant forms of PA that are resistant to monoclonal antibody neutralization [219]. The recombinant proteins were able to protect mouse macrophages from cell death in a dose dependent manner and protected 100% of rats from lethal toxin challenge when co-administered with toxin. Complete protection of rats was also observed when challenged with an antibody-resistant form of the toxin. The utility of these constructs at time points following *B. anthracis* challenge was not assessed, leaving therapeutic utility uncertain.

While significant effort has been put into the identification and characterization of pathogen-targeted small molecule therapeutics, most of the compounds are in very early stages of development for biological threat agent indications. The repertoire of existing anti-bacterials represents the class of drugs with many instances of efficacy established for non-biodefense pathogens, but often physiologically relevant disease states. This encourages anticipation that their efficacy may extrapolate to biodefense indications, which has in some fortunate cases been borne out in clinical practice of off-label use. For anti-viral and anti-toxin compounds, the ability to evaluate efficacy in other model systems is often limited. As a result, the developmental pipeline in these areas is overall less mature. Significant investment in a coordinated drug development campaign for these agents will be required to successfully advance candidates in the development pipeline. Many tantalizing new approaches to develop pathogen-targeted small molecule therapeutics report *in vitro* and even *in vivo* antimicrobial effects, but these results are frequently not rigorously tuned to the end requirements for a successful drug. Some experiments are conducted to show efficacy relying on a pre-dosing or coincident dosing of the candidate therapeutic with the infectious agent, which will not transfer to a PEP or treatment use. Others encounter problems ubiquitous in drug development where pharmacological properties of candidate compounds are not amenable for drug development – toxicity, poor bioavailability, compound metabolism in the host, etc.

5.3 HOST-TARGETED SMALL MOLECULE THERAPEUTICS

The host remains largely unexploited as a target for the interdiction of infectious diseases. This is due to a historical emphasis on drugs which attack aspects of the bacterial or viral life cycle. Given the low propensity for resistance that host targets would possess, coupled with a decreased likelihood of disrupting helpful microbiota, drugs which target the host may represent a class of therapeutics with increased tolerability. Challenges associated with the development of this class of therapeutics are not trivial, however. Inhibition of a cellular target may impact its normal function, with a resultant reduction in tolerability. Further, approval for a product of this class may be difficult under the FDA Animal Rule as the structure of the target may not be conserved in the animal species used to study efficacy against infection and support regulatory approval. In this section, recent advances in host-targeted therapeutics for biodefense agents are highlighted for viral pathogens and toxins, which typically derive from the virulence arsenal of bacterial pathogens.
5.3.1 Viral Therapeutics

Poxviruses are able to evade IFN-γ with the help of the B8R protein, a homolog of the extracellular domain of the IFN-γ receptor. B8R binds to IFN-γ and prevents its interaction with the receptor. A peptide mimetic of IFN-γ, called IFN-γ95-132, was able to divert the decoy viral protein B8R and inhibit vaccinia virus replication in vitro [7], presumably by preventing depletion of IFN-γ important for innate immune response. Intraperitoneal administration of the peptide mimetic completely protected mice challenged intra-nasally with a lethal dose of vaccinia when administered before and up to two days post-infection. When treatment was initiated six days post-infection, the peptide mimetic conferred 40% protection. The peptide mimetic was also effective in protecting mice against an intra-nasal, lethal challenge of the virus when administered orally on days -2, -1 and 0 prior to infection. The peptide mimetic also possessed adjuvant properties by boosting the adaptive response in vaccinated mice. It will next be important to study the effect of the peptide mimetic in NHPs.

Structural mimetics of cellular modulators have also been studied for anti-poxviral activity. A structural mimic of Suppressor Of Cytokine Signaling 1 (SOCS-1) protein was evaluated for its ability to protect against lethal vaccinia virus infection in mice [6]. Intraperitoneal administration of SOCS-1 mimetics lipo-KIR and lipo-Tkip provided complete protection when administered before, and at the time of, a lethal intranasal challenge of vaccinia virus and 80% protection when administered one day post-infection. Lipo-Tkip was protective against lethal intranasal vaccinia challenge even when administered orally.

Host proteins that are involved in Ebola Virus (EBOV) capsid assembly have been targeted using a cell-free-based screening system [61]. A small molecule library screened using this system identified hits with EC50 in the sub-micromolar range and high selectivity. Mice treated with one of the compounds, PAV-667, were completely protected from a lethal challenge of EBOV when PAV-667 was administered once daily for days 1 – 3 pre-infection in a 14 day study. The potential utility of this compound as a therapy will be determined in part by whether this compound has any efficacy when administered post-infection.

A novel anti-viral compound, FGI-106 was identified in a cell-based HTS for inhibitors of EBOV replication and was subsequently assessed for its ability to inhibit viral infection in mouse models. FGI-106 was also tested in cell-based assays against other virus families and was found to possess inhibitory activity against a wide range of viruses including dengue, Rift Valley fever, hepatitis C virus, and HIV [9]. Given the broad spectrum anti-viral activity that was observed, the authors speculate that FGI-106 targets a cellular pathway. Against a mouse-adapted strain of EBOV, the compound provided significant protection when given pre-exposure as well as 24 hours post-exposure. A single dose of FGI-101 administered 24 hours post-exposure provided 90% protection from lethal EBOV challenge in mice. Further, administration of FGI-106 reduced viral load in the kidney, liver, and spleen. Additional studies will be needed to determine if FGI-106 is effective if administered greater than 24 hours post-exposure, as would likely be required in a public health emergency.

Therapies targeting host inflammatory and coagulation response pathways resulting in the pathology of filovirus infection are also being examined. Recombinant Nematode Anti-coagulant Protein (rNAPc2) was evaluated for filovirus infection PEP efficacy in Guinea pigs [68]. When administered 10 minutes or 24 hours post-infection, rNAPc2 significantly increased survival time and rate by 33%. Reductions in markers for the coagulation cascade and pro-inflammatory response were also observed, suggesting that the administration of rNAPc2 could provide additional supportive care to patients beyond therapies targeting viral replication. Unfortunately, rNAPc2 has not been tested at periods beyond 24 hours post-infection.

Drugs targeting cellular factors have been examined for their utility in attenuating EBOV replication. Heat shock protein 90 (Hsp90), a molecular chaperone, has been identified as an important factor in the replication of several negative stranded enveloped viruses [40]. Smith et al. [225] evaluated seven Hsp90 inhibitors for their
ability to reduce EBOV replication. In the initial screen, geldanamycin, 17-AAG (a geldanamycin derivative), radicicol (a natural product), and four inhibitors from the benzamide class (AV-81 and its three derivatives AV-1, AV-2 and AV3) were evaluated for their ability to inhibit EBOV replication. These compounds significantly inhibited viral replication and demonstrated varying EC₅₀ values in the sub to low micromolar range. No drug-related toxicities were reported up to drug concentrations of 50 µM.

5.3.2 Toxin Therapeutics

Given the cellular persistence of BoNT, an examination of therapies which would interdict the intracellular establishment and persistence of intoxication has been pursued. Clinical observations and in vivo animal studies have brought to light significant differences in the duration of paralysis elicited by two of the toxin serotypes, with BoNT/A-induced paralysis averaging months in duration and paralysis induced by BoNT/E lasting days to weeks [114], [216]. These observations have been confirmed in in vitro studies of BoNT persistence, with cells requiring 11 weeks to recover from BoNT/A intoxication and 3 weeks to recover from intoxication with BoNT/E [60], [107]. This difference has been attributed to differences in BoNT Lc ubiquitination [183], [182]. Tsai et al. [243] demonstrated that the basis of the differential ubiquitination of the BoNT Lc was mediated by an association with TRAF2, a RING finger protein. Using chimeric forms of SNAP-25 containing ubiquitin ligase domains, BoNT/A was selectively targeted for degradation in cellular models of toxin persistence [243]. However, the mechanism of BoNT/A resistance to cellular degradation remains poorly understood. It may be possible that enzymes involved in the ubiquitination or deubiquitination could be selectively targeted as a means to treat BoNT intoxication. While chimeric SNAP-25:ubiquitin ligase constructs provide proof-of-principle in vitro data, there will be significant technical challenges in advancing these molecules to in vivo analysis due to issues with intraneuronal delivery.

Other candidate BoNT therapies aim to alleviate the symptoms of intoxication through mechanisms independent of direct host molecule interactions with the toxin. 3,4-Diaminopyridine (DAP) stimulates neurotransmitter efflux and has demonstrated the ability to restore neurotransmission in in vitro and ex vivo studies [2], [3]. 3,4-DAP is able to cross the blood-brain barrier and one drawback is the compound’s significant neurotoxicity. A medicinal chemistry campaign was undertaken to develop analogs of 3,4-DAP with improved efficacy and pharmacological properties [144]. Two aminopyridine analogs possessed similar potencies to 3,4-DAP in in vitro and ex vivo studies. One of these analogs did not readily penetrate the blood brain barrier and possessed efficacy equivalent to 3,4-DAP in rescuing BoNT intoxicated mice in the mouse lethality assay. These results suggest that aminopyridine derivatives may represent an alternative candidate therapeutic for the amelioration of the symptoms of BoNT intoxication.

To date, many of the screens conducted for small molecule inhibitors of BoNT intoxication were conducted using purified forms of the toxin light chain and were conducted ex vivo, outside of the context of the cell. This screening strategy fails to eliminate compounds with poor solubility or cellular toxicity and overlooks compounds which could target cellular proteins involved in the process of intoxication or intraneuronal persistence. Cell-based screens are being developed to identify compounds which may inhibit cellular processes related to the establishment and maintenance of BoNT intoxication [175]. Nuss et al. [176] has developed cell-based screening assays using antibodies that can differentially detect cleaved versus intact SNAP-25. These assays possess excellent sensitivity and reproducibility and appear amenable to adaptation to HTS platforms, providing new methods to identify candidate small molecule inhibitors of BoNT intoxication.

The development of small molecule inhibitors of ricin has been the focus of a modest research and development effort. Small molecules have been reported which are capable of inhibiting the retrograde transport of the toxin at the early Golgi apparatus interface [230]. These molecules protected 100% of mice from intranasal ricin
challenge when administered one hour prior to challenge at doses of 200 mg/kg. It is clear that further optimization of candidate compounds will be required to enhance potency and reduce the efficacious dose to pharmacologically acceptable levels. Further, the utility of ricin translocation inhibitors in a mass public health emergency is limited, as the technologies used to detect exposure are currently limited. Intoxication of exposure victims would likely be well underway before diagnosis, and past the point where inhibiting translocation of toxin into cells would be a viable intervention.

Staphylococcal Enterotoxin B (SEB) induces the promiscuous activation of T-cells by cross-linking the T-cell receptor with the major histocompatibility complex II molecule on antigen presenting cells [143], [232]. This results in the activation of a number of cell signaling pathways and the secretion of pro-inflammatory cytokines, culminating in the hyperinflammatory pathology of intoxication. SEB has been shown to induce signaling through MyD88, a protein induced by a number of pro-inflammatory cytokines that serves as a convergence point in cell signaling pathways [112]. Low molecular weight mimetics have been generated that inhibit the protein:protein interaction of the Toll-like receptor and MyD88. These mimetics demonstrated a significant attenuation of IL-1B production, suggesting that such an approach could have utility as an anti-inflammatory drug and candidate therapy for SEB intoxication [16]. It will be necessary to determine if these mimetics or others that inhibit more convergent points in the cell signaling pathway (e.g. MyD88 dimerization) are able to provide in vivo efficacy against SEB intoxication.

FDA-approved drugs have also been evaluated for their ability to prevent SEB-induced pathology and disease [115]. Rapamycin, which is FDA-approved for the prevention of graft rejection in renal transplant patients, was tested for its ability to protect against SEB challenge in a murine model due to its substantial immunosuppressive effects. Rapamycin decreased SEB-induced secretion of TNF-α, IL-1β, IL-6, IL-2, and IFN-γ from human Peripheral Blood Mononuclear Cells (PBMCs) and inhibited SEB-induced T-cell proliferation. Administration of rapamycin up to 5 hours following exposure to SEB completely protected mice from a lethal SEB challenge. When administration of the drug was delayed 24 hours post-exposure, 100% of mice survived challenge if intraperitoneal doses were administered every 12 – 24 hours up to the 96 hour time point. At 32 hours post-challenge, however, administration of rapamycin only protected 20% of mice, suggesting that the drug would only be useful in a public health or military exposure scenario if administered within a relatively short time window after exposure to toxin.

Host processes have been targeted selectively as a means of inhibiting B. anthracis toxin pathogenesis. A library of known phosphatase inhibitors was screened for the ability to protect against B. anthracis-induced cell death [187]. One compound with significant activity strongly inhibits the CD45 phosphatase. Testing of known CD45 inhibitors and CD45 knockdown using antisense technology protected macrophages from B. anthracis-induced cell death. Decreased CD45 expression did not affect LF-induced cell death of macrophages, suggesting the effect was independent of the action of LF. Targeted gene knockdown of CD45 with antisense technology prevented lethal anthrax infection in murine challenge models, when the knockdown was administered two days prior to challenge. This work provides the proof-of-concept data to support intercepting pathogeneses at host targets to counter bacterial threat agent infection, yet the requirement for pre-treatment will have to be improved upon with demonstration of post-exposure effectiveness if a useful therapeutic is to be developed.

Other mechanisms of blocking the host inflammatory response hijacked by LF appear to have prospects as host-targeted therapies. Inhibition of the Nlrp1b inflammasome in mouse macrophages by an organogold compound, auranofin inhibited LF-mediated toxicity [169]. This effect appeared not to involve direct inhibition of toxin entry or interaction with host targets, but rather blockage of downstream inflammatory responses that are activated by toxin. Effectiveness in an animal infection model is the next hurdle this prospective therapy would need to satisfy on the path as a candidate therapeutic.
The use of drugs with host target specificities is an immature field that faces additional challenges compared to drugs designed with pathogen specificity. It is reasonable to expect that off-target side effects will be greater when molecular designs target host molecules. In the CD45 study, it was also apparent that (defensive) targeting of host molecules may require much more fine tuning than (offensive) targeting of pathogen molecules. The study found that an optimal reduction in CD45 activity (around 65%) conferred protection, while greater reduction had little or no protective value. In classical pathogen-targeted therapies, such as antibiotics, doses are usually designed to go safely above a sub-therapeutic dose generalizable to populations within a certain body weight range. With host-targeted therapies it is likely that patient-specific maximum and minimum dosing will be required. Despite this, the strategy of targeting host molecules shows promise, but is likely a decade or more away from clinical use. The greatest potential use for host-targeted therapies may well lie in their use in combination with pathogen-targeted therapies to ameliorate results of infection such as toxic shock, giving the pathogen-targeted therapies time to take effect.

5.4 IMMUNOTHERAPEUTICS

Therapeutics which enhance or provide critical components of a protective immune response have been effective in the treatment or prevention of multiple diseases. Thus, the application of these technologies for the treatment of disease or prevention of infection caused by the biodefense agents is a logical and relatively lower risk approach compared to other, less mature, emerging technologies. One of these products, raxibacumab for the treatment of anthrax, has reached advanced development and represents one of the most mature candidate therapeutic MCMs.

5.4.1 Adaptive Immune Therapies

Passive immunization is accomplished by intravenous administration of pathogen-specific antibodies. The antibodies, either of human origin or humanized, then enable the host’s immune system to recognize and respond to a pathogen without a prerequisite priming experience (either prior infection or vaccination), or to neutralize components of the pathogen important for disease. This approach has been successfully fielded for multiple clinical uses, such as treatment of rabies virus infection.

5.4.1.1 Virus Countermeasures

EBOV is a strong candidate for development of passive immunization therapies. Human survivors of EBOV infection elicit a strong, long lasting humoral response whereas non-survivors are characterized by a suppressed humoral response [257], [258]. Passive immunization-mediated viral clearance is dependent upon antibody specificity. Neutralizing monoclonal antibodies have been shown to be protective in rodents [193], [237]. KZ52, a neutralizing antibody from a human survivor was able to protect guinea pigs, but was unable to protect NHPs [181]. Shedlock et al. [220] studied KZ52 and a survivor monkey antibody, JP3K11, and showed two distinct mechanisms to neutralize EBOV in vitro in which both antibodies differed in their ability to recognize proteolytically processed surface glycoprotein GP, thus affecting their neutralizing activity. Species-specific proteolytic processing of GP has been found to be responsible for the inability of the human antibody KZ52 to protect NHPs – NHPs process GP into a form not generated in infected humans, and thus not recognized by the human antibody. Any passive immunization therapy that is developed will have to account for these species-specific differences in epitope generation.

Neutralizing antibodies have also been reported against VEEV. Passive immunization using monoclonal antibodies against VEEV E3 glycoprotein were able to protect mice [192]. The antibodies were able to recognize protective epitopes within E3 which decreased viremia and provided a survival window where the host adaptive
immune response could be activated. Further, a purified humanized neutralizing antibody against the VEEV E2 protein was able to provide complete protection in mice when administered 24 hours before or after challenge with VEEV [88]. A similar humanized Mab, hu1A3B-7, has been reported recently with broad serogroup specificity [70]. Hu1A3B-7 showed neutralizing activity in vitro and protected mice infected via aerosol and subcutaneous routes with the VEEV TrD strain. Another advancement in this area has been the development of human Mabs (hMab) against VEEV by mapping the epitope of E1 and E2 proteins using phage display technology. The hMab F5 nIgG derived from VEEV-specific Fab from human donors showed neutralizing activity against VEEV in vitro [91]. Neutralization escape variants of F5nIgG helped map the binding sites of F5nIgG. This is the first report of a human epitope map for VEEV E1 and E2 proteins. Further studies will determine the ability of F5nIgG to confer protection in vivo.

Vaccinia Immune Globulin (VIG) was licensed to treat complications arising from vaccinations against smallpox with vaccinia virus. VIG has limitations, however, because it is a variable human product with poor efficacy in treating progressive vaccinia. This has stimulated the development of immune-therapeutics against poxvirus. The main hurdle in this development path is that poxvirions exist in two forms, the Intracellular Mature Virions (IMV) and Extracellular Enveloped Virions (EEV). Both virions are immunologically distinct and cannot be neutralized by a single antibody against any one of these forms. This demands the development of an effective Mab cocktail targeting both virion forms. This requirement has been addressed in a combination therapy with two human Mabs targeting the antigen in IMV (anti-H3 Mab hV26) and EEV (anti B5 Mab h101) [145]. Both antibodies exhibited significant efficacy against progressive vaccinia in mice compared to VIG. Combination therapy with both Mabs in mice infected with VACVNYCBOH showed improvement in survival compared to single Mab therapy. The combination therapy also showed improved protection in a murine model of eczema vaccinatum when administered 24 hours pre- and 12 hours post-infection [239]. It will be critical to study the effect of combination therapy post-infection to evaluate their utility post-exposure if there were a smallpox public health emergency.

5.4.1.2 Toxin Countermeasures

The development of monoclonal antibody therapy for the treatment or PEP of BoNT intoxication is underway. An oligoclonal combination of antibodies was developed and found to be required for efficient neutralization and clearance at high challenge doses [174]. Improving upon this paradigm, Sepulveda et al. [217] generated small anti-BoNT scFv, which possess epitope tags for recognition and clearance by a monoclonal antibody. The scFV and monoclonal antibody combination was able to clear BoNT from circulation by the liver, resulting in 100% protection of mice when administered two hours following exposure. Interestingly, the toxin neutralizing activity of the BoNT binding scFVs was dispensable for their activity, suggesting that removal from serum requires binding of the antibody, but not direct inhibition of toxin activity per se [217].

Further, small camelid single domain antibodies (VHHs) were developed that can bind and inhibit BoNT/A with nanomolar affinity [241]. When expressed in mammalian neuronal cells, the VHHs retained their ability to bind and prevent SNAP-25 cleavage, suggesting these proteins may be able to serve as therapeutic agents for botulism. However, efficient delivery of these proteins to the cholinergic neuron will remain a significant technical challenge in their development as a post-exposure BoNT therapy.

Passive immunization strategies for the treatment of SEB intoxication have been investigated. Monoclonal antibodies, identified during a screen of a phage display library using a detoxified form of SEB as the panning antigen, were characterized and evaluated for their ability to inhibit the pathological effects of SEB intoxication [123]. When pre-mixed with native SEB and administered intraperitoneally to mice, the anti-SEB monoclonal antibody provided 68% survival, while the control animals all succumbed to toxin challenge. It is currently
unknown if this antibody will be able to provide appreciable protection when administered following toxin challenge. In previous experiments, toxin neutralization with IgY anti-SEB antibodies generated in chickens was able to protect 100% of NHPs four hours following toxin exposure, suggesting that this therapeutic strategy may hold promise [125]. It will be critical to define the window of therapeutic opportunity for these candidate MCMs, as it will directly inform the utility of their use under a military operation or civilian public health emergency.

Anti-ricin monoclonal antibodies have been produced and have demonstrated protection against cell death in vitro [166]. Protection in a murine intestinal model of intoxication which examined levels of inflammatory markers as a correlate of mucosal tissue damage was demonstrated. However, in this experiment mice were administered the antibody 10 – 12 days prior to challenge. Since this study did not evaluate the PEP or treatment efficacy against a uniformly lethal aerosol challenge of ricin, any conclusion regarding its potential use as a candidate therapeutic is premature.

One immunotherapy for the treatment of inhalational anthrax has shown promise due to its efficacy in animal models and relative advanced stage of development. Raxibacumab, an anthrax monoclonal antibody targeting the protective antigen has been developed and tested for the treatment of inhalational anthrax [148]. Animals exposed via aerosol to a lethal challenge of B. anthracis spores were monitored for signs and symptoms of disease. Upon detection of protective antigen in serum, a rise in temperature, or both, animals were administered raxibacumab. In both rabbits and NHPs, there was a significant increase in survival at day 14 post-infection, with 44% of rabbits and 64% of NHPs surviving challenge (compared to 0% of placebo treated animals). These data suggest that a single dose of raxibacumab is able to increase survival in animal models of inhalational anthrax. It is currently unknown how the co-administration of antibiotics with this antitoxin therapy might further improve survival in these animal models.

The mechanism underlying antibody neutralization of toxins has long been attributed to the interruption of toxin – target substrate interactions. While this holds up as a relevant feature, recent research has also established a requirement in the case of LF toxin that neutralizing antibodies interact effectively with the immune complement system as well [1]. In these experiments, the effectiveness of a monoclonal antibody as an anti-toxin was dependent upon efficient binding of the constant region of the antibody to the Fc receptor. Swapping constant regions with less effective receptor binding reduced protection, and abolishing the receptor eliminated protection – in both in vitro macrophage assays and mouse infection experiments. This suggests that a nuanced understanding of neutralizing antibody interaction with immune components in addition to robust toxin binding will be required for optimal design of antibody-based anti-toxins.

Fusions of the extracellular domains of a major receptor of B. anthracis toxins, the human Capillary Morphogenesis protein-2 (CMG2) and human IgG Fc, were constructed to serve as a potential alternative to anti-PA antibodies [266]. Optimized expression in both transient and stable tobacco-based expression systems yielded fusion protein at high levels. When administered at the time of challenge, this construct was capable of fully protecting rabbits challenged with a lethal dose of B. anthracis spores. While promising, it will be imperative to challenge animals at later time points during the infection to fully assess the utility as a B. anthracis MCM.

5.4.1.3 Bacterial Countermeasures

A variety of bacterial pathogens are susceptible to interventions by passive immunization. Mice given a lethal, aerosol inoculation of Y. pestis can be protected by immediate aerosol administration of mouse monoclonal antibodies against both the F1 and V antigens [86]. Infection of mice with Y. pestis following administration of
human monoclonal antibodies allows the animals to survive a lethal inoculation [267]. The highest level of protection is dependent upon administration of multiple antibodies that target different pathogen antigens. What is uncertain from the experiments is how much the protection afforded by the treatment diminishes over a longer period beyond the 21 days tested in the published results. Further, the protection was conferred by administration of antibodies 24 hours prior to infection.

Monoclonal antibodies developed against \textit{F. tularensis} have also been successfully used to protect mice against infection. Mouse monoclonal antibodies prepared against the \textit{F. tularensis} Live Vaccine Strain (LVS) included two that provided protection, one against lipopolysaccharide, and one against the FopA gene [214]. The protection observed when antibodies were administered a day prior, the day of, and a day after infection was 100\% and 90\% for the two antibodies respectively against LVS. The protection afforded against the virulent SCHU S4 strain was limited to a delay in death provided by the anti-LPS antibody only. Administration of antibodies in three post-exposure doses (one, three, and five days after infection with LVS), only exhibited about half as much protection. The utility of passive immunization against bacterial pathogens is likely to be hampered by the need to administer antibodies before, or very soon after infection, and may also be constrained by high bacterial species and strain specificity.

Passive immunization promises in some cases to offer effective therapeutics against biodefense agents. Potential therapies will be eliminated for some pathogens or toxins where the administration of antibody therapy is required in advance of, or so soon after exposure as to be impractical in an emergency scenario. While many of the potential therapies discussed above have or appear likely to fail in this context, some appear to have a potentially useful therapeutic window of opportunity. Those candidates will then have to be found to have suitable pharmacological properties. Adaptive immune therapies will be constrained by narrow pathogen effects, with specificity to at best one species of pathogen, though possibly more narrow efficacy limited to strains or serotypes of a single species.

5.4.2 Innate Immune Therapies

Innate immunity involves a complex mechanism utilizing soluble cellular factors and immune cells which provides protection from invading pathogens. This section focuses on innate inhibitors that are currently being developed as potential therapeutic agents for viral or bacterial infections of biodefense relevance.

Therapeutic application of a recombinant human innate immune molecule, Mannose Binding Lectin (rhMBL), was evaluated for its ability to target glycosylated viruses [147]. rhMBL binds to EBOV and MARV envelope GP proteins and blocks the interaction with DC–SIGN. Mice administered with rhMBL resulting in rhMBL serum concentrations more than 7-fold above-average human levels survived lethal EBOV infection and became immune to virus re-challenge. The ability of rhMBL to target other glycosylated viruses and its use as a broad spectrum anti-viral needs to be studied further.

A similar immune-modulatory molecule, recombinant human-Activated Protein C (rhAPC) is currently licensed to treat patients with sepsis at a high risk of death. When rhAPC was administered to rhesus macaques 30 – 60 minutes post-infection with a lethal dose of EBOV-Zaire, it increased the mean survival time by 4.3 days [83]. Whether rhAPC would be effective when administered beyond 60 minutes post-infection is unknown. Thus, it is difficult to ascertain the utility of this treatment in a mass public health emergency when response times to administer MCMs will go beyond 60 minutes. The ability of these immune-modulatory molecules to mitigate the pathology of disease at later time points during infection may further limit their utility if they can only delay, but not prevent death.
The use of Cationic Lipsome-DNA Complexes (CLDCs) containing non-coding plasmid DNA to treat Western Equine Encephalitis Virus (WEEV) infection has been evaluated [136]. Mice were administered CLDCs prior to infection, at the time of infection, or treated following challenge with a subcutaneous or aerosol dose of WEEV. Mice treated with CDLCs had increased cytokine levels consistent with innate immune activation, which would lead to a robust Th1 response. Pre-treatment with CLDCs afforded the most protection from a uniformly lethal WEEV challenge. These data suggest that a non-specific activation of the innate immune response can provide protection from encephalitic alphavirus exposure and/or infection.

CLDC treatments have similarly demonstrated a protective effect against bacterial infections, but in one case this effect is only apparent when it potentiates other anti-bacterial treatments. In a mouse model of infection with *B. pseudomallei*, a sub-therapeutic dose of the antibiotic ceftazidime became protective through the addition of CLDC; the combination provided 90% survival in a short-term (20-day) timeframe compared to a 10% survival rate for controls [197]. While this treatment boosted short-term survival, continued evaluation for 60 days revealed that half of the animals eventually succumbed to latent infections. What remains to be determined is whether potentiation of a therapeutic dose of anti-bacterial can reduce latent infection significantly over anti-bacterial treatment alone.

Administration of CLDC can also provide protection of mice against aerosol *F. tularensis* infection [242]. One hundred percent protection and clearance of *F. tularensis* LVS occurred in mice given intranasal CLDC 24 hours prior to challenge. CLDC administration at 24 hours after challenge through an intravenous route, or intranasally were roughly half as effective; other routes of administration merely delayed death from LVS challenge. Unfortunately, the optimal administration of CLDC only delayed death when the challenge was the virulent *F. tularensis* SCHU S4 strain. CLDCs have been combined with the crude membrane protein fraction from *F. tularensis* to confer protection against bacterial infection [95]. The cocktail showed an *in vitro* effect with pre-treatment and at 4, but not 12 hours post-infection reduced *F. tularensis* establishment of infection in mouse and human macrophages. An *in vivo* protective effect was found in mice, but only with pre-treatment at 3 (but not 1, 2, 7) days before challenge. The appropriately pre-administered cocktail conferred 50 – 60 % survival at 28 days compared with 100% lethality in unprotected mice challenged with *F. tularensis*. A different cocktail combining CLDCs and lipopolysaccharide from *F. tularensis* membrane fractions did not work. Finally, CLDCs may have a broad capacity to protect animals from other pathogens since pre-treatment with the CLDC cocktail could also reduce infection of human macrophages *in vitro* by *Y. pestis*, *B. pseudomallei*, and *Brucella abortus*. This study also uncovered likely differences in the mechanisms conferring protection in different animal hosts – *in vitro* protection in mice macrophages relied on Reactive Oxygen Species (ROS) and reactive nitrogen species, but in human macrophages only ROS were involved.

Protecting against *B. anthracis* infection by activation of the innate immune response through stimulation of interferon production or addition of recombinant interferon has had limited efficacy. Pre-treatment of mice with Poly-ICLC (an inducer of Type I interferons IFN-α and IFN-β) or with recombinant IFN-β protected 60% and 40%, respectively, of mice challenged with an intranasally delivered, lethal dose of *B. anthracis* [251]. The experimental promise of this approach is still quite limited, since the pre-treatment occurred 24 hours prior to challenge. Furthermore, the mice in the experiments were only followed for 16 days post-infection, so it is unclear whether latent infection may have resulted in later lethality. Finally, of interest and also concern in these experiments was that the dose seemed subject to the same fine-tuning requirements as with the CD45 interference therapy described above. Namely, the best dose of IFN-β provided 40% protection at 50,000 units, while a dose of 150,000 units provided higher protection during the first five days of the experiment, but then mice in this cohort steadily began to succumb until all eventually died.
Use of CpG Oligodeoxyribonucleotide (ODN) pre-treatment has been shown to confer protection against parenteral *F. tularensis* LVS and parenteral *B. pseudomallei* challenge. The CpG ODN is thought to stimulate an immune response through TLR-9. The most recent work in this area found CpG ODN able to protect mice against aerosol challenge with *B. pseudomallei*, but not aerosol challenge with *F. tularensis* SCHU S4 [209]. The CpG ODN was effective when administered 48 hours prior to infection. Results were presented only for the lowest inoculum in experiments where CpG ODN was given one hour after bacterial challenge, which conferred a partial protective effect for up to 28 days. These results show dependency on pathogen strains and timing of administration, with pre-treatment being most effective and immediate PEP being less effective. Of most concern is that the authors point to slightly exacerbated infection in the CpG ODN treated mice challenged with *F. tularensis* SCHU S4. Failure to pre-administer can reduce or eliminate protection, but use against an inappropriate bacterial pathogen or strain could even worsen the effect.

An innate immune response protecting against bacterial infection can also be activated by stimulating TLR-3 via synthetic double stranded RNA (Poly I:C (PIC)). PIC administration to mice 24 hours and 1 hour pre-infection as well as 1 hour post-challenge delayed lethality by four days against inhaled *F. tularensis* [198]. Tests demonstrated that antibiotic treatment 5 days post-challenge results in 100% lethality, while 100% of mice survive for 15 days if PIC is administered 1 hour after infection and then antibiotic is given after five days. PIC-induced cytokine expression and enhanced *in vitro* phagocytosis and killing of *F. tularensis* by human monocytes. The authors hypothesize that PIC could extend the time available for administration of antibiotic in the event of human infection with threat agent bacteria. However, it is unclear how having a separate medication that allows later anti-bacterial administration has any advantage over simply having the effective antibiotic available instead.

In addition to stimulation of the innate immune response through nucleic acid signatures that are indicative of pathogens, stimulation using lipopolysaccharide signatures has been achieved. Application of synthetic Aminoalkyl Glucosaminide Phosphate (AGP) molecules stimulates TLR-4-mediated inflammatory responses. *Francisella spp.* is capable of evading host immune responses in part due to the weak TLR-4-mediated recognition of their lipopolysaccharide profile. Administration of AGP was shown to confer 40% survival to mice receiving a lethal dose of *F. tularensis* novicida [129]. Similar to other TLR-mediated protection, this approach required pre-treatment with the compound to be effective. The result suggests that the utility of innate immune stimulation as a protection against bacterial pathogens will be limited in utility due to a required foreknowledge of exposure and delivery of a pre-exposure course of therapy.

Targeting the innate immune response as a therapeutic approach faces challenges. Innate immunity is critical to the defence against disease. It is also a target of pathogen virulence factors along with other aspects of the host molecular machinery. Successful use of drugs to modulate innate immunity and restrict pathogenesis will require delicate and individualized fine-tuning. The wrong dose or timing could be ineffective, or even exacerbate infection. In addition to these challenges, the stimulation of innate immune responses seems largely ineffective unless performed in advance of (or in some cases almost immediately after) exposure to pathogen. The efficacy of each innate immune modulating strategy also seems to depend on the pathogen species, with possible differences even between strains. These features would require an innate immune modulating drug to be deployed and administered in advance of an exposure/attack with foreknowledge of when, and to what pathogen exposure would occur – very unlikely in a battlefield or public health scenario. Targeting the innate immune response will be an unsuccessful approach to protecting against biodefense threats unless these limitations can be overcome.
5.5 ANTI-MICROBIAL PEPTIDES

Anti-Microbial Peptides (AMPs) are generally considered part of the host defence mechanism and are conserved components of the innate immune response. For therapeutic purposes, AMPs can be extracted from bacteria that naturally generate them. Alternatively, natural AMPs can be synthetically modified or generated de novo. Few investigations into the use of AMPs as a treatment or PEP for threat agent exposure have been published. The Sheep Myeloid Anti-microbial Peptide-29 (SMAP-29) was tested for in vitro activity against *B. globigii*, *B. anthracis*, and *B. thailandensis*. SMAP-29 inhibited both *B. globigii* and *B. anthracis* at sub-micromolar concentrations, yet was less effective against *B. thailandensis* [44]. Unfortunately, SMAP-29 was also found to be highly haemolytic and cytotoxic to human blood and tissue culture cells. A second therapeutic example is a synthetic structural mimic of endogenous AMPs, Ceragenins (CSAs). CSAs were evaluated for anti-viral activity against vaccinia virus [87]. Topical application of one such peptide, CSA -13, to vaccinia lesions in a murine model reduced the development of satellite lesions. In addition, CSA-13 also stimulated the expression of endogenous AMPs against vaccinia virus in a SCID mouse model. The anti-viral mechanism of CSAs remains to be determined. Synthetic peptides like CSAs have the advantage of being resistant to human protease degradation which provides an opportunity to improve stability and toxicity issues associated with AMPs. In order to design new anti-microbials for use against biodefense pathogens, it may be possible to selectively enhance the specificity and potency of AMPs while reducing their toxicity [43].

5.6 NUCLEIC ACID SEQUENCE-BASED THERAPEUTICS

The emergence of nucleic acid based therapies in recent years has provided new therapeutic strategies against infectious diseases, including biodefense agent exposure. While it has been readily established that antisense molecules can inhibit the translation of viral or host mRNAs, only one antisense therapy for the treatment of an infectious disease has reached the commercial market. Fomivirsen has obtained FDA-approval for the treatment of CMV retinitis, providing regulatory precedent for the use of this therapeutic modality. Several nucleic acid based therapies are in pre-clinical and clinical development of the treatment of HIV, HBV, HCV, RSV, and influenza viruses [150], [173], [45], [122], [272], suggesting substantial interest on the part of biotechnology and pharmaceutical industries to utilize this technology for the treatment of infectious disease. It should be noted, however, that several significant limitations with antisense therapies exist. Because knowledge of the agent’s nucleic acid sequence is required, nucleic acid-based therapeutics will lack utility in a public health emergency with an unknown or genetically altered agent. Further, technical challenges with efficient delivery and tissue distribution of these molecules exist and may complicate their development as effective MCMs.

5.6.1 Viral Therapeutics

Antisense therapies have been evaluated for their ability to suppress filovirus replication. Administration of Phosphorodiamidate Morpholino Oligomers (PMOs) that target viral genomic sequences 30 – 60 minutes following virus challenge protected 60% of NHPs against a lethal EBOV challenge and 100% of NHPs against a MARV challenge [255]. While this is a novel breakthrough for the amelioration of infection with these extraordinarily pathogenic viruses, it will be necessary to improve the therapeutic window of opportunity with these candidate MCMs to truly offer utility during a military operation or public health emergency. Additionally, whether these products possess additive benefit or synergistic efficacy when combined with other candidate therapies is currently unknown. If the PMOs’ efficacy is limited to one hour post-exposure and there is no synergy with other products, PMOs will not be helpful in an emergency.

Other small interfering RNAs (siRNAs) have been evaluated for their ability to protect against EBOV infection. siRNAs targeting the EBOV-Zaire RNA polymerase L coding sequence formulated in Stable Nucleic Acid-
Lipid Particles (SNALPs) provided 100% protection of guinea pigs when administered one hour after a lethal EBOV challenge [67]. Further, SNALP-formulated siRNAs were evaluated for their ability to protect NHPs from EBOV challenge [66]. When given intravenously 30 minutes following exposure two of three rhesus macaques given four post-exposure treatments of the pooled anti-EBOV-Zaire siRNAs were protected from lethal ZEBOV infection, whereas all macaques given seven post-exposure treatments exhibited 100% protection. It will be necessary to conduct dose delay studies in order to ascertain the therapeutic window for siRNAs and ascertain the utility of this countermeasure.

RNA interference (RNAi) has been evaluated as a potential therapy for orthopox viruses [8]. Using Monkeypox (MPX) as a model system, 48 siRNA constructs were screened in vitro against genes essential for viral replication or entry. These constructs were able to inhibit viral expression by 65 – 95 % without apparent signs of cytotoxicity. One construct was capable of a prolonged inhibition of viral replication (7 days) at a pharmacologically relevant concentration of 10 nM. In addition to further substantiating the critical role of these genes in the viral lifecycle, this study provides in vitro proof-of-principle for the use of RNAi as a therapeutic platform for these viruses.

Antiviral agents based on RNAi have also been explored against VEEV. A combination of four siRNAs was tested against six strains of VEEV in vitro [177]. The siRNAs were able to inhibit viral replication in vitro. However, the study also reports the emergence of resistance to RNAi. Peptide-conjugated Phosphorodiamidate Morpholino Oligomers (PPMOs) evaluated against VEEV have been reported to inhibit viral replication [184]. Vero cells infected with different strains of VEEV when treated with 5’+P7-PPMO resulted in modest reduction in viral titer at 2.5 µM and significant reduction at 5, 7.5 and 10 µM. Mice receiving 5’+P7-PPMO pre- and post-infection resulted in 100% (8/8) protection whereas 63% (5/8) protection was observed in mice receiving only post-infection treatment.

5.6.2 Bacterial Therapeutics

Nucleic acid sequence specific anti-bacterial treatments are being developed employing chemically modified, antisense oligomers, but have yet to be directed at biodefense bacterial agents. In multi-drug-resistant Pseudomonas aeruginosa, expression of the drug efflux pump OprM can be greatly reduced by antisense phosphorothioate oligodeoxynucleotide packaged in polycation polyethylenimine complexes [254]. When OprM is thus downregulated, a wide variety of resistant isolates are restored to antibiotic sensitivity in vitro. A similar technology, using antisense phosphorodiamidate morpholino oligomers as peptide conjugates was demonstrated against B. cepacia complex [71]. Antisense downregulation of AcpC, the carrier protein (AcpC), important for lipoprotein biosynthesis, inhibited bacterial growth in culture. Use of antisense to reduce AcpP expression in vivo also conferred protection to 60% of mice against a lethal dose of B. multivorans. While antisense technology as a countermeasure against threat agent bacteria has yet to be tested, the results so far are encouraging. The technology will not likely provide a reliable anti-bacterial drug on its own, since the downregulation of bacterial genes does not appear to be completely penetrant. However, it has great potential to augment or potentiate other anti-bacterial drugs either by weakening resistance mechanisms, or bacterial metabolism.

Use of siRNA molecules and various chemical derivations of RNAi shows powerful in vitro effects against viral, and in some cases bacterial pathogens. In a few cases the effects have been extended to in vivo systems. The utility of this approach as a therapeutic will be constrained by typical pharmacological challenges, but also by a requirement for high sequence specificity of the RNAi mechanism. Any therapies that are developed will be limited in the viral or bacterial strains they can affect, and development of resistance may be unacceptably frequent. A further challenge is that with the few in vivo tests that have occurred, indications are that the therapeutic window of opportunity has been absent (pre-treatment required) or very short.
5.7 CONCLUSION

Since the institution of the U.S. FDA Animal Rule in 2002, only two threat agent MCMs have been approved for human use. These products, Cyanokit® and pyridostigmine bromide, are for the treatment of cyanide and nerve agent exposure, respectively. Given the level of investment in MCMs, approvals for biological agent MCMs under the Animal Rule have been discouragingly few. One factor contributing to this is the status of the animal models for many of these agents, which lack defined endpoints and fail to completely recapitulate the pathogenesis of the disease in humans. Given the absence, to date, of drug approval for biological agents under the Animal Rule, there are significant doubts about how mature state-of-the-art technologies must be before they can be incorporated into MCM development.

Due to the difficulty associated with getting conventional technologies (monoclonal antibody, polyclonal antibody, antibiotics) approved under the Animal Rule, at least as much difficulty can be expected for the development of technologies that have not been FDA-approved for more clinically testable applications. A dedicated, traditional drug or biologic development campaign which focuses on established technologies is likely to produce a lower developmental risk profile, and in the long-term yield products that are suitable of use in the U.S. Strategic National Stockpile (SNS). Innovation in drug and therapeutic development that remains segregated to a sole biodefense focus is unlikely to capture the advantages of many crucial market-driven advances. Instead, innovation in this space should build on advances in related fields and be adapted or extrapolated as appropriate. While this approach may not allow easy categorization of programs, it is pragmatic in the face of diminishing budgets and limited successes to date.

In order to expedite the development of therapeutic MCMs against these agents, it will be necessary to rapidly evaluate these products in models and at time points which accurately reflect the logistical realities of a public health response or field deployment of MCMs. In many studies, as highlighted by this article, the investigators conduct experiments to demonstrate the highest levels of efficacy to publish in scientific journals, but fail to adequately define the therapeutic parameters of their products. Given that in a mass casualty setting MCMs will likely not be administered until 24 – 48 hours following a release or detection of a sentinel case, it is necessary that developers target realistic therapeutic windows for their products.

The major gap in the state-of-the-art for therapeutic MCMs is that the current pipeline will not overcome the significant rate of attrition in biodefense drug development. Given that many candidates have not been evaluated in models adequate to assess their therapeutic efficacy, the developmental pipeline is left with only a handful of products that possess true therapeutic efficacy. If the domestic and international biodefense community seeks to develop and procure products for civilian or military use in a timely manner, rigorous down selection criteria need to be established and exercised early in product development. Concurrently, animal models for the therapeutic evaluation of products to the satisfaction of regulatory agencies need to be developed and refined.

5.8 REFERENCES


STATE-OF-THE-ART THERAPEUTIC MEDICAL COUNTERMEASURES FOR BIOLOGICAL THREAT AGENTS


---

5 - 30 STO-TR-HFM-186


Neurotoxin/A Intoxication with Aminopyridines: A New Twist on an Old Molecule. ACS Chem Biol. [Epub ahead of print]


**Disclaimer:** The contents of this article are the sole responsibility of the authors and do not necessarily represent the official views of the Biomedical Advanced Research and Development Authority, the Office of the Assistant Secretary for Preparedness and Response, or the U.S. Department of Health and Human Services.
Chapter 6 – LABORATORY IDENTIFICATION OF BIOLOGICAL THREATS

Timothy D. Minogue, David A. Norwood, Randal J. Schoepp and Mark J. Wolcott
Diagnostic Systems Division, USAMRIID, Fort Detrick, MD
UNITED STATES

Corresponding Author (Minogue): timothy.d.minogue.civ@mail.mil

6.1 INTRODUCTION

Medical diagnosis is the process by which clinicians attempt to deduce the cause of a particular disease or disorder in a sick individual. The goal of diagnosis is to assist in making correct medical decisions about the treatment and prognosis of the patient. For infectious diseases, a variety of medical information is used to make a diagnosis; including physical examination, interview with the patient, medical history of the patient, and clinical findings as reported by laboratory tests. While the focus of this chapter will center on laboratory tests used to diagnose biological threat agents, these tests represent a piece of the diagnostic puzzle and should not be used solely for diagnosis and treatment. Physical and clinical findings as well as medical history are critical to accurate diagnosis, and integrating all available medical information as well as all available laboratory information reduces the chance for misdiagnosis. Diagnosis requires the synthesis of multiple pieces of information into a medical judgment that will be used to impact patient care; therefore, getting the right answer must always take priority over getting a quick answer. The content of this review will focus on the current and future state of in vitro diagnostics, as defined by the FDA [1]: “those reagents, instruments, and systems intended for use in diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body.”

For the purposes of this chapter, a biological threat is any infectious disease entity or biological toxin encountered, either through natural distribution or intentionally delivered by an opposing force to deter, delay, or defeat the U.S. or allied military forces. The majority of biological threats of military and public health relevance are contained in the Health and Human Services (HHS) non-overlap select agents and toxins and the high consequence livestock pathogens and toxins/select agents (overlap agents) within the list of regulated biological select agents and toxins (Table 6-1). Many of these biological threats were part of offensive biological weapons programs at one time. Biological threat laboratory assays are subject to the same requirements and regulations as other infectious diseases, to be regulatory compliant, meaning that the test can be used for patient care. For laboratory assays, two critical elements must meet minimal standards to be considered regulatory compliant, the laboratory performing the test must be qualified and the test being performed must be validated. The quality of the clinical laboratory doing the testing is regulated by the Clinical Laboratory Improvement Amendments, which were passed in 1988 (CLIA ’88); while in vitro diagnostic tests are regulated by Section 210(h) of the Federal Food, Drug, and Cosmetic Act. As such, the FDA has oversight and regulatory authority to clear in vitro diagnostic tests (medical devices) for commercial sale and use. The combination of a CLIA accredited laboratory performing a FDA-cleared diagnostic test results in a regulatory compliant diagnostic result that can be used for treatment and prognosis of the patient. For the DoD, maintaining regulatory compliance in performing in vitro diagnostic tests in a deployed environment poses a significant challenge.
Table 6-1: Regulated Biological Select Agents and Toxins.

<table>
<thead>
<tr>
<th>HHS NON-OVERLAP SELECT AGENTS AND TOXINS</th>
<th>USDA HIGH CONSEQUENCE LIVESTOCK PATHOGENS AND TOXINS (NON-OVERLAP AGENTS AND TOXINS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crimean-Congo hemorrhagic fever virus</td>
<td>Akabane virus</td>
</tr>
<tr>
<td><em>Coccidioides posadasii</em></td>
<td>African swine fever virus</td>
</tr>
<tr>
<td>Ebola viruses</td>
<td>African horse sickness virus</td>
</tr>
<tr>
<td><em>Cercopithecia herpesvirus</em> 1 (Herpes B virus)</td>
<td>Avian influenza virus (highly pathogenic)</td>
</tr>
<tr>
<td>Lassa fever virus</td>
<td>Blue tongue virus (Exotic)</td>
</tr>
<tr>
<td>Marburg virus</td>
<td>Bovine spongiform encephalopathy agent</td>
</tr>
<tr>
<td>Monkeypox virus</td>
<td>Camel pox virus</td>
</tr>
<tr>
<td><em>Rickettsia prowazekii</em></td>
<td>Classical swine fever virus</td>
</tr>
<tr>
<td><em>Rickettsia rickettsii</em></td>
<td>Cowdria ruminantium (Heartwater)</td>
</tr>
<tr>
<td>South American haemorrhagic fever viruses</td>
<td>Foot and mouth disease virus</td>
</tr>
<tr>
<td><em>Junin</em></td>
<td>Goat pox virus</td>
</tr>
<tr>
<td><em>Machupo</em></td>
<td>Lumpy skin disease virus</td>
</tr>
<tr>
<td><em>Sasha</em></td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td><em>Fiecal</em></td>
<td>Malignant catarrhal fever virus (Exotic)</td>
</tr>
<tr>
<td><em>Guanarito</em></td>
<td>Menangle virus</td>
</tr>
<tr>
<td>Tick-borne encephalitis complex (flavi) viruses</td>
<td><em>Mycoplasma capricolum</em></td>
</tr>
<tr>
<td><em>Central European tick-borne encephalitis</em></td>
<td>M.F38/M. mycoides capr1</td>
</tr>
<tr>
<td><em>Far Eastern tick-borne encephalitis</em></td>
<td><em>Mycoplasma mycoides mycoides</em></td>
</tr>
<tr>
<td><em>Russian spring and summer encephalitis</em></td>
<td>Newcaelast disease virus (VVND)</td>
</tr>
<tr>
<td><em>Omsk hemorrhagic fever</em></td>
<td>Peste Des Petits Ruminants virus</td>
</tr>
<tr>
<td><em>Variala major virus</em> (Smallpox virus)</td>
<td>Rinderpest virus</td>
</tr>
<tr>
<td><em>Variala minor virus</em> (Alastrim')</td>
<td>Sheep pox virus</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>Swine vesicular disease virus</td>
</tr>
<tr>
<td><em>Abrin</em></td>
<td>Vesicular stomatitis virus (Exotic)</td>
</tr>
<tr>
<td>Conotoxins</td>
<td></td>
</tr>
<tr>
<td>Diacetyloxyclenol</td>
<td></td>
</tr>
<tr>
<td>Ricin</td>
<td></td>
</tr>
<tr>
<td>Saxtoxin</td>
<td></td>
</tr>
<tr>
<td>Shiga-like ribosome inactivating proteins</td>
<td></td>
</tr>
<tr>
<td>Tetrodotoxin</td>
<td></td>
</tr>
<tr>
<td><strong>HIGH CONSEQUENCE LIVESTOCK PATHOGENS</strong></td>
<td><strong>LISTED PLANT PATHOGENS</strong></td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
<td><em>Liberobacter afric anus</em></td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td><em>Liberobacter asiaticus</em></td>
</tr>
<tr>
<td><em>Brucella melitensis</em></td>
<td><em>Peronosclerospora philippinensis</em></td>
</tr>
<tr>
<td><em>Brucella suis</em></td>
<td><em>Phakopsora pachyrhizi</em></td>
</tr>
<tr>
<td><em>Burkholderia mallei</em> (formerly <em>Pseudomonas mallei</em>)</td>
<td><em>Plum Pox Potyvirus</em></td>
</tr>
<tr>
<td><em>Burkholderia pseudomallei</em> (formerly <em>Pseudomonas pseudomallei</em>)</td>
<td><em>Ralstonia solanacearum race 3, biovar 2</em></td>
</tr>
<tr>
<td>Botulinum neurotoxin producing species of <em>Clostridium</em></td>
<td><em>Schlerophthra rayssiae var zeae</em></td>
</tr>
<tr>
<td><em>Coccidioides immitis</em></td>
<td><em>Synchytrium endobioticum</em></td>
</tr>
<tr>
<td><em>Coxiella burnetii</em></td>
<td><em>Xanthomonas oryzae</em></td>
</tr>
<tr>
<td>Eastern equine encephalitis virus</td>
<td><em>Xylella fastidiosa</em> (citrus variegated chlorosis strain)</td>
</tr>
<tr>
<td>Hendra virus</td>
<td></td>
</tr>
<tr>
<td><em>Francisella tularensis</em></td>
<td></td>
</tr>
<tr>
<td><em>Nipah Virus</em></td>
<td></td>
</tr>
<tr>
<td>Rift Valley fever virus</td>
<td></td>
</tr>
<tr>
<td>Venezuelan equine encephalitis virus</td>
<td></td>
</tr>
<tr>
<td>Botulinum neurotoxin</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> epsilon toxin</td>
<td></td>
</tr>
<tr>
<td>Shigatoxin</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcal enterotoxin</em></td>
<td></td>
</tr>
<tr>
<td>T-2 toxin</td>
<td></td>
</tr>
</tbody>
</table>

CLIA sets the standards for any laboratory that performs testing on human samples for use in diagnosis and treatment of disease. The goal of CLIA was to improve the quality of any testing conducted for medical purposes, and DoD facilities are not exempt from the requirements. However, the DoD was allowed to develop a separate plan for ensuring quality and standards in diagnostic testing, the Clinical Laboratory Improvement Program (CLIP, DoDI 6440.2) [2]. CLIP is very similar to CLIA with certain exceptions to meet military operational requirements. Both CLIA and CLIP govern the quality of the laboratory performing the diagnostic test, and include standards for personnel, quality control, quality assurance, procedure manuals, proficiency testing and inspections for adherence to the standards. CLIA and CLIP require laboratory registration to perform testing, and registrations are based on the level of test complexity that the laboratory is accredited to perform. Minimal complexity tests (waived) are simple tests that do not require significant quality oversight, such as tests cleared by the FDA for home use. Moderate and high complexity tests require increased knowledge, training and experience, quality control, and interpretation and judgment. Moderate tests are typically more automated while high complexity tests require significant technical manipulation by personnel. The currently cleared diagnostic system, the Joint Biological Agent Identification and Diagnostic System (JBAIDS), is a high complexity test. As such, it can be difficult to maintain a high complexity CLIA registration in a deployed setting. Movement of laboratory diagnostic capabilities to forward locations is driving long-term goals for DoD medical diagnostic devices to be CLIA-waived devices. While this is unlikely to happen quickly, the Next-Generation Diagnostic System (NGDS) will likely be a moderate complexity device, an incremental improvement over the currently deployed system.

While CLIA/CLIP requirements are based on test complexity, FDA requirements for clearance are based on the risk associated with the test, and risk is dependent upon the potential harm associated with obtaining the wrong diagnostic result. The FDA classifies in vitro diagnostic tests as either Class I (lowest risk), Class II (moderate to high risk), or Class III (highest risk) medical devices. The currently fielded JBAIDS system is an FDA-regulated Class II device and currently resides in Combat Support Hospitals within the U.S. Army and within other medical treatment facilities for the Air Force and Navy. For the DoD, the challenge remains maintaining regulatory compliance in far forward operational settings. The availability of FDA-cleared assays for biological threats remains somewhat limited. In vitro diagnostic tests for biological threats are orphan products, meaning there is not a large enough market to incentivize private industry to develop the tests. This is because these tests are performed on an infrequent basis and, therefore, sales are limited. Consequently, most of the cleared diagnostic tests for biological threats have resulted from DoD acquisition programs. Currently cleared biological threat assays on the JBAIDS system include tests for Bacillus anthracis, Yersinia pestis, Francisella tularensis, and Coxiella burnetii. The absence of useful and cleared infectious disease assays on JBAIDS hampers the utility of the system for clinicians. Future diagnostic devices for DoD would benefit from expanded capabilities for common infectious diseases of military relevance, not just those that are most likely to be used in a biological attack.

While biological science technology continues to advance by leaps and bounds, it must be emphasized that the DoD currently fielded, regulatory compliant, in vitro diagnostic tests for biological threat agents are based on nucleic acid amplification chemistry that is 30 years old and a rapid cycling Polymerase Chain Reaction (PCR) platform that is more than 10 years old. While the NGDS acquisition program is underway, the platform is likely to be only an incremental improvement over the currently fielded system. The two most likely improvements will be onboard integrated sample processing and a sample in/answer out analysis flow. In essence, the system is likely to be an automated nucleic acid amplification in vitro diagnostic platform. During this time, microarrays, mass spectrometry, and DNA sequencing have advanced significantly for the identification of infectious agents [3]. Yet none of these approaches has matured to the point of receiving FDA clearance for medical diagnostic use or offer the hope of a simplified test that can be performed in a deployed setting for biological threat agents. Unlike technology, the ability of military laboratories to identify and confirm the presence of biological threats
using regulatory compliant diagnostics matures at a much slower rate. This is not to discount the use of newer technologies by the DoD for environmental testing, vector surveillance, and population surveillance. While these results can be used to make operational decisions, the results cannot be used for individual patient treatment, a concept often lost within the research and development community. The combination of using multiple diagnostic devices, multiple diagnostic markers, medical intelligence, clinical signs and symptoms, and classical microbiology (Figure 6-1) still provide the most reliable approach for medical diagnosis of diseases to impact medical treatment or response after a biological threat attack.

Figure 6-1: Orthogonal Diagnostic Testing Uses an Integrated Testing Strategy where More than One Technology, Technique, or Biomarker is Used to Produce Diagnostic Results, which are then Interpreted Collectively. While orthogonal diagnostic testing is a statistically independent approach, the combination of independent sensitivity and specificity values becomes highly valuable when combined. Orthogonal diagnostic testing improves the probability of reaching a “correct” result when the assays are less than 100% specific independently.

To enhance readiness and ensure the availability of laboratory testing capabilities, military and civilian clinical laboratories are linked into a series of laboratory response networks. The preeminent Laboratory Response
Network (LRN) for bioterrorism is sponsored by the Centers for Disease Control and Prevention (CDC). More recently, the DoD has established the Defense Laboratory Network (DLN) to further enhance military readiness as well. Together, these efforts have improved the national preparedness for biological threat identification, but continuing research and development are needed to improve the speed, reliability, robustness, and user friendliness of the new diagnostic technologies. This chapter will review currently available and future capabilities for agent identification and diagnostic technologies available to protect and sustain the health of military personnel.

6.2 THE LABORATORY RESPONSE

6.2.1 Role of the Military Clinical and Field Laboratories

Military clinical and field laboratories play a critical role in the early recognition of biological threats. Intentionally delivered biological agents can also be used in bioterrorism scenarios to create terror or panic in civilian and military populations to achieve political, religious, or strategic goals. Although the principal function of military clinical laboratories is to confirm the clinical diagnosis of the medical officer, laboratory staff also provides subject-matter expertise in theaters of operation on the handling and identification of hazardous microorganisms and biological toxins. In addition these laboratories have a global view of disease in the theater and they play an important sentinel role by recognizing unique patterns of disease. Military field laboratory personnel may also evaluate environmental samples and veterinary medicine specimens as part of force health protection or preventive medicine surveillance system in a theater of operations [4]. Military biological laboratory capabilities also exist to provide Chemical, Biological, Radiological, and Nuclear (CBRN) response, elimination and remediation activities [5].

6.2.2 Military Field Laboratories

Military field laboratories come in many different configurations and are often incorporated into most of the services basic deployable treatment facilities. If a complete medical treatment facility is part of a deployment, its intrinsic medical laboratory assets can be used. However, a medical laboratory may not be available for short duration operations. In this case, medical laboratory support would be provided by a facility outside the area of operations [6]. A typical Army medical treatment facility in a theater of operations will have a limited initial microbiology capability even with the intrinsic laboratory component. Following the removal of the microbiology capability from most Army medical treatment facilities under the 1994 Medical Reengineering Initiative (MRI), the capability has been restored with addition of a microbiology augmentation set (Medical Materiel Set [MMS], laboratory [microbiology] augmentation UA N403 NSN 6545-01-505-2714 LIN M48987) and JBAIDS (UA 9409 NSN 6545-01-537-1100 LIN J00447). The N403 set contains necessary equipment and reagents to identify commonly encountered pathogenic bacteria. Although this medical set does not contain an authoritative capability for identifying biological warfare agents, it supports diagnostics of common bacterial infections. The current laboratory capability includes routine bacterial identification but susceptibility testing is not currently being included. The capability for bio threat agent detection through is through the JBAIDS. Specimens requiring more comprehensive analysis capabilities still require forwarding to the nearest reference or confirmatory laboratory, including the currently deployable assets for each service.

6.2.2.1 Army

The Area Medical Laboratory (AML) is a modular, task-organized, and corps-level asset providing comprehensive laboratory support to theater Commanders [5], [7]. The AML has transitioned from the original mission of testing primarily clinical specimens, with a capability for environmental samples (supporting force
health protection), to strictly an environmental sample lab. They can test for a broad range of biological, chemical, and radiological hazards. For biological agents, the laboratory uses a variety of rapid analytical methods, including molecular methods (such as real-time PCR), immunoassays (such as Electrochemiluminescence (ECL) and Enzyme-Linked Immunosorbent Assay (ELISA)), and more advanced analyses involving bacterial culture, fatty acid profiling, and immunohistochemistry. The AML is the largest of the service deployable laboratories and can typically staff missions with a mix of microbiologists, biochemists, veterinary pathologists, and physicians. They maintain a degree of redundant equipment for long-term or split-base operations.

The 20th CBRNE Command (CBRNE – Chemical, Biological, Radiological, Nuclear and Explosives), previously called 20th Support Command CBRNE, they consolidated a multitude of assets under a single operational headquarters. Their mission is to detect, identify, assess, render-safe, dismantle, transfer, and dispose of unexploded ordnance, improvised explosive devices and other CBRNE hazards, including biological warfare agents. The CARA Mobile Expeditionary Laboratory, a unit within the 20th, provides high-throughput chemical, explosives, and biological sample analysis and has three mobile lab packages: a Light Mobile Expeditionary Lab (LMEL), a Heavy Mobile Expeditionary Lab (HMEL), and a Chemical Air Monitoring System (CAMS) platform. LMEL, HMEL, and CAMS deploy to support Weapons of Mass Destruction (WMD) elimination and remediation efforts in forward deployed areas.

6.2.2.2 Navy

The Navy’s Forward Deployable Preventive Medicine Units (FDPMUs) are medium-sized mobile laboratories using multiple rapid techniques (PCR and ELISA) for identifying biological warfare agents on the battlefield. The FDPMUs are also modular and have the ability to analyze samples containing chemical and radiological hazards. These laboratories specialize in identifying biological threat agents in concentrated environmental samples (high confidence), but they can also identify endemic infectious disease in clinically relevant samples.

6.2.2.3 Air Force

Air Force Biological Augmentation Teams (FFBATs) and Homeland Defense Laboratory Response Team (HLD LRT) use rapid analytical methods (such as real-time PCR) and immunological methods to screen environmental and clinical samples for threat agents [8], [9]. FFBAT teams are small (two members), easily deployed, and typically housed in a separate facility designed to be collocated with pre-existing or planned medical facilities. The units are capable of providing early warning to Commanders about the potential presence of biological threat agents, typically in support of installation protection programs. The theater Commander, in conjunction with the theater surgeon and nuclear, biological, and chemical officer, must decide which and how many of these laboratories are needed, based on factors such as the threat of a biological attack, the size of the theater, the number of detectors and sensitive sites in the theater, and the confidence level of results needed.

6.2.3 Defense Laboratory Network (DLN)

The response to future chemical and biological threats will require an integrated military laboratory network that can respond with agility and competence. The logistical and technical burden of preparing for all possible health threats will be too great for the military clinical or field laboratories, which have limited space and weight restrictions. The most important role of these laboratories is to provide rapid and accurate laboratory support to medical diagnosis, rule out the most common of threats, and alert the command about suspicious disease occurrences. The military DLN consists of the front-line medical treatment facility clinical laboratories or deployed military laboratories backed by regional medical treatment facilities or military reference laboratories with access to more sophisticated diagnostic capabilities. The clinical laboratories in the regional medical centers
or large medical activities are the gateways into the civilian LRN sponsored by the CDC. At the top of the military response capability are research laboratories, such as USAMRIID (Fort Detrick, MD) and the Naval Medical Research Center (Silver Spring, MD). Other laboratories, like the U.S. Air Force Institute for Operational Health (Wright Patterson AFB, OH) and the Naval Health Research Center (San Diego, CA) also provide reference laboratory services for a myriad of endemic infectious diseases. Military research laboratories have traditionally solved some of the most complex and difficult diagnostic problems, but they are not routinely organized to perform high-throughput clinical sample processing and evaluation. Sentinel laboratories are generally supported by the network’s designated confirmatory laboratories but may communicate directly with national laboratories if necessary. The network of military laboratories with connections to federal and state civilian response systems provides unparalleled depth and resources to the biological threat response (Figure 6-2). The DLN is a standing member of the federal Integrated Consortium of Laboratory Networks (ICLN). The ICLN was established in 2005 under a Memorandum of Agreement signed by senior officials of federal agencies including the Departments of Agriculture, Defense, Commerce, Energy, Health and Human Services, Homeland Security, Interior, Justice, State, and the Environmental Protection Agency (https://www.icln.org/ valid February 2014). The ICLN was charged with promoting enhanced commonality and integration of network functions. While the ICLN does not direct resources or operations, the ICLN provides an environment for integrating network operations and strategies. The Department of Homeland Security is charged with overall leadership and coordination.

Figure 6-2: The Network of Military Laboratories with Connections to Federal and State Civilian Response Systems Provides Unparalleled Depth and Resources to the Biological Threat Response. National Laboratory Response Network for Bioterrorism.
6.2.4 Identification Levels

Rapid infectious disease diagnostics are not quantitative, not linked to traceable standards, and, overall, are not as well developed as other laboratory technologies. The inherent biological variability that exists between any two organisms (mammalian and microbial) complicates the ability to discern with absolute certainty the perpetrator of an infectious disease event. Laboratory tests for many infectious agents are not highly automated and still rely on decades-old technologies and techniques. Culture remains the gold standard for identifying organisms, but not all infectious diseases can be cultured, or are difficult to culture in routine microbiology laboratories, making alternative methods necessary. These constraints significantly impact the confidence at which results on diagnostic or detection assays for infectious agents can be reported. It often goes unstated that the best can be done in biology is that, with high confidence, what is incriminated as the infectious disease agent has high probability of being correct.

When culture is difficult or not available (i.e. virus cultures in field laboratories), serological diagnosis of the antibody response to the organism has been, and is still, a useful method, and sometimes the only way to discern some infections. The problem with both traditional culture and immunodiagnoses (serodiagnoses) is the time required to results. Culture may take several days and serodiagnosis is limited by the time required to mount an antibody response, often a week or more (Figure 6-3). Within the past few decades molecular and immunodiagnostic technologies have been developed to improve the specificity and time to obtain diagnostic and detection information on infectious agents. Immunodiagnostic technologies are based on the use of antibodies, proteins produced by an immune response to harmful substances, called antigens. By using antibodies produced against specific infectious disease agents, diagnostic and detection assays can be generated that can detect the presence of those infectious disease agents within minutes. Molecular diagnostics are based on the detection of specific nucleic acids characteristic of the infectious disease agent. Often the molecular diagnostic assay has to rely on the amplification of specific DNA sequences from extracted nucleic acids, DNA or RNA. Amplification techniques take tiny amounts of nucleic acid material and replicate them many times through enzymatic reactions, some that occur through cycles of heating and cooling. With these techniques comes more ambiguity on interpreting the results of the assays. Unlike culture, which is definitive if present, immunodiagnostic and molecular diagnostic assays have various levels of false-positive and false-negative results. Being able to discern false-positive and false-negative results from true results becomes a risk management effort, aided through the implementation of different levels of “identification” to express the degree of certainty or confidence that the assay results represent.
Figure 6-3: The Typical Infection and Response Time Course Begins with the Initial Pathogen Encounter and Leads to the Formation and Maintenance of Active Immunological Memory (IgM and IgG) where Serological Detection is Useful. Clinical disease, however, typically occurs around days 3 – 5 where detection of the infectious agent is possible. Often, by the time clinical disease is manifest, especially for the bio threat agents, clinical intervention to insure survivability is not as effective as one would like. To provide the most effective medical intervention on infectious agents, the closer to time 0 laboratory data is available, the more successful the outcome.

6.2.4.1 Civilian

For the civilian laboratory system, there are two levels of identification, presumptive and confirmed, codified in the CDC LRN program [10]. In 1998 [11], following demonstration that Iraq sponsored state activities involving production and use of biological weapons, President Clinton issued Presidential Decision Directive 62 (PDD-62), titled Combating Terrorism, assigned specific missions to Federal departments and agencies. That directive included a request to Congress to provide funding to the DHHS to support a renewed program of public health preparedness. In 1999, the LRN was established by the Department of Health and Human Services, Centers for Disease Control and Prevention CDC in collaboration with founding partners, the Federal Bureau of Investigation, DoD, and the Association of Public Health Laboratories. The mission statement is: “The LRN is a critical national security infrastructure asset that, with its partners, will develop, maintain and strengthen an integrated domestic and international network of laboratories to respond rapidly to biological, chemical and radiological threats and other high priority public health emergencies through training, rapid testing, timely notification and secure messaging of laboratory results.”
Identification within the LRN-B (biologic portion of the LRN system) is in part based on the level of testing; sentinel, reference, and national laboratories (Figure 6-1) and the protocols they use. **Sentinel laboratories** represent the thousands of community-based hospital laboratories that have direct contact with patients and would probably be the first to spot atypical infectious disease presentations. Sentinel laboratories do not actually confirm the presence of biological agents but rather are trained to recognize and appropriately handle biological agents that could potentially be an extremely dangerous pathogen. They are responsible for referring presumptive samples to a reference level lab for more definitive testing. **Reference laboratories** perform standardized tests to detect, and typically confirm, the presence of biological agents that could represent a biological threat. **Reference laboratories** are normally the state public health laboratories and include the ability to work with biological agents at a biosafety Level 3 [12], but also include some county public health, animal health/veterinarian, and food safety laboratories. Most of the reference laboratories are capable of producing identification results allowing local authorities to respond quickly to emergencies; public health actionable results that allows for broad treatment of patient populations. The CDC LRN protocols, however, are currently limited to bacterial agents, orthopoxviruses, and a couple biologic toxins, and only some of the protocols have full “confirmation” methods for reference laboratory use (Table 6-2). A recent programmatic change to the LRN-B system will subdivide the network configuration for the reference laboratories. The LRN-B reference laboratories will be broken down into Limited (RL3), Standard (RL2), and Advanced levels (RL1). The standards for each level will be based in part on the minimum operational biosafety level, the core instrumentation and equipment available (including advanced platforms), and testing capabilities (number of agents and technologies). Limited laboratories (RL3) will typically do limited, specialized testing not meeting RL2 standards. Standard laboratories (RL2) will be typical state public health laboratories capable of the full agent testing capability on clinical and high risk environmental samples. Advanced laboratories (RL1), typically state public health laboratories in regional locations that cover a risk-based, priority population center (under the U.S. Department of Homeland Security (DHS) Urban Area Security Initiative [13]), will be capable of additional testing capabilities using advanced detection and characterization methods such as mass spectrometry. The three **National laboratories** have unique resources to handle highly infectious agents (typically at the biosafety Levels 3 and 4 [12]) and the ability to identify and characterize more agents to include BSL4 viruses.
### Table 6-2: Presumptive and Confirmation Methods.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Disease</th>
<th>Presumptive</th>
<th>Confirmatory (LRN)</th>
<th>Key Identity Markers</th>
<th>BSL-2</th>
<th>BSL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>Anthrax</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Culture and gamma phage with capsule or PCR of a culture with three assays</td>
<td><em>B. anthracis</em> is one of over 260 different Bacillus spp. but is readily distinguishable from the others by the production of beta-hemolysin that is readily apparent on blood agar plates. <em>B. anthracis</em> exists as both a vegetative cell and as an environmentally stable spore. <em>B. anthracis</em> contains 2 plasmids, pXO1 and pXO2 that impart virulence characteristics and serve as diagnostic markers for both immunoassay and nucleic acid assays. Immunoassays will differ when testing for the vegetative cell or the spore. While immunoassay and nucleic acid analysis can be used for diagnostic confirmation, culture is required for confirmation.</td>
<td>Culture; PCR; immunoassay</td>
<td>Not needed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunoassay (spore vs. cell) – HHA- or plate-based</td>
<td></td>
<td>Gramp-positive rod; spore-forming; aerobic; non-motile catalase positive; large, gray-white to white; non-hemolytic colonies on sheep blood agar plates;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td>Brucellosis</td>
<td>Nucleic acid amplification (PCR) (not species-specific)</td>
<td>Culture with biochemical testing</td>
<td>Depending on the taxonomy being used, <em>Brucellae</em> contain 10 recognized species that include <em>B. abortus</em>, <em>melitensis</em>, and <em>suis</em>, the most common and important human pathogens. Differentiating the human pathogenic species from the other <em>Brucellae</em>, however, is not easy and requires several growth and biochemical determinations. Immunoassay and nucleic acid assays are currently not helpful in distinguishing the pathogens from the non-pathogens. Culture and biochemical testing is required for confirmation.</td>
<td>Initial culture; PCR; immunoassay</td>
<td>Culture confirmation</td>
</tr>
<tr>
<td><em>Brucella melitensis</em></td>
<td>Brucellosis</td>
<td>Immunoassay (not species-specific) – HHA- or plate-based</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Disease</td>
<td>Presumptive</td>
<td>Confirmatory (LRN)</td>
<td>Key Identity Markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brucella suis</em></td>
<td>Brucellosis</td>
<td></td>
<td></td>
<td>Gram-negative coccobacilli or short rods; white, non-motile, non-encapsulated, non-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>spore forming, slow-growing, nonhemolytic colonies on sheep blood agar plates; some</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>species require enhanced CO₂ for growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia mallei</em></td>
<td>Glanders</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Culture with biochemical testing</td>
<td><em>B. mallei</em> and <em>B. pseudomallei</em> are two of the 60 currently recognized species that</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>include other human pathogens. As part of their environmental saprophytic lifestyle,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>the <em>Burkholderia</em> are complex organisms that are readily culturable but often display</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>colony morphology variations that confound routine microbiological analysis.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Biochemical differentiation, including gentamicin and polymyxin susceptibility,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>determination of arginine dihydrolase and lysine decarboxylase, and arabinose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>fermentation are required for differentiation and confirmation.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia pseudomallei</em></td>
<td>Melioidosis</td>
<td></td>
<td></td>
<td>Gram-negative rod; oxidase-positive to variable, small, non-motile, non-spore-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ultrating, non-encapsulated; primary isolation requires 48 – 72 h at 37°C;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>non-hemolytic, typically about 1 mm in width, white (turning yellow with age);</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>B. pseudomallei</em> grows well on MacConkey agar but <em>B. mallei</em> does not;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Botulism A-E</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Mouse testing</td>
<td>Gram-positive rod; spore-forming; obligate anaerobe catalase negative; lipase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunoassay – HHA- or plate-based</td>
<td></td>
<td>production on egg yolk agar; 150,000 dal protein toxin (Types A,B,C,D,E,F,G); 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>sub-units</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**BSL-2**

Initial culture; PCR

**BSL-3**

Culture confirmation

Initial culture; PCR; immunoassay; Toxin-antitoxin neutralization test
## LABORATORY IDENTIFICATION OF BIOLOGICAL THREATS

<table>
<thead>
<tr>
<th>Organism</th>
<th>Disease¹</th>
<th>Presumptive²</th>
<th>Confirmatory (LRN)³</th>
<th>Key Identity Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>Epsilon toxin</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Not in LRN</td>
<td>Gram-positive rod; spore-forming; obligate anaerobe catalase negative; 5 types (A-E) but only types B and D produce the epsilon toxin; on a blood agar plate produces double zone beta hemolysis; Initial culture; PCR; immunoassay</td>
</tr>
<tr>
<td><em>Coxiella burnetii</em></td>
<td>Q-fever</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Send to CDC</td>
<td><em>Coxiella burnetii</em> is an obligate intracellular parasite that makes routine culture difficult. Culture in eggs or cells has previously been required so routine laboratory diagnostics are not common. While highly infectious, <em>Coxiella burnetii</em> is typically not fatal and often serology is used for diagnosis. Direct fluorescent antibody and nucleic acid assays are often used for presumptive and confirmatory diagnostics. PCR; immunoassay</td>
</tr>
<tr>
<td><em>Francisella tularensis</em></td>
<td>Tularemia</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Culture with direct fluorescent antibody stain</td>
<td><em>F. tularensis</em> sub-species tularensis (Type A) and <em>F. tularensis</em> sub-species holarctica (Type B) are the two most virulent strains of this expanding group of organisms. Until recently, <em>F. tularensis</em> Type A or B were restricted to the northern hemisphere where <em>F. tularensis</em> Type A or B is common in North America but only <em>F. tularensis</em> Type B is typically found in Europe and Asia. <em>F. tularensis</em> is relatively easy to grow and growth is required for confirmation, typically by the direct fluorescent antibody assay. Extremely small, pleomorphic, Gram-negative coccobacilli; non-spore forming; facultative intracellular parasite; non-motile; catalase positive opalescent smooth colonies on cysteine heart agar. Initial culture; PCR; immunoassay</td>
</tr>
<tr>
<td><em>Rickettsia prowazekii</em></td>
<td>Louse-borne typhus, Typhus exanthematicus</td>
<td>JBAIDS nucleic acid amplification (PCR)</td>
<td>Not in LRN</td>
<td>Gram negative, obligate intracellular parasitic, aerobic bacteria</td>
</tr>
</tbody>
</table>
## LABORATORY IDENTIFICATION OF BIOLOGICAL THREATS

<table>
<thead>
<tr>
<th>Organism</th>
<th>Disease¹</th>
<th>Presumptive²</th>
<th>Confirmatory (LRN)³</th>
<th>Key Identity Markers</th>
<th>BSL-2</th>
<th>BSL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rickettsia rickettsii</td>
<td>Spotted fever</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Immunoassay – HHA- or plate-based</td>
<td>Gram-positive, cocci; facultative anaerobic, large round white to yellow, beta-hemolytic colonies on sheep blood agar; characteristic ‘grape-cluster’ on Gram stain; catalase and coagulase-positive; multiple toxins depend on strain</td>
<td>Initial culture; Not needed immunoassay</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Enterotoxin A and B SEA and SEB</td>
<td>Immunoassay – HHA- or plate-based</td>
<td>Not in LRN</td>
<td>Y. pestis belong to a smaller group of organisms but is much more difficult to correctly identify. Y. pestis have several plasmids that confer various virulence traits and are useful diagnostic assay targets but the plasmids are promiscuous and can be found in non-Y. pestis causing the potential for false-positive assays. Capsule (F1) is a good marker for the diagnosis of Y. pestis but does not get produced at the optimal growth temperature for Y. pestis (28°C) but instead is produced at 35-7°C making this marker less reliable for environmental Y. pestis detection. Immunoassay and nucleic acid assays are available for diagnostics but confirmation of Y. pestis is done using phage on cultural growth. Gram-negative coccobacilli often pleomorphic; non-spore forming; facultative anaerobe; non-motile beaten copper colonies (MacConkey’s agar)</td>
<td>Initial culture; PCR; immunoassay</td>
<td>Culture confirmation</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>Plague</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Culture with phage testing</td>
<td>Y. pestis belong to a smaller group of organisms but is much more difficult to correctly identify. Y. pestis have several plasmids that confer various virulence traits and are useful diagnostic assay targets but the plasmids are promiscuous and can be found in non-Y. pestis causing the potential for false-positive assays. Capsule (F1) is a good marker for the diagnosis of Y. pestis but does not get produced at the optimal growth temperature for Y. pestis (28°C) but instead is produced at 35-7°C making this marker less reliable for environmental Y. pestis detection. Immunoassay and nucleic acid assays are available for diagnostics but confirmation of Y. pestis is done using phage on cultural growth. Gram-negative coccobacilli often pleomorphic; non-spore forming; facultative anaerobe; non-motile beaten copper colonies (MacConkey’s agar)</td>
<td>Initial culture; PCR; immunoassay</td>
<td>Culture confirmation</td>
</tr>
<tr>
<td>Organism</td>
<td>Disease¹</td>
<td>Presumptive²</td>
<td>Confirmatory (LRN)³</td>
<td>Key Identity Markers</td>
<td>BSL-2</td>
<td>BSL-3</td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
<td>--------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Crimean-Congo hemorrhagic fever virus (CCHF) / bunyaviruses</td>
<td>Viral hemorrhagic fevers</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Not in LRN</td>
<td>Single negative-stranded, tripartite genomes [large (RNA-polymerase), medium (glycoproteins), small (nucleocapsid protein)] exist in a helical / pseudo-circular structure; enveloped RNA viruses</td>
<td>PCR</td>
<td>Culture confirmation/BSL4</td>
</tr>
<tr>
<td>Ebola, Marburg virus / filoviridae viruses</td>
<td>Viral hemorrhagic fevers</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Not in LRN</td>
<td>Linear, negative-sense single-stranded RNA virus; enveloped; filamentous or pleomorphic, with extensive branching, or U-shaped, 6-shaped, or circular forms; limited cytopathic effect in Vero cells</td>
<td>PCR</td>
<td>BSL-4</td>
</tr>
<tr>
<td>Lassa / Arenaviruses</td>
<td>Viral hemorrhagic fevers</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Not in LRN</td>
<td>Two single-stranded RNA segments ambisense RNA virus; beaded nucleocapsid, spherical with glycoprotein spikes</td>
<td>PCR</td>
<td>BSL-4</td>
</tr>
<tr>
<td>Variola major</td>
<td>Smallpox</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Send to CDC</td>
<td>Large double-stranded DNA virus; enveloped, brick- shaped morphology; Guarnieri bodies (virus inclusions) under light microscopy</td>
<td>PCR; immunoassay</td>
<td>BSL-4 (CDC ONLY)</td>
</tr>
<tr>
<td>Venezuelan Equine Encephalitis virus / alpha viruses</td>
<td>Viral encephalitic disease</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Not in LRN</td>
<td>Linear positive-sense single-stranded RNA virus; enveloped, spherical virions with distinct glycoprotein spikes; cytopathic effect in Vero cells</td>
<td>PCR</td>
<td>Culture confirmation</td>
</tr>
<tr>
<td>Yellow fever virus / flaviruses</td>
<td>Viral encephalitic disease</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Not in LRN</td>
<td>Linear positive-sense single-stranded RNA virus; enveloped, icosahedral nucleocapsid; cytopathic effect in Vero cells</td>
<td>PCR</td>
<td>Culture confirmation</td>
</tr>
<tr>
<td>Organism</td>
<td>Disease¹</td>
<td>Presumptive²</td>
<td>Confirmatory (LRN)³</td>
<td>Key Identity Markers</td>
<td>BSL-2</td>
<td>BSL-3</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Ricin toxin</td>
<td>Ricin intoxication</td>
<td>Nucleic acid amplification (PCR)</td>
<td>Send to CDC</td>
<td>60,000–65,000 dal protein toxin; 2 sub-units castor bean origin</td>
<td>PCR; immunoassay</td>
<td>Not needed</td>
</tr>
</tbody>
</table>

The CDC LRN, as a system, includes laboratories, secure communications, training, protocols, reagents, and proficiency testing. LRN member laboratories include federal laboratories (including laboratories at CDC, the U.S. Department of Agriculture, the Food and Drug Administration (FDA), and other facilities run by federal agencies), state and local public health, military (Department of Defense laboratories located both within the United States and abroad), food testing (FDA and USDA laboratories), environmental (water and other environmental samples), veterinary (USDA and state), and international laboratories (Canada, the United Kingdom, Australia, Mexico, and South Korea). As the LRN-B continues programmatic maturation, they will not only continue to address biological terrorism preparedness and response (national security and public health emergency preparedness), but also address emerging infectious disease preparedness and response (such as newly emerging viruses like MERS CoV) and biosurveillance.

6.2.4.2 Military

Military identification levels differ from the civilian system in two specific aspects:

1) Current military doctrine identifies four levels of identification based in part on what level or what unit does the testing. The military identification levels consist of four levels, denoted as:
   - Presumptive;
   - Field confirmation;
   - Theater level validation; and
   - Definitive.

2) The concept of testing for “biological markers” (biomarker) rather than culturing the specific agents.
The military concept of testing for biomarkers follows the logic that if the biomarker is present, that is an indication that the agent of interest is also present.

There are some complications with using biomarkers. First, there is a problem with the definition of what a biomarker is. While the term is used in military doctrine [5], there is no current definition in doctrine to guide the application. Multi-service Tactics, Techniques, and Procedures (MTTP) for Biological Surveillance [14] provided a definition, but that document was replaced by ATP 3-11.37 [7] in 2013 and the definition was lost. In upcoming doctrinal revisions, probably in ATP 4-02.7, “MTTP for Health Services Support in a CBRN Environment”, a definition will be re-established. The definition in the old FM 3-11.86, Appendix B, paragraph 3.a stated “A biomarker refers to the characteristics of a biological agent (microorganism or toxin) that are specific or unique to that agent”. That reference also went on to provide additional information regarding the usefulness or accuracy in identifying the biological agents, the sensitivity of scientific devices and/or methodologies for biomarkers, and examples of biomarkers. The types of biomarkers listed included nucleic acid sequences, antigens or toxins for immunological methods, growth properties (as demonstrated on biochemical tests or selective media), and microscopic characteristics. A good doctrinal definition will help guide correct application in the absence of specific details. Another scientific concern with the use of biomarkers is that some biomarkers are present from non-threat infectious agents. While these are typically considered false positive analyses, they really aren’t false positives but do complicate the ability to determine the true presence or absence of biological agents of concern. Biomarkers also don’t necessarily reflect viability of the infectious agent. While the simple presence or absence of an agent can be important, determination of viability may be a significant component, especially in non-clinical samples where the biomarker could be simply background flora. Another consideration in the reliance on biomarkers without culture, more definitive information on the infectious agent, like anti-microbial resistance, epidemiological strain typing, or as legal evidence for forensic science and attribution purposes, is not done. Concentrating on biomarkers could lead to a myopic result that limits the full understanding of medical implications for an incident or outbreak.

The military identification levels are well-defined in doctrine as follows [5], [7] (Figure 6-4).
Presumptive identification of a biological threat agent is achieved by the detection of a biological marker using a single test methodology (for example, Hand-Held Assay (HHA)). Presumptive identification employs technologies with limited specificity and sensitivity by general-purpose forces in a field environment to determine the presence of a biological hazard with a low level of confidence and but with a degree of certainty necessary to support immediate tactical decisions. Since identification at this level is based on specific technologies, they are limited to the assays deployed and cannot detect or identify new or emerging infectious disease agents for which the technologies assays are not available.

Field confirmatory identification is achieved when two or more independent technologies confirm the identification of a biological agent. This may be an immunoassay (for example, Hand-Held Assay (HHA); Electrochemiluminescence (ECL); Enzyme-Linked Immunosorbent Assay (ELISA)), PCR result, and/or culture growth/microscopy. According to doctrine, a single result from JBAIDS can be used as a field confirmatory identification. A genomic biomarker must be included. Field confirmatory identification employs technologies with increased specificity and sensitivity, by technical forces in a field environment, to identify the presence of biological agents with a moderate level of confidence and a degree of certainty necessary to support follow-on tactical decisions. Depending on the technologies deployed (i.e. culture), there is some limited ability to detect or identify infectious disease agents beyond the limits of deployed assays.
**Theater validation** is achieved using devices, materials, or technologies that detect biomarkers using two or more independent biomarker results (for example, one biomarker is detected by two or more independent methodologies or more than one biomarker is detected by a single methodology). Examples are:

1) Hand-held immunological assay plus nucleic acid amplification; or
2) Nucleic acid amplification using two different biomarkers (for example, gene targets).

Theater validation identification employs multiple independent, established protocols and technologies by scientific experts in a controlled environment of a fixed or mobile/transportable laboratory to characterize biological materials with a high level of confidence and the degree of certainty necessary to support operational level decisions. After a preventive medicine detachment, a Combat Support Hospital (CSH), and/or CBRN reconnaissance assets identifies a biological/clinical specimen as a biological threat agent, the specimen is sent by courier to those specialized laboratories/teams with advanced microbiological capabilities and highly skilled medical personnel. These could include laboratories/teams such as an AML, 20th SUPCOM CARA, United States Air Force (USAF) biological augmentation team, or United States Navy (USN) FDPMU when available in the operational area. While the units listed here have the potential to produce theater validation level results, they may not inherently have that capability deployed in all circumstances. The theater validation laboratories must implement a quality assurance program, preferably with independent audits, proficiency testing, scientist level data review, document control, demonstration of procedure traceability, some level of electronic sample management, documentation of personnel training, and accreditation (if available). These laboratories would typically conduct initial field confirmatory analysis (quick report) followed by theater validation (more testing and time). If these specialized laboratories/teams are unavailable, biological specimens that are presumptive positive for a biological threat agent will have to be forwarded to the nearest reference laboratory, even if this is in CONUS.

**Definitive** identification is the correlation of a biological agent to a known substance, or in the case where the substance is previously unknown, the substance is type classified and analyzed. Definitive identification is the employment of multiple state-of-the-art, independent, established protocols and technologies by scientific experts in a nationally recognized laboratory to determine the unambiguous identity of a biological agent with the highest level of confidence and degree of certainty necessary to support strategic-level decisions. It also supports the initiation of attribution to implicate or point to the source of the identified material. In all cases a definitive identification occurs at a U.S.-based and sanctioned reference laboratory specifically equipped to perform detailed analysis on the type of suspect material to be identified. Definitive identification typically includes the ability to propagate the biological agent so that there is sufficient material available for analysis by the multiple methods and protocols, the ability to look at strains by epidemiological methods, but also so that there is material available to initiate attribution analysis. Definitive identification is performed using the highest level quality assurance measures in a controlled laboratory. Definitive identification or “confirmation” testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC Laboratory Response Network (LRN) as appropriate. Specific LRN protocols and reagents are proprietary but any definitive identification or confirmation typically follows a well-established scheme, including the use of well-characterized reagents by well-practiced personnel.

Like biomarkers, there are also inherent problems with the application and details involved in the identification levels that need to be understood in order to correctly apply the inherent concepts contained within the definitions and an appropriate application of the term “confirmed”. In one definition of “confirmation” it states “the occurrence of two or more indicators corresponding with one another and thereby corroborating the predicted outcome.” Confirmation of an identification of a biological agent, however, often needs to be grounded in more information, especially given the consequences of an incorrect identification to both the military
member as well as the military operation being conducted. In addition, identification of a biological agent based on non-metabolic methods, in the absence of morbidity or mortality, always presents with the possibility that the identification is detecting inactive materials [15]. Biological materials, microbes and toxins, are fragile compared to nuclear or chemical agents. They can be inactivated during the course of dispersal (especially dissemination from munitions), through natural biocidal activity (sunlight both desiccates as well as inactivates through ultraviolet irradiation), ineffective weaponization processes, or a myriad of other physical or chemical activities. The confidence in an identification of a biological attack is also affected by how it has been detected [7], [8]. While doctrinally, low, medium, and high confidence are part of the identification levels, the level of confidence an assay provides is also governed by factors that include the scientific quality and accuracy of the test methods, the target or purpose of the assay(s), experience and knowledge of testing personnel, and the environment in which the lab is operating [8]. Detection by one biological detector system has a lower confidence level than if two detectors have made the detection. Theater validation identification (including two biomarkers) endorses and bolsters those automated detections, but “confirmation” should still be viewed with a level of suspicion due to inherent biological diversity. Until a full characterization of the agent can be undertaken, the term “confirmed” should be used with some level of reservation, and military Commanders, responsible for both the mission and the service members, should proceed with the realization of the ambiguous nature that biological threats present.

6.2.4.3 Allies
U.S. allies, especially those members of the North Atlantic Treaty Organization (NATO), have different doctrinal identification levels. Prior to 1995, NATO recognized the need for common approaches for Sampling and Identification of Biological and Chemical warfare Agents (SIBCA). Within the NATO doctrine, there are also three levels of identification [15]:

1) **Provisional Identification** – A biological agent may be considered provisionally identified when one of three criteria are met (presence of a unique antigen, presence of a unique nucleic acid sequence, or positive culture or multi-metabolic assay);

2) **Confirmed Identification** – The identification of a biological agent is confirmed when any two of the three criteria for provisional identification have been met in the presence of authentic reference standards (positive and negative controls) under identical experimental conditions; and

3) **Unambiguous Identification** – The unambiguous identification of a biological agent provides the highest level of certainty required for the development of strategic and political positions.

Confirmed identification becomes unambiguous under four criteria (positive response is obtained by a genetic identification method; positive response is obtained by an immunological method; positive match is obtained by *in vitro* culture or multi-metabolic assay; and the disease properties of the microbial agent are confirmed in an accepted animal model).

6.3 IDENTIFICATION APPROACHES

6.3.1 Specimen Collection and Processing
Clinical specimens can be divided into three different categories based on the ability to affect the disease course:

1) Early post-exposure;

2) Clinical; and

3) Convalescent/terminal/post-mortem [5], [16].
Common specimens for biological warfare agents are similar to those collected for diagnosis of any infectious disease and typically correspond to clinical manifestations (Table 6-3). Specimens often include swabs, induced respiratory secretions, blood cultures, serum, sputum, urine, stool, skin scrapings, lesion aspirates, and biopsy materials [5], [17]. Nasal and facial swab samples should not be used for making decisions about individual medical care, however, they could support the rapid identification of a biological threat (post-attack) and help direct force health protection efforts [18], [19]. Baseline serum samples (pre-symptomatic) should be collected on all potentially exposed personnel after an overt attack. These samples will help to both define the forces exposed but could also provide diagnostic information in the event of non-traditional agents being used. In cases of sudden or suspicious deaths, autopsy samples should be taken. Specimens and cultures containing possible highly infectious agents should be handled in accordance with established biosafety precautions. Specimens should be sent rapidly (within 24 hours) on wet ice (2°C to 8°C) to an analytical laboratory capable of handling them. Blood cultures should be collected before the administration of antibiotics. If necessary, the blood cultures should be shipped to the laboratory within 24 hours at room temperature (21°C – 23°C). Overseas (OCONUS) laboratories should not attempt to ship clinical specimens to CONUS reference laboratories using only wet ice unless the provisions for re-icing the samples is made with the carrier. Shipments requiring more than 24 hours should be frozen on dry ice or liquid nitrogen if possible. Specific shipping guidance should be obtained from the supporting laboratory before shipment. Specimens should not be treated with permanent fixatives (i.e. formalin or formaldehyde) unless that is the only way to insure sample stability. Storage and shipping of samples at -20°C – 25°C is contraindicated.
### Table 6-3: Specimen Collection for Select Biological Warfare Agents.

<table>
<thead>
<tr>
<th>Pathogen(s)</th>
<th>Incubation Period</th>
<th>Post-Exposure</th>
<th>Clinical</th>
<th>Convalescent/Terminal/Post-Mortem</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus anthracis</strong></td>
<td>1 – 3 days; 3 days</td>
<td>0 – 72 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Serum for toxin assays; whole blood (blood cultures); and tissue smears for direct fluorescent antibody</td>
</tr>
<tr>
<td><strong>Brucella</strong></td>
<td>5 – 60 days; 5 days</td>
<td>0 – 36 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Whole blood (blood cultures); NOTE: Notify laboratory for extended culture incubation protocol.</td>
</tr>
<tr>
<td><strong>Burkholderia pseudomallei / mallei</strong></td>
<td>1 – 21 days; 3 days</td>
<td>0 – 48 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Serum for capsular polysaccharide assays; whole blood (blood cultures)</td>
</tr>
<tr>
<td><strong>Clostridium botulinum / botulinum Toxin A/B/E</strong></td>
<td>0 – 24 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions for toxin detection</td>
<td>24 – 72 hours</td>
<td>Blood or serum for toxin detection</td>
</tr>
<tr>
<td><strong>Coxiella burnetii</strong></td>
<td>7 – 41 days</td>
<td>0 – 72 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Whole blood (blood cultures) and direct molecular detection</td>
</tr>
<tr>
<td><strong>Encephalitic viruses / alpha viruses / VEE, etc.</strong></td>
<td>2 – 6 days</td>
<td>0 – 24 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Throat swabs up to 5 days then cerebrospinal fluid and serum</td>
</tr>
<tr>
<td><strong>Francisella tularensis</strong></td>
<td>1 – 21 days; 3 days</td>
<td>0 – 24 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Whole blood (blood cultures); direct fluorescent antibody</td>
</tr>
<tr>
<td><strong>Hemorrhagic fever viruses/ Ebola / Marburg/ Dengue, etc.</strong></td>
<td>4 – 21 days; 3 days</td>
<td>0 – 24 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Serum</td>
</tr>
<tr>
<td><strong>Ricin</strong></td>
<td>18 – 24 hours</td>
<td>0 – 24 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Serum/plasma for toxin assays; urine for ricin is questionable</td>
</tr>
<tr>
<td><strong>Staphylococcus Enterotoxin A/B/C</strong></td>
<td>3 – 12 hours</td>
<td>0 – 4 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Blood or serum</td>
</tr>
<tr>
<td><strong>Vesicular and pustular rash illnesses/ Orthopox (Variola)</strong></td>
<td>7 – 17 days</td>
<td>0 – 72 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Serum and lesions/scrapings for microscopy and viral culture</td>
</tr>
<tr>
<td><strong>Yersinia pestis</strong></td>
<td>1 – 7 days; 2 days</td>
<td>0 – 72 hours</td>
<td>Nasal and throat swabs, and induced respiratory secretions</td>
<td>Whole blood (blood cultures); direct fluorescent antibody</td>
</tr>
</tbody>
</table>
Environmental samples, while not patient-specific, are often highly useful to medical decision-making. These samples include several different categories of materials such as buffers and filters from air sampling devices, powders, soil and vegetation, animals (including rodents and insects as potential vectors), food samples from both fresh and packaged materials if ingestion is suspected, and just about everything else that isn’t a clinical sample. These samples, when taken prior to any overt disease onset, can help identify a causative agent and potentially lead to prophylactic treatment. Non-clinical samples represent the biggest challenge in the detection of biological agents due to the vast repertoire of sample types and microorganisms in the environment that will cause false-positive and false-negative detection reactions in many laboratory assays. There is a substantial amount guidance, both military-specific [5], [15] and general [20], so details of taking and processing of environmental samples is beyond the scope of this chapter. Environmental samples will contain a myriad of physical and chemical agents that can potentially interfere with detection technologies and cause false negative results. Environmental samples include samples that are both highly stable as well as samples that will degrade with time similar to clinical samples. Guidelines for the submission of environmental samples are not as well-detailed as those for clinical samples. In general, environmental samples should be maintained at nearly the same state as when they were collected. Dry samples should be kept dry, moist or wet samples should be preserved from desiccation, and cold samples should be kept cool. One especially critical requirement for any environmental sample is the initiation and maintenance of “chain of custody” documentation [7], [15], [21]; from the sample collection through to the analysis laboratory. Again, like shipping clinical samples, guidance should be obtained from the supporting laboratory before shipment.

There are a multitude of international, domestic, and commercial regulations that mandate the proper packing and documentation (including labelling) of biological materials (Table 6-4). Biological samples, infectious agents, and Biological Select Agents and Toxins (BSAT) all represent some level of dangerous goods that need special handling to protect the public, airline workers, couriers, and other persons who work for commercial shippers and who handle the dangerous goods within the many segments of the shipping process. In addition, proper packing and shipping of dangerous goods reduces the exposure of the shipper to the risks of criminal and civil liabilities associated with shipping dangerous goods, particularly infectious substances. Each of the regulations deals with specific shipping requirements but in general, all define an infectious substance as a material known or reasonably expected to contain a pathogen (a microorganism that can cause disease in humans or animals). Universal examples of pathogens include bacteria, viruses, fungi, and other infectious agents. An infectious substance is assigned to one of the following three potential categories:

1) **Category A** – An infectious substance transported in a form capable of causing permanent disability or life-threatening or fatal disease in otherwise healthy humans or animals when exposure occurs. A Category A infectious substance is assigned the identification number UN 2814 or UN 2900, based on the known medical history or symptoms of the source patient or animal, endemic local conditions, or professional judgment concerning the individual circumstances of the source human or animal.

2) **Category B** – An infectious substance that does not meet the criteria for inclusion in Category A. Category B infectious substances bear the shipping term “Biological substance, Category B” and are assigned the identification number UN 3373.

3) Toxins from plant, animal, or bacterial sources that do not contain an infectious substance, and are not contained in an infectious substance, may be considered for classification as toxic substances; UN3172.
### Table 6-4: International and Domestic Standards for Shipping.

<table>
<thead>
<tr>
<th>International</th>
<th>Domestic</th>
</tr>
</thead>
<tbody>
<tr>
<td>International Civil Aviation Organization (ICAO) Regulations ([<a href="http://www.icao.int/safety/DangerousGoods/Pages/default.aspx">http://www.icao.int/safety/DangerousGoods/Pages/default.aspx</a> valid February 2014](<a href="http://www.icao.int/safety/DangerousGoods/Pages/default.aspx">http://www.icao.int/safety/DangerousGoods/Pages/default.aspx</a> valid February 2014))</td>
<td><strong>DoD 4500.9-R, Defense Transportation Regulation;</strong> Part II, Chapter 204</td>
</tr>
<tr>
<td>Army Regulation 190-17, Biological Select Agents and Toxins Security Program (2009)</td>
<td></td>
</tr>
</tbody>
</table>
In addition, other requirements may exist, including requirements for dry ice (dry ice is classified by DOT and IATA as a ‘miscellaneous’ hazard, class 9). The International Air Transport Association (IATA) Dangerous Goods Regulations (DGR) is the leading guide to shipping dangerous goods, including infectious agents by air, which generally includes most shipments from CONUS and OCONUS. The DGR provided requirements for packaging a shipment to classify, mark, pack, label and document dangerous to meet international requirements. Key issues in shipping biological materials include, at a minimum, maintaining the sample integrity (especially metabolic viability), some identification of the sample if possible (determining appropriate Category A, Category B, or toxin), packaging requirements (packaging corresponding to category such as Category A must consist of three components: a primary receptacle(s); a secondary packaging; and a rigid outer packaging), and documentation (IATA Shipper’s Declaration for Dangerous Goods, DD Form 2890, DoD Multimodal Dangerous Goods Declaration, APHIS/CDC Form 2, Request to Transfer Select Agents and Toxins, and any import or export permits required). Other considerations for shipping biological samples may exist [5], [22], [23] and typically require personnel that have been trained and are certified to package hazardous materials for shipment (including but not limited to “Transport of Biomedical Materials,” http://usaphcapps.amedd.army.mil/traincon/Describe.aspx?Name=tbmed valid February 20147). Specific specimen collection and handling guidelines for the bioterrorism agents are available from CDC and the American Society for Microbiology (see http://www.bt.cdc.gov/bioterrorism/ or http://www.asm.org/index.php/guidelines/sentinel-guidelines both valid February 2014).

6.3.2 Culture-Based Microbiological Methods

Microbes that cause infectious disease are an example of a classic host-parasite relationship. Suspecting, or even having some evidence of a microbe’s ability to produce disease, is still inferential science. Having unequivocal proof of a specific etiological agent as the cause of an infectious disease requires the application of conventional microbial culture in order to validate Koch’s postulates (the four standards of a logical chain of experimental evidence designed to establish a causal relationship between a causative microbe and a disease). Microorganisms can cause tissue damage (disease) by releasing a variety of toxins or destructive enzymes into the host. While there are number of ways to obtain indirect evidence of a microbe’s effect on the host, propagating the causative microbial agent is still considered the gold standard for linking a specific microbial agent to the disease status.

Specific guidelines for identifying bioterrorism agents can be obtained from the CDC (http://www.bt.cdc.gov) or the American Society for Microbiology (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Guidelines for identification of additional agents that cause other infectious diseases can be found in the plethora of diagnostic microbiology textbooks. While the ability to propagate infectious disease microbes in routine culture has been available for over a century, many bioterrorism and infectious disease agents, especially the viruses, are not always easily culture. In addition, culturing a specific microbial agent from a clinical sample is often routine; culturing the same microbial agent from an environmental sample is more difficult. In either case, having an idea of which of the myriad of microbial agents you are looking for will greatly aid in an attempt to set up right conditions for propagation. A physician’s clinical observations or medical intelligence should help guide the analytical plan (see Table 6-3) [17], [24].

The bioterrorism and infectious disease agents are broken out into aerobic and anaerobic bacterial agents along with viruses. Fungal and parasitic microbial agents are not often encountered as bioterrorism and infectious disease agents against humans. Most aerobic bacterial threat agents can be isolated by using three common clinical bacteriological media:

1) 5% sheep Blood Agar (SBA);
2) MacConkey agar (MAC); and
3) Chocolate agar (CHOC).

Cystine Heart Agar (CHA) supplemented with 5% sheep blood has been suggested as a preferred medium for *Francisella tularensis*, but CHOC agar usually suffices in clinical samples. While Brucella agar was developed as a preferred medium for Brucella, improvements in SBA and CHOC agars to support the growth of fastidious microorganisms such as Brucella. Non-selective SBA supports the growth of most bacterial agents, including *Bacillus anthracis*, Brucella, *Burkholderia*, and *Yersinia pestis*. MAC agar, which is the preferred selective medium for Gram-negative *Enterobacteriaceae*, supports *Burkholderia* and *Yersinia pestis*. Liquid medium, such as trypticase soy broth, can also be used followed by sub-culturing to SBA or CHOC when solid medium initially fails to produce growth. Anaerobic organisms (those organisms that do not require oxygen for growth; some of which may react negatively or even die if oxygen is present), such as *Clostridium* species, require the use of anaerobic media and methods. Anaerobic methods reduce the exposure of microorganisms to molecular oxygen (O$_2$) through the use of anaerobic jars or anaerobic chambers, and use culture media that especially designed to dissolve or deplete O$_2$, allowing the anaerobes to propagate. The liquid medium thioglycollate readily supports anaerobic microorganisms and should be considered a routine media if *Clostridium* species could be encountered. The use of multiple bacteriological media is recommended both for redundancy as well as an aid to initial notification. Propagation of viruses is more complex and usually takes longer than those for bacteria. Since viruses are obligate intracellular parasites, propagation in various host systems are required. Most readily viruses are typically propagated in cultures of various cell lines but laboratory animals and embryonated eggs are also used. While there is no single cell culture that is sensitive to all the viruses likely to be encountered, Vero (African green monkey kidney) cells are commonly used for many of the viruses (Table 6-5). Cells used for propagating viruses require growth at an appropriate temperature and gas mixture (typically, 37°C, 5% CO$_2$ for mammalian cells) in an incubator. In addition, cell cultures also require special growth medium that have stringent requirements for pH, glucose, antibiotics, growth factors, and other nutrients. Growth factors used to supplement media are often derived from the serum of animal blood, such as fetal bovine serum. Cell plating density (number of cells per volume of culture medium) and inoculation density of the virus are critical factors. Viruses manifested their presence in cell culture by different mechanisms including cellular degeneration (Cytopathic Effect – CPE), plaque formation, and metabolic inhibition testing. Some viruses require other means to demonstrate their presence in cell culture including fluorescent antibody testing or nucleic acid amplification methods.

### Table 6-5: Viral Hemorrhagic Fever (VHF) Culture Information.

<table>
<thead>
<tr>
<th>Viral Hemorrhagic Fever (VHF)</th>
<th>Virus Endemic Area Mortality</th>
<th>Cells and Incubation Time</th>
<th>Growth Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arenaviruses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lassa virus</td>
<td>West Africa</td>
<td>1% to 2%</td>
<td>Vero E6 – Vero 3 – 5 days</td>
</tr>
<tr>
<td>Junin</td>
<td>Argentinean pampas</td>
<td>30%</td>
<td>Vero – 3 – 5 days</td>
</tr>
<tr>
<td>Machupo</td>
<td>Bolivia</td>
<td>25% to 35%</td>
<td>Vero E6 – 3 to 5 days</td>
</tr>
<tr>
<td><strong>Bunyaviruses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crimean-Congo Hemorrhagic Fever Virus (CCHFV)</td>
<td>Africa, SE Europe, Central</td>
<td>30% &lt; 0.5%</td>
<td>SM 3 to 14 days Possible to passage in E6, SW13 or CER cells after initial isolation but may require &gt; 1 blind passages.</td>
</tr>
</tbody>
</table>

Plaque assays just as difficult.
### Viral Hemorrhagic Fever (VHF)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Endemic Area</th>
<th>Mortality</th>
<th>Cells and Incubation Time</th>
<th>Growth Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rift Valley fever</td>
<td>Asia, India Africa</td>
<td>Vero – 2 to 4 days</td>
<td></td>
<td>CPE/plaques.</td>
</tr>
<tr>
<td>Hanta-virus</td>
<td>Europe, Asia, South America</td>
<td>5% for HFRS</td>
<td>Vero E6 – 10 to 14 days</td>
<td>No CPE – requires 2nd assay such as IFA or PCR. Often requires blind serial passages to isolate. Hard to plaque.</td>
</tr>
<tr>
<td>(Hantaan, Dobrava, Seoul, Puumala, Sin Nombre Andes)</td>
<td>(rare)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ebola virus</td>
<td>Africa, Philippines (Ebola Reston)</td>
<td>50% to 90% for Sudan/Zaire</td>
<td>Vero E6 – 6 to 12 days</td>
<td>CPE/plaques.</td>
</tr>
<tr>
<td>Marburg virus</td>
<td>Africa</td>
<td>23% to 70%</td>
<td>Vero E6 – 6 to 12 days</td>
<td>CPE/plaques.</td>
</tr>
<tr>
<td>Yellow fever virus</td>
<td>Africa, South America</td>
<td>Overall 3% to 12%, 20% to 50% if severe second phase develops</td>
<td>MK2 cells (also BHK21) – 3 to 6 days</td>
<td>Little to no CPE – requires 2nd assay such as PCR or IFA to confirm. Plaques fine in Vero cells</td>
</tr>
<tr>
<td>Kyasanur Forest disease virus</td>
<td>Southern India</td>
<td>3% to 5%</td>
<td>Vero/Vero E6 SM – 3 to 6 days</td>
<td>CPE/plaques</td>
</tr>
<tr>
<td>Omsk Hemorrhagic Fever virus</td>
<td>Siberia</td>
<td>0.2% to 3%</td>
<td>Vero/Vero E6 SM – 3 to 6 days</td>
<td>CPE/plaques</td>
</tr>
</tbody>
</table>

#### 6.3.2.1 Automated Identification Systems

There are a number of automated identification systems commercially available that all have some capability in the identification of the major bacterial biological threat agents (*Bacillus anthracis, Brucella* spp., *Burkholderia mallei, Burkholderia pseudomallei, Francisella tularensis, and Yersinia pestis*). These systems include the BioMérieux VITEK® 2, Siemens MicroScan®, MIDI Sherlock® Microbial Identification System, Trek ARIS2X®, Biolog, and Bruker Biotyper matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). The Becton Dickinson Phoenix™ Automated Microbiology System does not appear to be capable of identification of the major bacterial biological threat agents listed. An advantage to the automated identification systems is that, if a laboratory is routinely using one of these commercial systems, personnel are already trained and reagents are typically on-hand. The primary disadvantage is that often, false-positives or false-negatives, including misidentifications as another organism, occur (Table 6-6). While some identifications on some systems are problematic, identification of some agents by the automated systems are very accurate and often highly discriminatory. The identification of *Bacillus anthracis* and *Francisella tularensis* by the MIDI Sherlock® Microbial Identification System are very specific and accepted methods [25]-[27]. Blind acceptance of results from one of the automated commercial system, however, needs to be avoided and substantiated, or refuted, by other assay information.
Table 6-6: Automated Identification Systems for Biological Threat Agents.

<table>
<thead>
<tr>
<th>Biological Threat Agent</th>
<th>BioMérieux VITEK® 2</th>
<th>Siemens MicroScan®</th>
<th>MIDI Sherlock® Microbial Identification System</th>
<th>Trek ARIS2X®</th>
<th>Biolog</th>
<th>Bruker Biotyper®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus anthracis</td>
<td>Rapid Neg ID Type 3 plate</td>
<td>Neg ID Type 2 plate</td>
<td>Biodefense Library 3.0/ BTR3 and RBTR3 Instant FAME</td>
<td>GNID plate</td>
<td>Dangerous Pathogen Identification Database³</td>
<td>Security – Relevant (SR) library</td>
</tr>
<tr>
<td>Brucella spp.</td>
<td>Yes – BCL Card</td>
<td>–</td>
<td>–</td>
<td>Yes²</td>
<td>–</td>
<td>Yes – GP plate</td>
</tr>
<tr>
<td>Burkholderia mallei / pseudomallei</td>
<td>Yes – GN Card¹</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes – GN plate</td>
<td>Yes – GN plate</td>
</tr>
<tr>
<td>Francisella tularensis</td>
<td>Yes – GN Card¹,²</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Yes – GN plate</td>
<td>Yes – GN plate</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>Yes – GN Card</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes – GN plate</td>
<td>Yes – GN plate</td>
</tr>
</tbody>
</table>

1 – Noted as a species that may be non-reactive.
2 – AOAC INTERNATIONAL cleared for Bacillus anthracis ID.
3 – GENIII plate has been evaluated for all biological threat agents but database is not commercially available.
4 – Known false results for this organism on this system.
5 – Differentiation of Burkholderia mallei and Burkholderia pseudomallei may not be possible.
6 – Another system, BioMérieux VITEK MS is similar.

While not an automated identification system, identification of bacteria with sequence data of rRNA genes (16S or 23S) needs to be mentioned. Carl Woese pioneered this use of 16S rRNA in the late 1970s for use in phylogenetic studies [28]. 16S rRNA gene sequencing has become a standard reference method for identification of many microbes. Bacterial 16S rRNA gene sequences are available on public databases such as the National Center for Biotechnology Information (NCBI) and the Michigan State University Ribosomal Database Project. Commercially, Applied Biosystems sells 16S rDNA bacterial identification kits under the MicroSeq® name that provide standardized reagents and protocols, but they are not yet FDA-approved for direct patient care. While implementation in a routine clinical microbiology laboratory has several drawbacks for microbial identification (time and cost predominately), the accuracy and practicality for many of the biological threat agents is useful. But like all systems, there are limitations to full implementation, predominately in that Bacillus anthracis, Brucella species, and Yersinia pestis are often not able to be differentiated from near neighbors with sufficient resolution as to make the system practical.

6.3.2.2 Antibiotic and Anti-Microbial Susceptibility Testing

A principle reason for propagation of bioterrorism or infectious disease agents in culture is to screen the agent for antibiotic or anti-microbial agent resistance or susceptibility. While most of the bacterial biological threat agents have well-characterized susceptibility to antibiotics (Table 6-7), it will be critical to distinguish those organisms that acquire natural or laboratory modifications to normal or traditional anti-microbial susceptibility
There are strains of *Bacillus anthracis* [30]-[32], *Brucella abortus*, *Burkholderia* [29], *Francisella tularensis* [33], [34], and *Yersinia pestis* [35], [36] that have been reported to have natural anti-microbial drug-resistance, including multiple drug-resistances [37]-[42]. The Clinical and Laboratory Standards Institute (www.clsi.org/) has published standard protocols that include the biological threat agents, in order to ensure accuracy and reproducibility of results. For the biological threat agents classical minimum inhibitory concentration determinations are the preferred method [43]. While commercial antibiotic susceptibility testing devices are available [44]-[45], they have not been standardized to insure correspondence to the reference method. The CDC LRN does include the use of the Epsilometer test (E-test) for anti-microbial susceptibility testing of selected microorganisms. The E-test is a direct quantification agar dilution method [46] that has been adopted by many laboratories due to the ease of use and quantification capabilities. Molecular methods that screen for unique genetic markers of resistance have been developed [36], [47]-[53]; however, molecular analysis approaches can be cumbersome when multiple loci are involved [49], [50] and do not always correlate with therapeutic effectiveness nor laboratory data [29]. DNA microarrays offer the potential for simultaneous testing for specific antibiotic resistance genes, loci, and markers [48], [49], [54], but are not sufficiently developed for routine use.

**Table 6-7: Standard Antibiotic Susceptibility Testing for Biological Threat Agents.**

<table>
<thead>
<tr>
<th><em>Bacillus anthracis</em></th>
<th><em>Brucella spp.</em></th>
<th><em>Burkholderia mallei / pseudomallei</em></th>
<th><em>Francisella tularensis</em></th>
<th><em>Yersinia pestis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>Gentamicin</td>
<td>Doxycycline</td>
<td>Gentamicin</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Streptomycin</td>
<td>Tetracycline</td>
<td>Streptomycin</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Doxycycline</td>
<td>Imipenem</td>
<td>Doxycycline</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Tetracycline</td>
<td>Amoxicillin-clavulanate</td>
<td>Tetracycline</td>
<td>Tetracycline</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim-sulfamethoxazole</td>
<td>Trimethoprim-sulfamethoxazole</td>
<td>Ciprofloxacin</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Levofoxacin</td>
<td>Chlorampenicol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 6.3.3 Microbial Culture versus Rapid Methods

With the introduction of newer rapid methods for biological threat agent detection and the codification of the term “biomarkers” in the military doctrine, there has been avoidance on the discussion of classical microbiological culture in the detection of biological threat agents. Classical microbiology culture, whether for bacteria or viruses, has been stigmatized as archaic and overly time-consuming. The concept of obtaining a result in less than an hour, and be able to do something with that result, has taken center stage. While the newer rapid methods for biological threat agent detection have certainly matured over the past decade there are still problematic areas in the sole reliance on these newer methods.

Current CONOPS for theater validation laboratories are for multiple technologies that do not necessarily include culture of the organism. Most often, the use of nucleic acid amplification (through polymerase chain reaction) and immunoassays are the predominate methods for rapid identification. Operation of a theater validation laboratory with PCR and immunoassay technologies does not require the containment of a Biosafety Level 3 (BSL-3) facility.
In order to cause disease, microbial agents must be living or toxin agents must be biologically (metabolically) active. Unless and identification of a biological agent is based on some metabolic method, in the absence of morbidity or mortality, there is a possibility that the implicated agent has been inactivated [15]. Inactivation of biological materials, especially in non-clinical samples, readily occurs and culture (or multi-metabolic assays for toxins) is the only way to insure that an implicated biological agent is actually capable of causing disease.

CDC LRN reference laboratories typically include the use of BSL-3 facilities since the CDC LRN identification requires identification based on culture of the organism(s). Current DoD doctrine, at the theater validation level, does not include culture as a requirement. The CDC LRN, however, does not include viral diagnostics/detection capabilities other than the inclusion of smallpox. In some areas of operations, consideration for viral threat agents is just as high, if not higher, then for the more traditional bacterial agents. While deployed assets for the diagnostic/detection of viruses is not robust for practical reasons, consideration for those agents must be included in operations planning. While bacterial culturing, even for the majority of the biological threat agents, can be done in BSL-2 facilities, DA PAM 385-69 [55], however, invokes enhanced requirements on facilities engaged in culturing any organisms, even those less than BSL-3. Any laboratory doing culture work will have to comply with all the provisions of that reference. Laboratories not doing culture work do not invoke the requirements of the DA PAM 385-69.

Another consideration for inclusion of microbial culturing technologies includes the ability to provide sufficient samples for forensic science analysis and attribution. Without the propagation of the causative agents, the ability to conclusively confirm the agent as well as the ability to share samples amongst attribution laboratories will be greatly hindered.

### 6.3.4 Integration of *In Vivo* and *In Vitro* Diagnostic Tests

Integrated diagnostics or orthogonal testing, is a recommended testing strategy for both clinical as well as environmental samples. Orthogonal diagnostic testing is the key to improving the reliability of rapid diagnostic technologies. Orthogonal testing refers to tests that are statistically independent or non-overlapping but, in combination, provide a higher degree of certainty of the final result. While orthogonal testing is not a standard perspective in the clinical diagnostic industry, the concept and its application are paramount when investigating some infectious agents. Any single detection technology has a set of limits with regard to sensitivity and, most importantly, specificity. Orthogonal testing seeks to overcome the inherent limitations of individual test results with the strength of data combinations [17]. The application of orthogonal diagnostic testing uses an integrated testing strategy where more than one technology, technique, or biomarker is used to produce diagnostic results, which are then interpreted collectively (Figure 6-1).

### 6.3.5 Immunodiagnostic Methods

An integrated approach to agent detection and identification, utilizing both immunological and nucleic acid-detection will provide the most reliable laboratory data and is essential for a complete and accurate disease diagnosis [17]. Understanding the strengths and weaknesses of each assay is paramount in the interpretation of results. Nucleic acid-detection assays are exquisitely sensitive and specific; this is the strength of the assay, but can also be a weakness in particular situations. Immunodiagnostic assays are comparatively less sensitive, but have broader specificity; this is a weakness of the assay, but can also be a strength in situations. In an orthogonal system, the advantages of the nucleic acid and immunological assays will offset the disadvantages of each. Detection of an endemic pathogen will rely on the high sensitivity of the nucleic acid-detection assay; however for a newly emerging genetic variant the specificity of the nucleic acid-detection assay may result in a false negative. A detection system that incorporates immunodiagnostic assays will detect the variant with the
broader specificity of antibodies. This can be illustrated with the detection of the newest ebolavirus, Bundibugyo. Initially, PCR-based assays failed to detect the virus due the genetic variation, only when the less sensitive but more broadly reactive antigen detection and capture IgM Enzyme-Linked Immunosorbent Assays (ELISAs) were employed was the virus detected and identified as an ebolavirus [56]. Clearly, both immunodiagnostic and nucleic acid-detection assays are vital when detecting pathogens that exhibit genetic variation whether natural or intentionally engineered.

Immunodiagnostic techniques diagnose disease by detection of agent-specific antigens and/or antibodies present in clinical samples. The most significant problem associated with development of an integrated diagnostic system is the inability of immunodiagnostic technologies to detect agents with sensitivities approaching those of more sensitive nucleic acid-detection technologies. These differences in assay sensitivity increase the probability of obtaining disparate results, and could therefore actually complicate medical decisions. However, continued advances in immunodiagnostic technologies provide the basis for developing antigen- and antibody-detection platforms capable of meeting requirements for sensitivity, specificity, assay speed, robustness, and simplicity. Detection of specific proteins or other antigens or host-produced antibodies directed against such antigens constitutes one of the most widely used and successful methods for identifying biological agents and for diagnosing the diseases they cause. Nearly all methods for detecting antigens and antibodies rely on production of complexes made of one or more receptor molecules and the entity being detected (Figure 6-5).

![Figure 6-5: Representation of Common Enzyme-Linked Immunosorbent Assay (ELISA) Formats.](image)

The assay can be configured to detect antigen or antibodies. The target of interest (Direct and Indirect Assay) or a capture antibody (Sandwich Assay) is immobilized by direct adsorption to a solid support such as a 96-well plate or magnetic bead. Detection of the target is accomplished using an enzyme-conjugated primary antibody (Direct Assay) or a matched set of unlabeled primary and conjugated secondary antibodies (Indirect and Sandwich Assay).

Diagnosing disease using immunodiagnostic technologies is a multi-step process involving formation of complexes bound to a solid substrate. This process is like making a sandwich where detecting the biological agent or antibody depends on incorporation of all of the sandwich components. The assays are relatively simple and robust, but elimination of any one part of the sandwich results in a failure and a negative response. Primary
ligands used in most immunoassays are polyclonal or monoclonal antibodies or antibody fragments. Generally, the first step in an immunodiagnostic assay is binding one or more antibodies for the target of interest onto a solid support. Immunoassays are either heterogeneous or homogeneous depending on the nature of the solid substrate. A heterogeneous assay requires physical separation of bound from unbound reactants by using techniques such as washing or centrifugation. These types of assays can remove interfering substances and are, therefore, usually more specific. Heterogeneous assays require more steps and increased manipulation that cumulatively affect assay precision. A homogeneous assay requires no physical separation but may require pre-treatment steps to remove interfering substances. Homogeneous assays are usually faster and more conducive to automation due to their simplicity. However, cost of these assays is usually greater due to the types of reagents and equipment required.

Once the test sample is reacted with the capture element, the final step in any immunoassay is detection of a signal generated by one or more assay components. This detection step is typically accomplished by using antibodies bound to (or labeled with) inorganic or organic molecules that produce a detectable signal under specific chemical or environmental conditions. The earliest labels used were molecules containing radioactive isotopes. However, radioisotope labels have generally been replaced with less cumbersome labels such as enzymes. Enzymes are effective labels because they catalyze chemical reactions, which can produce a signal. Depending on the nature of the signal, reactants may be detected visually, electronically, chemically, or physically. A single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction; therefore these labels are effective at amplifying assay signals. Most common enzyme-substrate reactions used in immunodiagnostics produce a visual signal that can be detected with the naked eye or by a spectrophotometer.

Fluorescent dyes and other organic and inorganic molecules capable of generating luminescent signals are also commonly used labels in immunoassays. Assays using these molecules are often more sensitive than enzyme immunoassays, but require specialized instrumentation and often suffer from high background contamination due to intrinsic fluorescent and luminescent qualities of some proteins and light-scattering effects. Signals in assays using these types of labels are amplified by integrating light signals over time and cyclic generation of photons. Other commonly used labels include gold, latex, and magnetic or paramagnetic particles. Each can be visualized by the naked eye or by instruments and are quite stable under a variety of environmental conditions. However, these labels are essentially inert and therefore do not produce an amplified signal. Signal amplification is useful and desirable because it results in increased assay sensitivity.

Advances in the fields of biomedical engineering, chemistry, physics, and biology have led to an explosion of new diagnostic platforms and assays systems that offer great promise for improving diagnostic capabilities. Here we provide an overview of technologies that are currently being used for identification of biological agents and are either being used or being developed for use in diagnosing the diseases they cause.

### 6.3.5.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Since the 1970s, ELISA has remained a core technology for diagnosing disease caused by a wide variety of infectious and non-infectious agents. As a result, ELISA is perhaps the most widely used and best understood immunoassay technology. Assays have been developed in many formats and can be designed to detect either antigens associated with the agents themselves or antibodies produced in response to infection. ELISAs that detect biological agents or agent-specific antibodies are heterogeneous assays that capture agent-specific antigen or host-derived antibody onto a plastic multi-well plate by an antibody or antigen previously bound to the plate surface (capture element). Complexed antigen or antibody is then detected using a secondary antibody (detector antibody). The detector antibody can be directly labeled with a signal-generating molecule such as in a direct
ELISA or it can be detected with another antibody that is labeled with an enzyme such as in an indirect or capture (sandwich) ELISA formats. These enzymes catalyze a chemical reaction with substrate that results in a colorimetric change. Intensity of this color can be measured by a modified spectrophotometer that determines the optical density of the reaction using a specific wavelength of light. In many cases, the assay can be interpreted without instrumentation by simply viewing the color that appears in the reaction vessel.

The major advantages of ELISA are their ability to be configured for a variety of uses and applications. ELISAs can be used in field laboratory settings, but they require power for temperature controlled incubators and refrigerators and other ancillary equipment needs. In addition, they are commonly used and understood by clinical laboratories and physicians, are amenable to high-throughput laboratory use and automation, do not require highly purified antibodies, and are relatively inexpensive to perform. The major disadvantages are that they are labor intensive, temperature dependent, have a narrow antigen concentration dynamic range that makes quantitation difficult, and are relatively slow.

At USAMRIID antigen-detection ELISAs have been developed for nearly 40 different biological agents and antibody-detection ELISAs for nearly 90 different agents. All of these assays were developed to utilize the same solid phase, buffers and other reagents, incubation periods, incubation temperatures, and general procedures (Table 6-8). Although there is significant variation in assay limits of detection, ELISAs typically are capable of detecting as little as 1 ng of antigen per ml of sample.

**Table 6-8: Comparison of Immunodiagnostic Methods.**

<table>
<thead>
<tr>
<th>Antibody Requirements</th>
<th>ELISA</th>
<th>ECL</th>
<th>Luminex</th>
<th>HHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purity</td>
<td>None</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>Labeling</td>
<td>None</td>
<td>Biotin/Ruthenium</td>
<td>Biotin/Beads</td>
<td>Beads</td>
</tr>
<tr>
<td><strong>Assay Parameter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coating Time</td>
<td>12 hrs</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>3.5 hrs</td>
<td>15 min</td>
<td>30 min</td>
<td>15 min</td>
</tr>
<tr>
<td>Read Time</td>
<td>1 sec/well</td>
<td>1 min/tube</td>
<td>20 – 120 sec/well</td>
<td>30 sec</td>
</tr>
<tr>
<td>Number Steps</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td># Buffers Required</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Specialized Reagents</td>
<td>Conjugate</td>
<td>Assay Buffer</td>
<td>Sheath Fluid</td>
<td>Sample Buffer</td>
</tr>
<tr>
<td>Solid Phase Used</td>
<td>Microtiter well</td>
<td>Magnetic Bead</td>
<td>Colored latex bead</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>Reaction</td>
<td>Bound</td>
<td>In solution</td>
<td>In solution</td>
<td>Bound</td>
</tr>
<tr>
<td>Detector Label Used</td>
<td>HRP</td>
<td>Ru</td>
<td>PE</td>
<td>Gold</td>
</tr>
<tr>
<td>Detection Method</td>
<td>Colorimetric</td>
<td>Chemiluminescence</td>
<td>Fluorescence</td>
<td>Visual</td>
</tr>
<tr>
<td>Amount of Sample per Test</td>
<td>100 ul</td>
<td>50 ul</td>
<td>50 ul</td>
<td>200 ul</td>
</tr>
<tr>
<td>Prozone</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Sample Matrix Effects</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
6.3.5.2 Electrochemiluminescence

Immunodiagnostic technologies based on Electrochemiluminescence (ECL) detection are of continued military interest. ECL technology was commercially developed by BioVeris (Gaithersburg, MD) and was incorporated into a field ready immunodiagnostic system, the M1M. The assay formats are similar to those of ELISA, however magnetic beads serve as the solid support and magnets are used to concentrate target agents. The detection of target uses a chemiluminescent label (ruthenium, Ru). The small size of Ru (1057 kDa) makes it easily conjugated to any protein ligand (antigen or antibody) using standard chemistries without affecting immunoreactivity or solubility of the protein. The heart of the M1M ECL analyzer is an electrochemical flow cell with a photo-detector placed just above the electrode. A magnet positioned just below the electrode captures the magnetic bead-Ru-tagged immune complex and holds it against the electrode. Application of an electric field results in a rapid electron transfer reaction between the substrate (tripropylamine) and the Ru. Excitation with as little as 1.5 v results in light emission, which in turn is detected by a Charge-Coupled Device (CCD) camera. The system’s strengths come from its speed, sensitivity, accuracy, and precision over a wide dynamic range. Magnetic beads provide a greater surface area than conventional surface-binding assays like ELISA. The reaction does not suffer surface steric and diffusion limitations encountered in solid-phase immunoassays; instead it occurs in a turbulent bead suspension, thus allowing for rapid reaction kinetics and short incubation time. Detection limits as low as 200 fmol/L are possible with a linear dynamic range that can span six orders of magnitude [57], [58].

Assay configurations can be identical to ELISA, direct, indirect, or sandwich assays. For antigen detection assays, the beads are coated with capture antibody while for antibody detection assays, the beads are coated with antigen or capture antibody. The coated paramagnetic beads, in the presence of biological agent (target) form immune complexes that are detected by the Ru-conjugated detector antibody. After a short, 15 min incubation period the analyzer draws the sample into the flow cell, captures and washes the magnetic beads, and measures the electrochemiluminescent signal (up to 1 min per sample cleaning and reading time). Conveniently, the reagents can be lyophilized. The system uses 96-well plates that allow high sample throughput.

The ECL system effectively can detect staphylococcal enterotoxin B, ricin toxin, botulinum toxin, Francisella tularensis, Yersinia pestis F1 antigen, Bacillus anthracis Protective Antigen (PA) and capsule, and Venezuelan equine encephalitis virus [17], [59]-[62]. The system was demonstrated in field settings and was used as one part of an integrated diagnostic system in several deployable and/or deployed laboratories. In 2007, Roche...
LABORATORY IDENTIFICATION OF BIOLOGICAL THREATS

(Basel, Switzerland) acquired BioVeris to expand its ECL-based Elecsys Systems, which will ultimately lead to the demise of the M1M platform and its use by the DoD. The platform remains in use but Roche is no longer producing reagents and the system will be forced into obsolescence when supplies are no longer available. Critical assay performance characteristics and detection limits from three typical ECL agent-detection assays are shown in (Table 6-8).

Meso Scale Diagnostics (MSD), Rockville, MD, has developed a line of immunodiagnostic instruments based on the ECL technology. Unlike the M1M that was singleplex, analyzing a single sample for a single target, the MSD instrument is capable of multiplex analysis, analyzing for multiple targets on a single sample. The MSD MULTI-ARRAY® technology utilizes electrochemiluminescence to detect binding events on patterned arrays. In multi-well microplates, capture antibodies are bound to carbon electrodes integrated into the bottom of the plate. The plates can have up to 10 electrodes per well, with each electrode coated with a different capture antibody. Similar to the sandwich ELISA, the target of interest is captured on the electrode and detected by the target-specific Ru-conjugated detector antibody. As in the M1M system, electrochemical stimulation results in the Ru label emitting light at the surface of the electrodes from which the concentration of target associated the particular electrode can be determined. Evaluation of the technology at USAMRIID found sample testing in simple matrices, like high volume air handler buffer worked well, but the assays suffered from increased backgrounds in more complex matrices, like blood or serum. The ECL analyzer PR2 is available in a manual configuration, Model 1800 and a fully automated configuration, Model 1900 each of which is capable of high-throughput analysis. For environmental testing, the Model 1500 is designed for automated aerosol sample testing. This multiplexed immunoassay platform has over 400 assays commercially available for use in clinical, environmental, and research applications, with kits that are specifically designed for biodefense. MSD assays can be customized, however antibody printing onto the electrodes must be done by the company, rendering Laboratory-Derived Tests (LDTs) less flexible, more complicated, and most likely more expensive. The NGDS acquisition program has identified the MSD PR2 instruments for possible inclusion as the immunodiagnostic component in their portable human diagnostic system. Dependence on any single company for both instrument and assays increases the risk to the DoD diagnostic and detection programs, which is reminiscent of the BioVeris experience [59].

6.3.5.3 Flow Cytometry

Flow cytometry, the measurement of physical and chemical characteristics of small particles, has many current applications in areas of research and health care and are commonplace in most large clinical laboratories. Applications include cytokine detection, cell differentiation, chromosome analysis, cell sorting and typing, bacterial counting, hematology, DNA content, and drug discovery. The technique works by placing biological samples (i.e. cells or other particles) into a liquid suspension. A fluorescent dye, the choice of which is based on its ability to bind to the particles of interest, is added to the solution. The suspension is made to flow in a stream past a laser beam. Light is scattered and the distribution and intensity of scattered light is characteristic of the sample passing through. The wavelength of light is selected such that it causes the dye, bound to the particle of interest, to fluoresce. A computer counts and/or analyzes the fluorescent sample as it passes through the laser beam. Using the same excitation source, fluorescence may be split into different color components so that several different fluorophores can be measured simultaneously and signals interpreted by specialized software. Multiplexed flow cytometry assays have been demonstrated for a variety of cytokine targets [63]. Particles can also be sorted from the stream and diverted into separate containers by applying a charge to the particles of interest.

The Luminex® xMAP technology (Austin, TX) has resulted in significant improvements in multiplex flow cytometry-based diagnostics. The xMAP technology is based on polystyrene bead sets encoded with different
intensities of red and infrared dyes (unique address to a bead set) and coated with a specific-capture antibody against one of the analytes of interest. Interrogation of the beads by two lasers identifies the spectral property of the bead (address) and hence associated analyte, in addition to the phycoerythrin-labeled secondary antibody against the specific analyte.

The Luminex® 100/200™ and the FLEXMAP 3D® systems are flow cytometry-based instruments that can rapidly perform up to 100 tests simultaneously on a single sample. They incorporate three familiar technologies; bioassays, microspheres, and fluorescence. Assays occur in solution thus reaction kinetics are rapid and incubation times are shorter. Capture antibodies or ligands are bound to microspheres labeled with two spectrally distinct fluorochromes. By adjusting the ratio of each fluorochrome, microspheres can be distinguished based on their spectral address. Bioassays are conducted on the surfaces of these microspheres. Detector antibodies are labeled with any of a number of different green fluorescent dyes. This detector-bound fluorochrome measures the extent of interaction that occurs at the microsphere surface, i.e. detects antigen in a typical antigen-detection assay. The instruments employ two lasers, one for detection of the microsphere itself, and the other for the detector. Microspheres are analyzed individually as they pass by two separate laser beams and are classified based on their spectral address and are measured in real time. Thousands (20,000) of microspheres are processed per second resulting in an assay system theoretically capable of analyzing up to 100 different reactions on a single sample in just seconds. The manufacturer reports assay sensitivities in the femtomole level, dynamic range of 3 to 4 orders of magnitude, and claim results are highly consistent and reproducible [64]. Because the intensity of the fluorescent label is read only at the surface of each microsphere, any unbound reporter molecules remaining in solution do not affect the assay making homogeneous assay formats possible. The system can employ tubes as well as 96- and 384-well plates and can be automated. In addition to the Luminex instrument, a plate shaker and liquid handling devices are required to complete assays. As with most technologies, a number of different formats can be used. Many multiplexed assay kits are commercially available from a number of different manufacturers for various cytokines, phosphoproteins, and hormones.

The FLEXMAP 3D® instrument is capable of high throughput and can be automated making it better suited for a large clinical laboratory. Currently, there are no field-ready versions of the Luminex® 100/200™ available, limiting the practical use of this instrument in deployment situations. There are currently no commercial or DoD sources for biological threat agent assays available for this platform.

6.3.5.4 MAGPIX®

Flow cytometry-based systems can be accommodated in large diagnostic laboratories where environmental conditions are controlled and preventative maintenance by qualified technicians is available to ensure the flow cells and lasers are clean, aligned, and functioning properly. Recently, the MAGPIX® instrument based on the Luminex® xMAP technology was introduced. The instrument eliminates some of the shortcomings of the flow cytometry-based instruments and has tremendous potential for forward laboratory applications in such resource-limited environments. MAGPIX uses magnetic color-coded microspheres to perform multiplexed assays. There are 50 different individually addressable bead sets that can be used on instrument. Instead of interrogating individual microspheres sequentially through flow cytometry, MAGPIX uses magnetic force to move the microspheres to a stage and then images all the magnetic microspheres from that sample at once using a Charge-Coupled Device (CCD) camera. Three images, each taken with a different filter, are used to discriminate bead sets and determine assay signals. Two images are used to identify the unique bead address and the third image measures the presence of tracer fluorophore, indicating the presence of target analyte. The MAGPIX carries sufficient drive fluid onboard (650 mL) to analyze eight full microtiter plates (768 samples) and has a throughput rate of ~ 96 samples per hour, or 1.6 samples per minute. The system is fully compatible with all magnetic bead-based assays currently performed on the Luminex flow cytometers; all assay, sample, and reagent preparation
protocols for both systems are analogous. The sensitivity of the MAGPIX system is similar or identical to the Luminex 100/200 instrument, being able to detect ricin in the pg/mL range.

Sensitivities of bead-based assays are typically in the same range as, or in some cases superior to, those obtained in ELISAs [65], [66]. Previous limitations in fieldability for the Luminex flow cytometric instruments (large size, susceptibility of the laser alignment to shock or vibration) have also been largely overcome in the new MAGPIX instrument; this latter platform is smaller and more rugged. Per-instrument cost has also been significantly decreased, which may also make it more affordable for widespread deployment in forward facilities. Featuring a flexible, open-architecture design, xMAP technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately. Six assays are commercially available for biodefense toxin targets, *Botulinum* toxins A, B, E, F, Staphylococcal Enterotoxin B (SEB), and ricin.

### 6.3.5.5 Hand-Held Assays (HHAs)

Hand-held assays are immunodiagnostic assays that are ideally suited for field-based diagnostics. They are commonly found on the commercial market and are simple enough to use and interpret that some types are even approved for over-the-counter use by the FDA; the best known being the home pregnancy test. Hand-held assays are typically designed on natural or synthetic membranes contained within a plastic or cardboard housing. A capture antibody (for antigen detection) or antigen (for antibody detection) is bound to the membrane and a second antibody labeled with some visible marker element is placed on a sample application pad. As sample flows across the membrane, antigen or antibody present in the sample binds to labeled antibody and is captured as the complex passes the bound antibody or antigen (Figure 6-6). Colloidal gold, carbon, paramagnetic, or colored latex beads are commonly used particles that create a visible line in the capture zone of the assay membrane.

![Figure 6-6: Illustration of a Typical Hand-Held Immunoassay. (By U.S. National Aeronautics and Space Administration (Lateral_Flow_Assay.jpg) [Public domain], via Wikimedia Commons.](image)
Hand-held assays are advantageous because they are relatively inexpensive, they are simple and require little training to use, and results can be obtained in only 5 to 15 minutes. One of the greatest advantages of hand-held assays is the lack of reliance on instrumentation and logistical needs associated with those instruments. However, this lack of instrumentation decreases the utility of the tests because results cannot be quantified. To respond to this deficiency, several technologies are available to make these assays more quantitative and have the added benefit of increasing their sensitivity. One technology, produced by Response Biomedical Corporation, allows for quantitative interpretation of the hand-held assay [67]-[70]. The Rapid Analyte Measurement Platform (RAMP®) cartridges for biodefense can detect **Bacillus anthracis**, ricin, **Botulinum** toxin, and smallpox virus. Another method for quantitative detection of antibody/antigen complex formation in hand-held assays is use of up-converting phosphors [71], [72]. Paramagnetic particles have similarly been used in assays and instruments capable of detecting changes in magnetic flux within the capture zone (Quantum Design, San Diego, CA) have proven useful by improving sensitivity by as much as several orders of magnitude over more traditional hand-held assays.

Hand-held assays are commonly used by DoD for detection of biological threat agents. The DoD Medical Countermeasure Systems, Critical Reagent Program, a repository for DoD diagnostic reagents offers a number of LFAs for the detection of biological threat agents. In addition, several commercial companies have begun to market a variety of threat agent tests for use by first responders. However, independent evaluation of these assays has not typically been performed so data acquired from the use of these assays must be interpreted carefully. Another common disadvantage of hand-held assays is their inability to incorporate the capability to run a full spectrum of control assays on a single strip assay. Recently, the U.S. Food and Drug Administration (FDA) approved two LFAs for the detection of **Bacillus anthracis** for use in clinical settings [73]. As with any diagnostic test, understanding its strengths and weaknesses will aid in proper interpretation of the results. Hand-held assays are useful in initial screening of samples for biological threat agents, but results should be followed with confirmatory testing using an orthogonal system.

**6.3.6 Future Perspectives**

Traditionally, assays for detecting proteins and other non-nucleic acid targets, including antigens, antibodies, carbohydrates, and other organic molecules were conducted using antibodies produced in appropriate host animals. As a result, these assays were generically referred to as immunodiagnostic or immunodetection methods. In reality, numerous other non-antibody molecules, including aptamers, peptides, and engineered antibody fragments are now being used in affinity-based detection technologies [74]-[82].

Since an immunodiagnostic assay is directly related to the characteristics of the antibody components used, improved antibodies or antibody-like elements have the potential to significantly improve the sensitivity, specificity, and robustness of the assays. Naturally occurring single domain Antibodies (sdAbs) derived from camelids and sharks possess unique properties that could improve present-day immunodiagnostics. Through convergent evolutionary processes, both camelid and shark immune systems naturally possess non-conventional antibody sub-sets composed only of heavy chain homodimers and a single variable domain [83], [84]. The variable (V) domains of these antibodies represent the smallest naturally occurring antigen binding domains known. These extremely small (12 – 15 kD) sdAbs, can target enzyme clefts and cryptic antigens that conventional antibodies cannot. Unique structural characteristics provide them high temperature (> 60 – 90 °C), proteolytic, and pH stability [85]-[92], high solubility [93], and efficient and economical expression in a variety of microorganisms (including *Escherichia coli*) [94]. The unique features of these naturally occurring molecules could vastly improve the utility of any immunodiagnostic assay.
Antibody-based biosensors provide the most reliable detection capability across the broadest range of biowarfare agents. They are, therefore, the preferred platform for DoD biosensor applications. However, the fragility of the antibody molecule together with the short shelf-life (typically two weeks or less) of antibody-based biosensors severely complicates their use outside of a clinical laboratory environment. In addition, the variability in affinity across various antibody systems has precluded the development of multiplexing antibody arrays for biosensor applications. The Defense Advanced Research Projects Agency (DARPA) sponsored the Antibody Technology Program (ATP) to develop and demonstrate approaches for achieving revolutionary improvements in the stability of antibodies while simultaneously demonstrating the ability to control antibody affinity for use in immunological detection [95]-[97]. Each performer was supplied with the same starting material, single chain Fragments (scFvs), and was asked to improve the antibodies by engineering them for improved stability and affinity. The desired metrics for improvements were decreasing the affinity of the antibody by at least 100-fold, and increasing the stability of the supplied antibody such that it maintained its activity at 70°C for one hour. Initially, the performers achieved these requirements in separate proteins before attempting to meet both requirements in one protein. Each group approached the solution differently, but resulted in antibodies with greater binding to the target ligand and improved thermostability. The ATP increased antibody affinity by a factor of 400. Temperature stability of antibody molecules was improved by a factor of 36, which translated into an increased shelf-life at room temperature from about one month to three years. Similarly, antibody survival at 70°C increased from five to ten minutes to 48 hours. By creating these stable antibodies, it was supposed that different variable regions could be grafted onto the developed backbone in order to increase the stability of antibodies in general, without altering the affinity. These improvements would translate into improved immunodiagnostic assays that would function well in more austere environments, as well as decreasing the cold chain needs for these reagents.

Often the Achilles heel of immunodiagnostic assays is the lower sensitivity when compared to PCR-based assays. Advances in antibody development or engineering can improve antibody characteristics and therefore the resulting assays, but there are other advancements that combine antibody detection with PCR to achieve sensitivity levels equivalent to PCR. Immuno-PCR assays are similar to ELISAs, but substitute the detector antibodies with conjugated to enzymes with antibodies that are labeled with DNA [98]. Using label-specific PCR primers, the DNA label is amplified and can result in increased sensitivity of 10^5-fold. These assays that relied on a single DNA-labeled antibody exhibited high background signals that frequently resulted in false positive results [99], [100]. The Proximity Ligation Assay (PLA) eliminated the background limitations of immune-PCR by requiring the binding of antibodies to at least two different epitopes on the target antigen [101]. Each antibody is labeled with a specific oligonucleotide containing a PCR primer site and having either a free 5’ or 3’ end (Figure 6-7). When the antibodies bind the target, the DNA labels are brought into proximity and the two complementary ends hybridize to a connector oligonucleotide with compatible ends. The hybridized strands are joined by DNA ligase and serves as a template for amplification and fluorescent probe detection. The amplified DNA is a surrogate marker for the target protein of interest. The 5’ or 3’ oligonucleotide ends that fail to hybridize completely with connectors cannot be amplified and reduces the background and the possibility of false positives. PLA detection of viruses and bacterium has proven to be more sensitive than ELISA and as sensitive as real-time PCR [102]. In addition, the assays work in a wide variety of biological matrices, serum, plasma, cerebrospinal fluid, cell culture media, and lysates of cells and tissues [101], [103]. Improvements in technology and the components of immunodiagnostic assays continue to close the gap in sensitivity between protein detection and nucleic-acid detection making an orthogonal system ever more powerful.
Figure 6-7: Generic Overview of PLA Reactants and Assay. (A.) In addition to PCR reagents, PLA consists of antibodies to two different epitopes, each labeled with a unique oligonucleotide (proximity probe) and a connector oligonucleotide complementary to the free ends of each proximity probe. Unique to our design is the inclusion of magnetic beads coated with antigen-specific antibodies. (B.) After formation of a bead/antigen/proximity probe complex, the free 5’ and 3’ ends of the antibody-bound oligonucleotides that are in close proximity to each other hybridize onto the connector oligonucleotide and are covalently joined by DNA ligase. Once joined these provide a template for PCR amplification.

6.3.7 Molecular Detection Methods

Currently, Polymerase Chain Reaction (PCR) is the predominant methodology for detection of molecular signatures. Originally conceived in 1983 by Kary Mullis [104], the first published application of PCR was by Saiki et al. amplifying beta-globin genomic sequences and thus hallmarking the advent of the molecular biology field [105]. In its simplest form, PCR consists of target genomic material, two oligonucleotide primers that flank the target sequence, a heat-stable DNA polymerase, a defined solution of salts, and an equimolar mixture of deoxyriboNucleotide TriPhosPhates (dNTPs). This mixture is subjected to repeated cycles of defined temperature changes that facilitate denaturation of the template, annealing of the primers to the target, and extension of the primers so that the target sequence is amplifying. With each cycle, there is a theoretical doubling of the target sequence. The whole procedure is carried out in a programmable thermal cycler that precisely controls the temperature at which the steps occur, the length of time the reaction is held at the different temperatures, and the number of cycles. Under ideal conditions, a single copy of a nucleic acid target can be amplified over a billion-fold after 30 cycles; thus, allowing amplification from targeted genomic signature with potential detection of etiologic agents down to a single copy [106]-[108]. Genomic material, DNA or RNA (in the form of cDNA),
can be targeted by this method amplification. Rapid detection methods typically rely on real-time PCR where targeted genomic signatures are amplified via primers and detection accomplished through oligonucleotide probe hybridization. To this end, numerous PCR-based technologies are currently implemented in the clinical setting for diagnosis of infectious agents.

6.3.7.1 Real-Time PCR

By far the most important development in rapid identification of biological agents was real-time PCR methods. Although traditional PCR is a powerful analytical tool that launched a revolution in molecular biology, it is difficult to use in clinical and field laboratories. As originally conceived, gene amplification assays can take more than 5 to 6 hours to complete, not including the sample processing required to remove PCR inhibitors [109]. The improvement of assay time-to-answer came with the development of assay chemistries that allowed the PCR reaction to be monitored during the exponential amplification phase, i.e. real time (Figure 6-8). In this context, Lee et al. and Livak et al. developed real-time assays for detection and quantification of fluorescent reporters where fluorescence increase was directly proportion to the amount of PCR product generated in the reaction [110], [111]. In this scenario, higher starting copy number of the nucleic acid target resulted in earlier amplification where significant increase in fluorescence is observed. There are three main probe-based fluorescence-monitoring systems for DNA amplification:

1) Hydrolysis probes;
2) Hybridization probes; and
3) DNA-binding agents.

Figure 6-8: Overview of Real-Time PCR Reactants and Reaction Conditions. Generic.
(A.) Real-time PCR reactions (TaqMan probes depicted) consist of the canonical PCR reactants such as forward and reverse primers as well as a DNA template. In addition to these reactants, real-time PCR contain either a fluorescently labeled probe or intercalating dye that is used to monitor amplicon quantities. In the depicted scenario, a sequence of DNA complementary to target sequence separates a fluorophore (F) and a quencher (Q). Fluorescence from the fluorophore in proximity to the quencher is greatly diminished compared to absence or distal fluorescence.

(B.) Similar to conventional PCR, real-time PCR reactions begin with a denaturing of the DNA template. Reducing the temperature allows amplicon-specific primers to anneal to the target sequence and amplification to begin. In some type of real-time reactions, amplified double stranded DNA is directly quantified through measurement of DNA intercalating dyes such as SYBR® green, which only fluoresce when intercalated. In the instance depicted, the probe anneals to the DNA template in similar fashion to the primers. When DNA polymerase encounters the probe, the enzyme's exonuclease function cleaves the probe liberating the fluorophore. No longer in proximity to the quencher, fluorophore fluorescence can be monitored and then correlated to target sequence concentration. Subsequent cycling and amplification yield progressively more DNA template and, consequently, more fluorophore fluorescence.

Hydrolysis probes most exemplified by TaqMan (Applied Biosystems, Foster City, California) chemistries have been the most successful for rapidly identifying biological threats [107]. Numerous assays have been developed against biological threat and infectious agents using these approaches by the DoD, the CDC, and the U.S. Department of Energy [107], [108].

The JBAIDS is the current DoD fielded platform for molecular diagnostic/real-time PCR detection in reference laboratory, combat support hospital and forward operating settings. This system supports assays primarily in the identification of several biological threat agents for clinical diagnostic application while also supporting assays for biosurveillance screening of biological threats as well as some infectious diseases. FDA-cleared assays for clinical diagnostics include *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, *Coxiella burnetti*, and several forms of influenza (H5N1, A, B and A sub-typing). Other assays for biosurveillance purposes cover additional biological threat targets, toxins, and food-borne pathogens. These assays can be run in approximately 30 minutes with up to 32 samples per run. With this system, a presumptive identification of most biological agents can be completed in 3 hours or less. An excellent system for detecting biological threat agents, this system suffers from lack of use in the field setting due to the lack of assays for more commonly acquired pathogens that are more routinely seen in the clinical setting. To mitigate this issue, future generations of molecular detection instruments should have regulatory cleared assays for common infectious diseases to make use and maintenance worthwhile.

### 6.3.7.2 Next-Generation Molecular Diagnostics

The Biofire JBAIDS device is currently fielded in DoD medical laboratories, several of the aforementioned problems exist with this system including: lack of routine usage due to limit assay availability and limited capability to run independent or replicate samples (32 samples per run). To address some of these issues, the Joint Program Executive Office (JPEO), the office that fielded the JBAIDS, is currently under source selection for the NGDS. Under consideration for NGDS are the FilmArray (Biofire Diagnostics), the Liat Analyzer (IQuum), and the 3M Focus Integrated Cycler. These instruments were chosen based on ease of use, sensitivity and available FDA-cleared assays for respiratory or other commonly acquired infectious diseases.

FilmArray is the next-generation pathogen detection instrument from BioFire DX under consideration as an NGDS solution. This instrument is an integrated sample prep and multiplex PCR diagnostic platform capable of detecting bacteria and viruses in a single reaction. This system can run FDA-cleared assays for common respiratory organisms or assays for biological threat detection in a pouch-based array, thus providing a routine
application for the instrument in a clinical setting. Several of the pouches have been evaluated, beyond the respiratory pouch, to include the blood culture and biological threat pouches verifying performance characteristics [112]-[115]. Up to 48 independent reactions can be run in a single run; however only a single sample can be run per pouch thereby limiting the throughput of this device. Overall, the system is a simple use instrument employing syringe and closed pouch-based system to bead-beat and extracts nucleic acid with downstream application to an array-based set of real-time PCR reactions. Due to the low complexity of operation, it is currently being evaluated at FDA for a CLIA-waver.

IQuum Liat is a PCR-based NGDS candidate similar in underlying chemistry to the FilmArray; however, the principal difference lies in this systems ease of use and time-to-answer. The Liat employs independent tube-based cartridges to extract the sample via bead-beating and run singleplex real-time PCR assays. Advantages of the system include:

- Integrated computing and monitoring of the real-time reactions;
- Light-weight platform (8 lbs.); and
- An overall time-to-answer of 20 – 60 minutes assay contingent.

Currently, the flu A/B assay is regulatory compliant with the FDA allowing routine clinical application of the instrument. Independent assays for filoviruses and Bacillus anthracis facilitate biological threat detection. Single tube application of discrete organism real-time assays makes this systems strong candidate for screening patients when known outbreaks arise; however the multiplex-ability of the FilmArray and the higher-sample through-put of the 3M Focus make these stronger candidates for screening in unknown etiologic agent conditions.

The last instrument under consideration by JPEO is the 3M Focus Integrated Cycler. This system is capable of running up to 96 patient samples in a single assay run or 8 samples across multiple assays. This is a substantial advantage over the other two instruments in the context of throughput; however this also induced a higher level of complexity to set-up and running the instrument. In addition, offline sample processing is required for extraction of nucleic acids. Currently, an assay for flu A/B and other respiratory viruses is cleared through the FDA although numerous other assays for infectious diseases exist in various states of clearance within the U.S. and internationally.

Overall, these systems provide an incremental step forward in technology compared to the instrument the selected device will replace, JBAIDS. Assay versatility will be sacrificed for integrated sample processing and clinically applicable assays upon deployment. While these additions to the DoD portfolio will augment current biosurveillance and biological threat detection capabilities, further development is required to truly progress the front-line military diagnostic applications. The current forerunner for filing this capability is next-generation sequencing applications.

### 6.3.7.3 The Horizon – Agnostic Diagnostic Applications

The current endpoint and desired capability for diagnostics in the DoD is an agnostic molecular platform. All the aforementioned technologies require some *a priori* knowledge of the organism; for instance, real-time PCR requires sequence information regarding the target of interest to design primers and probe. In addition, in the application of real-time PCR, guidance from medical intelligence, symptomology, or endemic diseases is required since there is limitation to the number of discrete targets and samples that can be queried in a single run. These limitations could be overcome by application of agnostic diagnostic approaches such as Next-Generation Sequencing (NGS) pathogen detection strategies.
NGS has many potential benefits over current molecular diagnostic approaches. In terms of agnostic detection, NGS has the capability to sequence an entire genome of an organism, thus obviating the need for specific \textit{a priori} knowledge of the pathogen. For example, the detection of novel filovirus variants such as Lujo virus was accomplished via NGS discovery [116]. While numerous methodologies have come and gone over the course of technology development, current field leaders are Illumina sequence-by-synthesis (Illumina Inc., San Diego, CA) and PacBio (Pacific Biosciences, Menlo Park, CA) single molecule real-time sequencing. Each system has advantages and disadvantages. Illumina is current leader with shorter sequence reads (72 – 250 bp), but generating significantly more sequence data (> 10 Gb). PacBio on the other hand generates much longer reads (1 – 10 kb), but has significantly higher error rates. Combinatory approaches between these two technologies have been applied to mitigate independent disadvantages while retaining platform-specific advantages [117]. In fact, numerous lab-derived tests and even 510 K submissions have cleared FDA for use detecting cancer. However, several steps and obstacles require mitigation before these technologies can be applied to regulatory compliant detection of pathogenic organism. Principal in these issues include: mitigation of high-amounts of background host-derived nucleic acid, lack of specificity due to agnostic-nature and sensitivity issues. Current efforts within academia and DoD show promise towards mitigating these issues and bringing NGS into the diagnostic toolbox.

6.4 BIOSURVEILLANCE AND EMERGING THREATS

The emergence of new biological threats is a particular challenge for the military clinical or field laboratory. In the past, the biological defence research program for diagnostics has focused agent-specific identification using collections of biological threats in the biological weapons programs of the United States (ended in 1969) and the former Soviet Union [118], [119]. However, several critical events have broadened the scope of the biological threat over the last three decades. The maturation and proliferation of biotechnology have resulted in several laboratory demonstrations of genetically engineered threats with new, potentially lethal characteristics [120]-[124]. Jackson \textit{et al.} demonstrated the virulence of orthopoxviruses was enhanced by the insertion of immunoregulatory genes, such as interleukin-4 [123]. In other work, Athamna \textit{et al.} demonstrated the intentional selection of antibiotic-resistant \textit{B. anthracis} [120]. Borzenkov \textit{et al.} modified \textit{Francisella, Brucella, and Yersinia} species by inserting beta-endorphin genes [121], [122]. As a result of the proliferation of these biotechniques, public health officials can no longer depend on an adversary choosing any of the 15 to 20 biological threats of past generations, but now must prepare for a future of an infinite number of threats, some of which may have been genetically engineered to enhance virulence or avoid detection. Secondly, the emergence of more virulent and/or infectious strains of naturally occurring infectious diseases has posed significant public health challenges to civilian and military populations. The emergence of the H5N1 and H1N1 variants of influenza are recent examples of the challenge that naturally occurring infectious diseases can present, the latter resulting in a pandemic from 2009 – 2010. These new threats will require the development of identification and diagnostic systems that can be flexibly used to allow early recognition of a unique biological threat, representing one of the next major research and development challenges for the DoD, Department of Health and Human Services (DHHS), and Department of Homeland Security (DHS). The ability to identify and characterize genetically engineered threats or naturally emerging infectious diseases before they negatively impact military and public health is the focus of new initiatives in biosurveillance.

A national effort on Biosurveillance was formally initiated on October 18, 2007 in Homeland Security Presidential Directive-21 (HSPD-21) [125]. HSPD-21 defines biosurveillance as “the process of active data-gathering with appropriate analysis and interpretation of biosphere data that might relate to disease activity and threats to human or animal health – whether infectious, toxic, metabolic, or otherwise, and regardless of intentional or natural origin – in order to achieve early warning of health threats, early detection of health events,
and overall situational awareness of disease activity.” The DoD community has accepted “biosurveillance” as defined in HSPD-21 as a working definition, and as synonymous with “health surveillance” as defined in Department of Defense Directive (DoDD) 6490.02E, Comprehensive Health Surveillance, which establishes policies and assigns responsibility for routine, comprehensive health surveillance of all military service members [126]. The DoD has an extensive health surveillance program for all military personnel, and this effort is executed by the Armed Forces Health Surveillance Center (AFHSC) [127]. However, in addition to human health surveillance, biosurveillance encompasses active data gathering and interpretation of data from the entire biosphere, including animal health surveillance, vector surveillance, and environmental surveillance [128]. The challenge seven years removed from HSPD-21 is accessing, collecting and interpreting all of the surveillance data that is available in a way that provides actionable information to impact public health. Specific challenges that must be addressed include information sharing, information technology tools to assimilate and analyze data, and algorithms to interpret and report the sub-set of data that impacts public health. Within the confines of biosurveillance, diagnostic testing results are a very small percentage of the health surveillance data, and an even smaller percentage of the biosurveillance data. Therefore, care must be exercised to ensure that diagnostic testing data feeds into biosurveillance without allowing the biosurveillance mission to become the critical requirements for diagnostic assay and platform development. Diagnostics must continue to focus on assisting clinicians in making correct medical decisions about the treatment and prognosis of individual patients. Translating the identification of a potential public health threat through biosurveillance to a medical countermeasure, such as an in vitro diagnostic test, is the ultimate goal and the significant challenge for the biosurveillance enterprise. Doing so in a timely manner will be critical to maintain military readiness and minimize public health impacts.

Ultimately, the success of biosurveillance depends upon the tools and technologies available to survey the biological space that impacts human health. These tools must move away from agent-specific identification, which is currently the foundation of most FDA-cleared in vitro diagnostic tests, to a more agnostic approach. Unlike diagnostic tests, which are typically chosen based on clinical suspicion of a particular disease, biosurveillance platforms must attempt to identify all agent(s) in a particular sample. This can be approached through the use of multiple complementary identification technologies or through the use of agent agnostic platforms. The service lab component of the NGDS acquisition program will deliver several complementary platforms to OCONUS research laboratories to enhance the DoD’s biosurveillance capability. The instruments will include The Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument, the Luminex MAGPIX®, and the Illumina MiSeq instruments. The 7500 Fast Dx is an FDA-cleared molecular diagnostic device for the detection of nucleic acids by real-time PCR while the MAGPIX® is a highly multiplexed combined immunoassay/molecular assay platform for the detection of proteins or nucleic acids. Combined, these instruments could potentially cover the nucleic acid and protein biological space, to include identification of viruses, bacteria, and toxins. The critical challenge for these two instruments will be the availability of assays that are capable of extensively surveying the infectious disease space. In addition, the Illumina MiSeq instrument is a nucleic acid sequencing instrument that could be potentially used as an agnostic approach to agent identification [129], [130]. Metagenomic sequencing has become a favored approach to identify all biological components in clinical and environmental samples, and significant investments have been made to stand up genomic sequencing centers within the DoD. The role out of MiSeq instruments in overseas laboratories is the DoD’s attempt to take this capability beyond reference laboratories. While sequencing has advanced significantly in the past decade, it has proven most useful in samples where the amount of organisms is not limiting. This is often not the case in clinical samples, where the concentration of organism is extremely low in relation to the host nucleic acid in the sample. Teasing out the sequences that are significant for biosurveillance and public health purposes is the critical biochemical and bioinformatic challenge for metagenomic sequencing approaches [131].
While sequencing provides a wealth of information, sequence data alone does not substitute for the need to propagate and maintain the viable organisms necessary for medical countermeasure development efforts. This capability is critical, especially for unknown or emerging threats, as all vaccine, therapeutic, and diagnostic development will require enough purified agent material to perform the necessary investigations. Overall, a rapid response capability from agent identification to therapeutic delivery to the Warfighter requires integration across program areas with logical transition from one capability area to another within DoD. A comprehensive biosurveillance plan will include sample acquisition, identification, and characterization capability that allows for rapid development of medical countermeasures. Transition of the deliverables from biosurveillance should bridge pathogen discovery with diagnostics, animal model development, and vaccine and therapeutic evaluation; thereby shortening the timeline between agent identification and fielding of medical countermeasures. Ultimately, data from biosurveillance efforts must lead to actionable information to respond rapidly with medical countermeasures such as vaccines, therapeutics, and diagnostics.

Ultimately, the information provided by biosurveillance needs to translate into products that can be used in an emergency situation to impact military readiness and public health. The Nation’s ability to react to a biological event to minimize casualties and impacts, or Biopreparedness, is critical during an emerging outbreak or intentional release of a biological threat agent. The emergence of H1N1 and H5N1 strains of influenza were valuable lessons for the U.S. government response to provide medical countermeasures, to include the availability of in vitro diagnostic tests. In 2004, the Project BioShield Act amended the Federal Food and Drug Cosmetic Act (21 U.S.C. 360bbb-3; sec 564) to include a process by which non-FDA-approved products or off-label uses of approved products could be rapidly fielded in declared emergency situations. Only the Secretary of Defense, Secretary of Homeland Security or Secretary of Health and Human Services can determine if an emergency situation meets the criteria established in the act. Once this occurs, the U.S. Secretary of Health and Human Services issues a declaration allowing Emergency Use Authorization (EUA) submissions to the FDA for consideration and potential use. Declared emergencies are not limited to ongoing emergencies, but also include situations that may present a heightened risk for potential attack or events. Any potential situation that would pose a significant risk to the public, U.S. military forces or have the potential to adversely affect national security could be declared an emergency situation. This process was activated, refined, and used for in vitro diagnostics during the H1N1 pandemic in 2009/2010 [132]. The typical process to use an in vitro diagnostic test during an emergency involves the declaration of emergency, the submission of performance data to the FDA, FDA review, and FDA authorization to use the test under the EUA. One outcome of the H1N1 EUA process for diagnostics was the development of a pre-EUA process to streamline this process. Based on the FDA’s H1N1 Guidance document [132], the DoD and FDA worked together to define a process for pre-positioning performance data for in vitro diagnostic tests that were not yet FDA-cleared but could be invaluable during a declared emergency. By allowing pre-EUA submissions for diagnostic tests, the FDA is able to review data, request additional data, and make preliminary decisions on utility before an emergency is declared; greatly reducing the time between the declaration of an emergency and the authorization to use the test. Pre-EUA approval does not grant permission to use or market the product under non-emergency conditions, but greatly enhances Biopreparedness because a biological threat event occur. The DoD submitted 73 assays for pre-EUA consideration to the FDA in July of 2010, to date 8 assays have been accepted after providing additional performance data on the JBAIDS and ABI 7500 FASTDX real-time PCR platforms. The pre-EUA process continues to expand the immediate availability of in vitro diagnostics during a declared emergency, and adds previously unavailable biopreparedness capability for the DoD and the Nation.

Success in responding to emerging or genetically engineered biological threats is dependent upon identifying, characterizing, and reducing the health impacts of the threat. This requires a continuum from identification of the threat at the point of presentation (clinically or environmentally) through rapid medical countermeasure deployment. Doing so quickly requires the assimilation of all available biological data, determination of which
data is meaningful, and identification of actionable information signifying a threat to public health. These are the underlying goals of biosurveillance. However, the collection of samples, characterization of the threat agent, development or identification of the appropriate countermeasures, and deployment of those countermeasures to be used under regulatory compliance are necessary to achieve the desired end state; minimizing the public health and military readiness impacts of emerging and engineered threats.

6.5 FUTURE APPROACHES

6.5.1 Early Recognition of the Host Response

Early recognition is critical for the diagnosis and treatment of biological threat agents because of their disease progression, persistence and lethality (Table 6-9). The host responds to microbial invasion immunologically and also responds to pathological factors expressed by the foreign organism or toxin. Identifying early changes in the host gene response may provide an immediate indication of exposure to an agent and subsequently lead to early identification of the specific agent, before the onset of disease. Several biological agents and toxins directly affect components important for innate immunity, such as macrophage or dendritic cell functions or immunomodulator expression. Studies suggest that anthrax lethal factor may induce apoptosis in peripheral blood mononuclear cells, inhibit production of pro-inflammatory cytokines in peripheral blood mononuclear cells, and impair dendritic cells [133], [134]. Poxviruses may possess several mechanisms to inhibit innate immunity [135]. Gibb et al. reported that alveolar macrophages infected with Ebola virus demonstrated transient increases in cytokine and chemokine mRNA levels that were markedly reduced after 2 hours post-exposure [136]. Others have shown that Ebola virus infections are characterized by dysregulation of normal host immune responses [137]. However, directly detecting these effects, especially inhibition of cytokine expression, is technically difficult to measure in potentially exposed populations.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Human to Human Transmission</th>
<th>Infective Dose (Aerosol)</th>
<th>Incubation Period</th>
<th>Duration of Illness</th>
<th>Lethality</th>
<th>Persistence of Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthrax</td>
<td>No</td>
<td>8000 – 1000 spores</td>
<td>1 – 6 days</td>
<td>3 – 5 days (fatal if untreated)</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Brucellosis</td>
<td>No</td>
<td>10 – 100 cells</td>
<td>5 – 60 days; usually 30 – 60 days</td>
<td>Weeks to months</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Glanders</td>
<td>Low</td>
<td>5000 – 10,000 cells (NHP)</td>
<td>10 – 14 days</td>
<td>7 – 10 days (fatal if untreated)</td>
<td>Moderate to high; &gt; 50%</td>
<td>High</td>
</tr>
<tr>
<td>Melioidosis</td>
<td>Low</td>
<td>50 – 80 cells (NHP)</td>
<td>1 – 21 days; up to years</td>
<td>2 – 3 days (fatal if untreated)</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Plague</td>
<td>Moderate</td>
<td>500 – 15000 cells</td>
<td>1 – 7 days; usually 2 – 3 days</td>
<td>1 – 6 days (fatal if untreated)</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>
New approaches that evaluate the regulation of host genes in microarrays may allow for early disease recognition [138], [139]. A complicated picture is emerging that goes beyond dysregulation of genes related to innate immunity. Relman et al. suggested that there are genome-wide responses to pathogenic agents [140]. Mendis et al. identified cDNA fragments that were differentially expressed after 16 hours of in-vitro exposure of human peripheral blood mononuclear cells to staphylococcal enterotoxin B [141]. By using custom cDNA microarrays and real-time analysis, these investigators found a unique set of genes associated with staphylococcal enterotoxin B exposure. By 16 hours, there was a convergence of some gene expression responses, and many of those genes code for proteins such as proteinases, transcription factors, vascular tone regulators, and respiratory distress. Additional studies are needed to characterize normal baseline parameters from a diverse group of individuals undergoing common physiological responses to the environment, as well as responses to the highest priority biological agents and toxins in appropriate animal models. Approaches that integrate detection of early host responses with the sensitive detection of biological agent markers can decrease morbidity and mortality by encouraging optimal therapeutic intervention.

### 6.6 SUMMARY

Military clinical and field laboratories play a critical role in the early recognition of biological threats, serving as unique sentinels in CONUS and OCONUS areas for biological threats and emerging infectious diseases. While
performing regulatory compliant patient diagnostics for biological threats is difficult in a theater of operation, some success has been realized with the fielding of the JBAIDS real-time PCR platform. The NGDS acquisition program will incrementally improve this capability by providing a highly multiplexed “sample in/answer out” capability for molecular biological threat identification. While these fielded platforms provide a diagnostic capability in theater, they are not definitive means of identification and are based on targets that are currently well understood. Definitive identification requires orthogonal testing to the improve reliability of rapid diagnostic technologies and reduce risk. The integration of culture as well as nucleic acid and immunological biomarkers for the identification of biological threat agents is critical to elevate the level of confidence in identifying these high consequence infectious diseases. The network of laboratories available for confirmatory and definitive testing is strong and has improved significantly within the past 5 years. Future technologies will further increase the orthogonal capabilities of diagnostic platforms and strive towards agent agnostic agent identification. The integration of molecular and immunological identification on a single platform using common analytical chemistries may be realized within the next 5 – 10 years, and whole genome metagenomic sequencing holds the promise of identifying all infectious agents in a given sample. These approaches will be critical to accommodate the identification of emerging as well as genetically engineered agents. While there are indications that these future approaches are making progress, there will be regulatory challenges for diagnostic use of highly multiplexed and sequencing technologies. Fortunately, the FDA has been forward thinking and is currently engaged in identifying the key standards required for both highly multiplexed and whole sample sequencing based approaches for clearance of diagnostics. Biosurveillance initiatives may provide a means to evaluate and improve future platforms that could ultimately transition to diagnostic devices if costs permit. In the meantime, medical diagnostics for biological threat agents will rely on proven technologies that incorporate incremental improvements to simplify and improve the reliability and robustness of diagnostic devices for use throughout the military clinical and field laboratories.

6.7 REFERENCES


[5] ATP 4-02.84, Multiservice Tactics, Techniques, and Procedures for Treatment of Biological Warfare Agent Casualties, Department of the Army, Editor March 2013: Washington, DC, USA.


[7] ATP 3-11.37, Multi-Service Tactics, Techniques, and Procedures for Chemical, Biological, Radiological, and Nuclear Reconnaissance and Surveillance, Department of the Army, Editor March 2013: Washington, DC, USA.


[16] U.S. Army Medical Research Institute of Infectious Diseases, Medical Management of Biological Casualties Handbook, 2011, Department of the Army: Fort Detrick, MD, USA.


[21] FM 4-02.7, Multiservice Tactics, Techniques, and Procedures for Health Service Support in a Chemical, Biological, Radiological, and Nuclear Environment Department of the Army, Editor June 2009: Washington, DC, USA.


7.1 INTRODUCTION

Smallpox is caused by the Variola Major Virus (VARV), which is a member of the Poxviridae family and the Orthopoxvirus genus. Despite the eradication of smallpox, VARV remains a public health concern, because of the possibility that clandestine stocks of VARV may exist. The impact of a VARV attack in the human population now would be even more catastrophic than it was during the previous century: vaccination programmes were abandoned, immunosuppressed populations are more prevalent, and people’s increased mobility (including intercontinental air travel) has accelerated the pace of viral spread around the world. For these reasons, considerable investment has been made in developing improved vaccines and anti-viral drugs, as reviewed in “Scientific Review of Variola Virus Research, 1999 – 2010”; (http://whqlibdoc.who.int/hq/2010/WHO_HSE_GAR_BDP_2010.3_eng.pdf). This chapter updates the status of currently available medical countermeasures against smallpox.

7.2 HISTORY OF SMALLPOX

Smallpox, so named to differentiate it from great-pox (syphilis), was described by Edward Jenner as “the most dreadful scourge of the human species.” Although the exact number of deaths during Jenners’ time is unknown, it is estimated to have been approximately 400 million people just in the 20th century alone. The origin of VARV remains unknown; first descriptions of smallpox were documented in 4th-century China, 7th-century India, and 10th-century Mediterranean and southwestern Asia. Moreover, Egyptian mummies buried over 3,000 years ago have skin lesions that are consistent with smallpox. Before the 15th century, smallpox was generally confined to the Eurasian landmass. However, colonialists introduced smallpox to the Americas, central and Southern Africa and Australia between the 15th and 18th century with devastating consequences, as the indigenous population was decimated with case fatality rates approaching 90%. By the end of the 19th century a milder and less lethal form of smallpox became apparent. The virus was named variola minor and it was first documented in South Africa during 1904, but it had been clinically apparent in the United States since 1896. Originally described as Amass (alastrim in South America), this virus eventually became recognized in Brazil during the 1960s and in Botswana, Ethiopia and Somalia during the 1970s. The variola minor derivatives of variola major (classical smallpox) are believed to have originated in several places throughout the globe as the virus adapted to humans. The case-fatality rates for variola major were up to 30% and 1% for variola minor [44].

7.3 CLINICAL FEATURES OF SMALLPOX

The virus was most often transmitted between humans by large-droplet respiratory particles inhaled by susceptible persons who had close, face-to-face contact. Clinically, smallpox in an unvaccinated person has a 7 to 19 day incubation period from the time infection is established within the respiratory tract until the first symptoms of fever, malaise, headache and backache occur, culminating in the start of the characteristic rash. The rash starts with papules which sequentially transform into vesicles and then pustules; a majority of these
lesions are located on the head and limbs (often confluent) compared to the trunk. The rash is typically centrifugal (head and limbs), but centripetal. Lesions range from 0.5 – 1 cm in diameter, skin lesions were deep-seated and in the same stage of development in any one area of the body. Once pustules have dried, scabs will form which eventually desquamate during the following 2 – 3 week period. The resultant feature of these cutaneous lesions is the formation of the classic pock scar [44]. Two clinical variations of smallpox have been identified. Flat-type smallpox is a rare form of the disease (about 6% in unvaccinated people) and it is characterized by lesions that remain level with the skin and usually resulted in death. Another variation of the disease is hemorrhagic-smallpox (< 2% in unvaccinated people); it also had a high mortality rate and is characterized by hemorrhages into the skin and/or mucous membranes early in the course of illness [23].

Although smallpox is typically spread by respiratory droplets over a short distance, some examples of long distance transmission do exist. One such case occurred in 1978 at the University of Birmingham, UK, where a woman died of smallpox. She is identified as the last human fatality of the disease. It is widely believed that VARV traveled up through an air duct that connected a smallpox virology laboratory to her workstation. Another case occurred in a hospital at Meschede, Germany. In this case, a recent returnee from Pakistan is believed to have initiated 19 other cases of smallpox on all levels of a large general hospital despite being isolated for the 5 days of his stay. Factors that enabled VARV to travel long distances in the hospital were likely: strong rising air currents, the patient’s severe cough, and the humidity level.

Prior to its eradication, smallpox as a clinical entity was relatively easy to recognize, although it was sometimes confused with other exanthematous illnesses [23]. For example, the severe chickenpox rash caused by varicella zoster virus was often misdiagnosed as that of smallpox. Other diseases confused with vesicular-stage smallpox included monkeypox, generalized vaccinia virus infection, disseminated herpes zoster virus infection, disseminated herpes simplex virus infection, drug reactions (eruptions), erythema multiforme, enteroviral infections, secondary syphilis, scabies, insect bites, impetigo and molluscum contagiosum. Diseases confused with haemorrhagic smallpox included acute leukaemia, meningococcaemia and idiopathic thrombocytopenic purpura. As a step to counter this type of diagnostic error, the Centers for Disease Control and Prevention (CDC) in collaboration with numerous other professional organizations, has developed an algorithm for evaluating patients (www.cdc.gov/nip/smallpox and www.bt.cdc.gov/EmContact/index.asp).

### 7.4 COLLECTION AND HANDLING OF SPECIMENS

A suspected case of smallpox should be reported immediately to the appropriate local or state health department. Current international recommendations state that work with VARV is to be done using biosafety Level 4 laboratories sanctioned by the WHO. Information about the safe collection and handling of specimens is available on the CDC web site (http://emergency.cdc.gov/agent/smallpox/response-plan/#guided). At least two to four scabs or material from vesicular lesions (or both) are considered suitable specimens. Lesions should be sampled so that both the vesicle fluid and the overlying skin are collected. If available, a series of three electron microscope grids can be applied to the lesion [42]. Sample storage in transport medium is discouraged. Specific recommendations for electron-microscopy sampling and specimen processing are available (http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf).

Lesion biopsies may also provide material suitable for direct viral evaluation. Blood and throat swabs obtained during the prodromal febrile phase and early in the rash phase are also a potential source of virus. In addition, patient serum can be obtained for serologic assays to substantiate a viral diagnosis or to infer a retrospective diagnosis. Virus-containing specimens should be stored and transported at –20°C or on dry ice. The exceptions are electron-microscopy grids and formalin-fixed tissues, which should be kept at room temperature. Standard refrigerator temperature (4°C) is acceptable for less than seven days’ storage. Packaging and transport of clinical
samples should follow international standards for packaging and international regulations for the transport of infectious substances. A triple packaging system must be used for transport of all clinical samples [119]. Clinical samples should be considered as infectious substances from Category A and should be assigned to United Nations number UN 2814. Practical guidance on complying with regulations for all modes of transport of infectious substances and patient specimens can be found on the WHO web site (http://www.who.int/ihr/biosafety/publications/en/index.html).

7.5 THE HISTORY OF SMALLPOX VACCINATION

Historically it was understood that humans who survived an initial smallpox infection never developed the disease again. Furthermore, persons infected with VARV by cutaneous scratches suffered a less severe form of the disease. For these reasons, the practice of inoculating naive persons with pustular fluid collected from smallpox victims became a common practice; it was called variolation. Variolation usually induced a milder form of the disease which was typified by a severe, local, cutaneous lesion with smaller satellite cutaneous lesions; however, variolation sometimes lead to generalized rashes with associated deaths. Variolation was likely developed in both India and China and was subsequently introduced to Egypt and the rest of Africa in the 17th century and Europe and their colonies in the 18th century. By the end of the 18th century, variolation had been widely accepted throughout the world as a means to prevent smallpox. The widespread use of variolation reduced the impact of the virus in the upper-classes but not in the general population. Despite its successes, the mortality rate and the frequent development of classical smallpox in many patients meant that variolation was less than ideal. Fortunately, a solution evolved from the observation that milkmaids were rarely susceptible to smallpox. This was attributed to a zoonotic disease, cowpox, acquired from cows. Based on these observations, Edward Jenner inoculated a boy with Cowpox Virus (CPXV). When Jenner subsequently challenged the boy by variolation, he resisted the infection. After additional study, Jenner published his Inquiry on the subject, marking the beginning of the vaccination era [52]. By the beginning of the 19th century this method of vaccination (vacca, Latin for cow) had become accepted as it afforded the same level of protection as variolation. Vaccination institutes were established and the vaccine had been transferred to all continents. However, widespread vaccination was limited due to technical problems and short supplies of the vaccine. Cowpox was a rare disease in Europe, and was absent in the Americas. Human-to-human vaccination was practised, but it caused the transmission of other pathogens and was eventually banned. In some countries vaccination was substituted with OPVs causing horsepox (a method called equination). Moreover, in a recent study it was found that the causative agent of horsepox was closely related to the Vaccinia Virus (VACV), supporting the hypothesis that horsepox, or close relatives, replaced CPXV in the mid-19th century. Subsequently, CPXV and then the closely-related VACV were produced in the skin of live animals. A further advance was the development of freeze-dried vaccine in 1950, which allowed the vaccine to be maintained, transported and used in the field without refrigeration or loss of potency. Finally, development of the bifurcated needle allowed unskilled personnel to administer the vaccine successfully. By the 1950s, endemic smallpox had been eliminated from most industrial nations.

7.5.1 The WHO Smallpox Global Eradication Programme

In 1959, the Twelfth World Health Assembly adopted a resolution, proposed by the Soviet Union, to achieve global eradication of smallpox [35]. Progress was slow; in 1967 the Intensified Smallpox Eradication Programme started which relied on a new concept composed of surveillance and ring vaccination: new cases of smallpox were thereby identified and quarantined, and close contacts were vaccinated and quarantined. This policy led to the eradication of smallpox, with the last naturally occurring case reported in Somalia in 1977 and the Thirty-third World Health Assembly declared on 8 May 1980 that smallpox eradication had been achieved. Success relied on the following key properties of both the vaccine and the disease:
i) Smallpox infections are restricted to humans, there is no animal reservoir in which the virus may persist;
ii) VARV cannot establish latent or persistent infections, because individuals recovering from the disease
clear the virus;
iii) The signs of smallpox were easily noticed and potential contacts could then be vaccinated; and
iv) The vaccine induced a long-lasting protective immunity, was easy to prepare, cheap and stable without
refrigeration.

7.5.2 Destruction of Variola Virus Stocks

In 1976 the WHO Smallpox Eradication Unit initiated attempts to reduce the number of VARV stocks held in
various laboratories. To avoid reintroduction of smallpox, all known stocks of VARV had to be either destroyed
or sent to two smallpox repositories: the CDC, Atlanta, United States of America, and the State Research Center
of Virology and Biotechnology VECTOR laboratory, Novosibirsk, the Russian Federation. Both labs are the
only official places where infectious VARV stocks are being maintained. The Russian Federation collection
comprises 120 strains, analysis of viability has been performed on 59 VARV strains and 32 strains proved to be
viable. The United States–maintained WHO collaborating centre repository contains samples of 451 isolates or
specimens.

The destruction of these stocks has been discussed at the World Health Assembly since 1986. As a result live
VARV material has been retained for critical public health research. Over the years destruction has been
postponed several times to complete work with live VARV. A tremendous progress has been made with regard
to the development of two excellent candidate anti-viral drugs with distinct mechanisms of action, new,
less reactogenic smallpox vaccines (both licensed vaccines and candidate vaccines); diagnostic tests for VARV
and other OPVs; and animal models. Therefore, in 2013 the members of the Advisory Committee on Variola
virus Research were, by and large, in agreement that live VARVs need no longer be retained for further essential
research for public health benefits, but saw the necessity to retain live VARVs for further work on anti-virals.
The destruction by the CDC of 70 of its 420 variola virus stocks in the process of approved research has set a
potential precedent for the progressive reduction of all live virus material as a means of meeting the request of
the World Health Assembly to destroy VARV stocks (http://www.who.int/csr/resources/publications/

7.6 THE ORIGIN OF VACCINIA VIRUS

Initially, CPXV was used for smallpox vaccination. This virus is infrequently found in cattle, and causes
sporadic infections in humans and a number of animals, but its natural reservoir is probably wild rodents.
In 1939, Downie showed that CPXV preparations used as smallpox vaccines contained a different virus, this was
named VACV [25]. Over the years, VACV obviously had replaced CPXV as the smallpox vaccine. Following
the extensive use of VACV for smallpox vaccination during the 20th century, the virus has come to infect
domestic animals, notably buffaloes in India and cattle in Brazil. These animals can, in turn, transmit the virus to
humans. Despite this, VACV is not considered a natural human pathogen.

Based on analyses of the viral genome sequences, it is unlikely that VACV is derived from either a CPXV or a
VARV. The favoured hypothesis for the origin of VACV is that it is an OPV species that previously infected
animals in which it is no longer endemic [10]. Horsepox virus has been suggested as the origin of VACV and
in support of that an OPV whose closest relative is VACV has been isolated from diseased Mongolian
horses [110]. The reasons for VACV becoming the 20th-century smallpox vaccine are not recorded. Possibly,
VACV had a higher prevalence when vaccines were established, or vaccinators selected VACV because CPXV produced a more severe reaction and VACV has lower virulence. Although the origin and natural host of VACV remain mysteries, this virus is the most intensively studied of the poxviruses [83].

7.6.1 The Vaccine Used to Eradicate Smallpox

Because of the long history of VACV as a vaccine, a wide variety of VACV strains have been used in different regions of the world [35]. The New York City Board of Health (NYCBH) strain was used in North America and West Africa. Wyeth Laboratories commercialized the Dryvax vaccine, which was prepared from the lymph fluid of the skin of calves infected with the NYCBH strain; it was available in the United States after the smallpox eradication campaign. The EM-63 strain was derived from the NYCBH strain, and was used in Russia and India, while the Lister/Elstree strain, developed at the Lister Institute in the UK, became the most widely used vaccine worldwide [94]. The Temple of Heaven/Tian-Tan strain (China) was also widely used. Other VACV strains used during the eradication programme include the Copenhagen strain (Denmark), the Bern strain (Switzerland), the Dairen strain (Japan), the Ankara strain (Turkey), the Tashkent strain (Uzbekistan) and the Paris strain (France). Most of the vaccines used during the smallpox eradication programme were grown on the skin of live animals – mainly calves, but also sheep, buffaloes and rabbits [83].

However, the safety record was not perfect since a number of adverse effects were associated with vaccination. Accidental infections occurred when virus was transmitted from the inoculation site in vaccinees or from contact persons; ocular and generalized infections were of greatest concern. The severe infection in individuals with eczema or immunological deficiency was a major complication. Both conditions are considered contraindications to smallpox vaccination. A small percentage of vaccinees had severe neurological adverse effects, such as encephalitis, and these cases were unpredictable. Post-vaccine adverse effects were more frequently associated with some vaccine strains than with others [65], [35] – the limited epidemiological data available suggest that the NYCBH and Lister strains were associated with lower frequencies of adverse effects, while the Copenhagen and Tashkent strains were more virulent. Some modelling studies have estimated that the number of deaths after vaccination with the NYCBH strain was one per million vaccinations [40], [126], [88]. A recent study reviewing the available epidemiological data estimates the number of deaths during a mass vaccination campaign in tens per million for the NYCBH strain, and up to two hundred per million for the Lister strain [61]. During a smallpox vaccination campaign in the United States, involving more than 700,000 individuals, the frequency of vaccination-related myopericarditis cases was higher than anticipated [6]. Even without adverse effects, less serious side-effects such as a skin lesion, low-grade fever and headaches are common. This can make people reluctant to be vaccinated.

7.7 TISSUE CULTURE AND CLONAL SMALLPOX VACCINES TODAY

In response to the risk of a deliberate release of smallpox as an act of terrorism, some countries have manufactured smallpox vaccines to replenish their stocks [94], and WHO has established stocks of smallpox vaccine. These vaccines have been prepared in tissue culture and in animals. Given their similarity to the traditional smallpox vaccine, it is likely that these manufactured vaccines will have the same efficacy and the same rate of adverse effects. Since genetic heterogeneity has been documented for the Lister and Dryvax vaccines [69], [37], the next generation vaccines are plaque-purified viral clones with properties similar to their parental strains. As a result the clonal smallpox vaccine, ACAM2000, was licensed for use in the United States in August 2007 [36]. This vaccine was derived by plaque purification from Dryvax, and has been grown in the Vero monkey cell line. Although they are believed to be as effective as the first vaccines used to eradicate smallpox, it is possible – although unlikely – that they have lost viral clones important for their efficacy in
SMALLPOX

humans. In addition, they may be associated with the same adverse effects as the standard Dryvax or Lister vaccines used in the smallpox eradication campaign.

7.7.1 Attenuated Smallpox Vaccines: MVA and LC16m8

Because of the infrequent but significant post-vaccine adverse events observed with smallpox vaccines based on replicating VACV, research has been directed towards the development of smallpox vaccines based on non-replicating highly attenuated VACV strains with improved safety that retain good immunogenic properties. A common method to attenuate VACV is by multiple passages in tissue culture, leading to genetic alterations, lower virulence and restricted host range. In the following data on two new, less reactogenic smallpox vaccines (both licensed) are presented.

Modified Vaccinia Ankara (MVA) is a VACV strain derived from Chorioallantois Vaccinia Ankara (CVA) in the late 1950s by 570 passages in chick embryo fibroblasts [76]. This passaging resulted in a virus of restricted host range that is unable to replicate efficiently in human cells but expresses most of the viral proteins [106], [127]. The virus was licensed as a vaccine in Germany, and safely used to vaccinate more than 100,000 people, but its field-effectiveness against smallpox was not tested. MVA has large deletions in the terminal regions of the genome, which contain non-essential genes that are often involved in evasion of the host immune response or in maintaining the broad host range of VACV [5]. As a result of these deletions, MVA replicates well in chick embryo fibroblasts and baby hamster kidney cells, but is restricted in human cells [19]. In most types of cells, MVA produces most of the viral antigens, but only immature virus particles are formed, and cell-to-cell spread does not happen. As a vaccine, MVA is considered effective because it is immunogenic, and safe because it does not replicate in human and most other mammalian cells as demonstrated by studies with immunodeficient monkeys [103] and mice. It induces an antibody profile similar to that induced by Dryvax and protective immunity against MPXV in non-human primates [27], [28]. Two doses of MVA are required to achieve the same peak immune responses produced using a single dose of replicating VACV. However, already a single dose is protective in animal models and compared to replicating VACV demonstrates an earlier onset of protection in non-human primates [28] and even post-exposure protection ability in mice [97]. Phase I and II clinical trials with MVA have been completed, and Phase III clinical trials started in 2013 [114], [121]. As a result of these investigations Imvanex® (Imvamune® in the USA) was licensed in the 28 Member States of the European Union, Iceland, Liechtenstein and Norway in July 2013, with the indication for active immunization against smallpox of all adults. In November 2013 it was also approved in Canada. In the USA, a pre-Emergency Use Authorization is in place that would allow use of the vaccine in certain immunocompromised populations (i.e. HIV and atopic dermatitis regardless of age and including pregnant women and children). It has been included in the U.S. Strategic National Stockpile, with an initial order of 20 million doses was made and, in April 2013, the U.S. government ordered another 8 million doses. The vaccine has been administered to more than 7000 people with no safety concerns, including individuals that are not considered candidates to receive replicating smallpox vaccines. The EMA and Health Canada regulatory approvals of Imvanex® and Imvamune® respectively offer a unique opportunity to upgrade existing smallpox preparedness plans by securing equal access to smallpox protection for citizens who are not candidates to receive replicating smallpox vaccines, such as LC16m8, ACAM2000 and DryVax. Further on it opens up an opportunity for active pre-event vaccination of 1st-line responder communities, to build resilience and secure an effective preparedness in case of an unexpected smallpox event.

LC16m8 is a VACV strain that was developed by passaging VACV Lister strain through primary kidney epithelial cells at low temperature (30°C) and was licensed in Japan in 1975 [41], [56]. The virus has a take rate similar to the Lister strain; it differs from the Lister strain in being temperature restricted, having a limited host range and showing greatly reduced adverse effects (in terms of both severity of adverse effects and number of
people suffering adverse effects). LC16m8 does not have large deletions in its genome, and most of the open reading frames appear to be functional. The small-plaque phenotype of LC16m8 was attributed to a mutation in the \textit{B5R} gene, encoding a protein with homology to complement regulatory proteins. This protein is essential for the formation of extracellular enveloped virus and is an important target antigen for antibodies that neutralize the virion [91]. Since this mutation may easily revert, a stabilized version of LC16m8 has been developed, with a deletion of the entire \textit{B5R} gene [59]. Other mutations responsible for the temperature restriction and \textit{in vivo} attenuation are likely to be found elsewhere in the viral genome. LC16m8 has been shown to protect monkeys against MPXV [96]. Today, LC16m8 is being stockpiled in Japan. It has been administered to some 90,000 infants in the past and recently to more than 8000 military personnel in Japan and 125 adults in USA without severe adverse effects. Given to people who had previously been immunized against smallpox (even several decades ago), LC16m8 induced an effective booster response irrespective of how many doses they had previously received. In animals vaccinated with LC16m8 at primary vaccination and revaccinated with a conventional vaccine (e.g. Lister), skin lesions were still observed in a diminished size but the immune response was comparable to that in animals vaccinated twice with a conventional smallpox vaccine. In other words, LC16m8 is an adequate vaccine to be stockpiled for preparedness against possible smallpox attacks and is expected to work effectively when used singly or in combination use with first- and second-generation vaccines. The manufacturing capacity in Japan is 80 million doses a year.

7.8 IMMUNOBIOLOGICAL PREPARATIONS

Although not carried out in a controlled manner, several studies suggest that the administration of Vaccinia Immune Globulin (VIG) from humans provided significant protection to smallpox contacts and alleviation of vaccine complications [46]. VIG has been licensed by the FDA for treatment of post-vaccination complications. However, because it has all the drawbacks of a donor blood-based preparation, use of specific human anti-OPV recombinant antibodies would be preferable, especially fully human recombinant (or monoclonal) antibodies. The key stage in developing fully human recombinant antibodies is selecting variable domains responsible for the antibody specificity, affinity and biological properties. One way to produce them is to select variable domains from phage libraries. By this, 34 antibodies were obtained at VECTOR and tested for their ability to neutralize the infectivity of VARV; five antibodies capable of neutralizing VARV were found (Tikunova, unpublished data, 2006). These antibodies will require evaluation in animal models of VARV infection.

7.8.1 Electron Microscopy for Rapid Diagnosis

Electron microscopy is regarded as a first-line method for laboratory diagnosis of poxvirus infections because of the typical morphology of the virion, the high number of particles usually present in poxvirus-induced lesions and the relative ease of acquiring samples. Clinical diagnosis of a poxvirus infection in humans is now infrequent, so electron microscopy observations may provide one of the first clues to the cause of an unknown rash illness [42]. OPVs share a brick-shaped virion morphology, which is irregularly covered by short, tubular elements resembling small stretches of tape. The size may vary from 250 nm × 290 nm up to 280 nm × 350 nm. Although individual OPV species cannot be distinguished morphologically, they are easily separated from herpesviruses, which are important differential diagnoses (e.g. to differentiate from chickenpox). Because poxviruses are tightly associated with the cellular matrix, samples had been grinded in a mortar with sterile sand or pulverized after flash-freezing it in liquid nitrogen. Now, commercially available tube systems (lysing matrices in combination with bead beaters or mixing mills) are more advantageous because they allow standardization of the procedure and avoid cross-contamination. Two freeze/thaw cycles or sonication (or both) facilitate disruption of cells in a closed tube system. A concentration of 10^5 viral particles/ml is required for successful diagnosis by the visualization of virions. Preparation and examination of samples requires patience.
and experience. Even when brick-shaped poxvirus particles are found rather quickly, it is worthwhile further scanning the sample because additional viruses might also be present. A sample may take 30 minutes to examine, so electron microscopy can take up to two hours to yield results after the samples are received. Descriptions of methods for negative-stain evaluation and pictures of negative-stained particles are available (http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf).

7.8.2 Genome-Based Diagnostic Assays

Taking into account the serious consequences of the diagnosis “Smallpox” or even the consequences of a misdiagnosis, there is a need to identify smallpox unambiguously, rapidly and reliably. This includes the need to differentiate VARV in an equally reliable manner from other similar clinical entities.

The rapid development of nucleic acid research in recent years has yielded many options for DNA-based detection methods. Techniques like Polymerase Chain Reaction (PCR), real-time PCR, microarrays and genome sequencing are no longer restricted to a few dedicated laboratories. PCR results in the in vitro production of large quantities of a desired sequence of DNA from a complex mixture of heterogeneous sequences. PCR does not differentiate between viable and non-viable organisms.

Assays described in the past to identify and differentiate species of the genus OPV are based on sequences of the hemagglutinin [92], the cytokine response modifier B [73] and the A-type inclusion protein gene [80]. These rather time-consuming conventional PCR methods are now being replaced by real-time PCR assays which combine amplification and detection of target DNA in one reaction vessel, thereby decreasing the risk of cross-contamination. In addition, real-time PCR provides quantitative information. The numerous advantages of the real-time PCR technique led to its introduction into the field of poxvirus diagnostics in 2002. Recent developments of portable real-time PCR machines and lyophilized reagents [2] raise the exciting prospect of these techniques being used for rapid diagnosis in the field.

However, for any PCR-based diagnostic test, the knowledge of at least part of the poxvirus genome is necessary to design valid and reliable assays. With regard to VARV a total of 45 epidemiologically varied VARV isolates from the past 30 years were sequenced [30]. Low sequence diversity suggests that there is probably very little difference in the isolates’ functional gene content. This increases the likelihood that sequence-based detection methods will efficiently identify a re-emerging VARV. Poxvirus genome sequences are accessible at www.poxvirus.org, representing all eight genera of the sub-family Chordopoxvirinae, including 46 VARV strains. It will be important, as additional sequence information becomes available from related viruses, that the various PCR primers and probes are periodically reviewed, in silico, if not via practical laboratory testing, for their actual specificity and sensitivity. For that purpose, screening of large OPV strain collections is essential.

Nevertheless, it needs to be stressed that a positive VARV PCR result must be confirmed by amplifying other parts of the genome. The use of multiple assays which target various portions of the genome, in addition to non-nucleic acid detection/diagnostic assays, will increase the confidence, especially in the context of the lack of naturally occurring disease. In our view a “state-of-the-art” real-time PCR strategy is a combination of:

i) A generic OPV assay which allows VARV identification by melting analysis; and

ii) A VARV-specific assay.

The parallel application of different detection formats, like 5’ nuclease and hybridization probes, can be beneficial. Of course, an internal control to monitor inhibition has to be included and a modified VARV DNA as positive control would be of great advantage. By following this strategy, VARV can be reliably detected even in mixtures of VARV and non-VARV OPVs.
7.8.3 Real-Time PCR Assays Useful for Specific VARV Identification

Olson et al. [85] designed a real-time LightCycler PCR assay that was compiled in a kit system under GMP conditions and still is commercially available. The applicability of this method was demonstrated by successful amplification of 180 strains belonging to the OPV species VARV, monkeypox, CPXV, VACV, camelpox, and mousepox viruses. A difference of 5°C in the respective melting temperatures differentiates VARV and non-VARV-OPVs. Nitsche et al. [84] published three LightCycler-based real-time PCR assays, with two assays being based on generic amplification and one VARV-specific assay. The assays were successfully validated with VARV-DNAs. Putkuri et al. [89] published a LightCycler assay using generic OPV primers to amplify part of the HA gene. Identification of VARV is achieved by FMCA. The presented melting curves and the respective Tm show that discrimination between VARV and non-VARV OPVs is based on a difference of 2°C only which may lead to difficulties in correct genotyping of VARV. In 2003 a VARV-specific assay targeting the HA gene was described and evaluated by Ibrahim et al. [49]. Later this assay was modified by Kulesh et al. [62] who used identical primers but a slightly modified, shortened probe. This assay had been successfully evaluated with 322 coded samples that included genomic DNAs from 48 different isolates of VARV and 25 different strains other than VARV, although based on the design of the probe, some VARVs may not be detected equally sensitively. In 2007 Scaramozzino et al. [98] used one probe matching perfectly with non-variola OPVs, while the other probe matches perfectly with VARVs. Since the VARV-specific probe reacts to some extent with monkeypox virus DNA, results have to be interpreted by analysis of the height of the fluorescence signal. Only the perfectly matching probe will give a higher fluorescence signal. Although the differences in signal intensity are shown to be significant for the presented viruses, in a screening run of unknown specimens every run should contain appropriate controls that allow a clear classification of VARV and non-VARV OPV. Fedele and colleagues [33] proposed an assay that targets the crmB gene with OPV-generic primers and two 5’ nuclease probes modified by MGB. One MGB probe is designed to recognize VARV only, whereas the other detects OPVs generically. A blast search showed that the VARV-specific probe will detect all VARV strains. A most recent paper [74] used a rather new format, called Eclipse probes, to detect VARV major and minor. The authors emphasise that this assay and a second one also described in their paper have not been developed for screening purposes but are to be used to differentiate VARV major from VARV minor strains when a positive VARV diagnosis has already been obtained from other assays. Taken together, there are a number of validated assays which can be used to specifically identify VARV. None of the assays is licenced, however, one assay is commercially available but for research purposes only.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Target Gene</th>
<th>Method</th>
<th>Validation with Genomic VARV?</th>
</tr>
</thead>
<tbody>
<tr>
<td>[49]</td>
<td>HA</td>
<td>TaqMan, VARV-specific probe cleavage</td>
<td>Yes</td>
</tr>
<tr>
<td>[84]</td>
<td>Rpo 18, VETF, A13L</td>
<td>LightCycler with hybridization probes; melting curve analysis differentiates VARV from other orthopoxviruses</td>
<td>Yes</td>
</tr>
<tr>
<td>[62]</td>
<td>HA</td>
<td>TaqMan, VARV-specific probe</td>
<td>Yes</td>
</tr>
<tr>
<td>[85]</td>
<td>14 kDa</td>
<td>LightCycler with hybridization probes; melting curve analysis differentiates VARV from other orthopoxviruses</td>
<td>Commercially available for research purposes</td>
</tr>
</tbody>
</table>
### Other Real-Time PCR Assays

In the following assays are described which based on *in silico* analysis today would led to false-positive signals, thereby – if not confirmed by a second assay – unnecessarily would cause a false-positive “smallpox” alert. Espy and colleagues [31] were the first to introduce real-time LightCycler PCR as a diagnostic tool for OPV differentiation. A generic OPV-specific PCR followed by Fluorescence Melting Curve Analysis (FMCA) allows discrimination of smallpox virus due to its different melting temperature as compared to non-v variola viruses. However, a blast search using the sequences of the probes revealed not only identity to sequences in the Hemagglutinin (HA) gene of VARV, but also to the HA gene of various OPVs. This would result in false-positive results and as a consequence lead to considerable confusion. The same is true for an assay described by Panning et al. [86] targeting the HA gene: several CPXVs would be misidentified as VARV. Two assays designed to be VARV-specific, target short stretches of the B9R and B10R genes [62]. Taken together, with the increasing availability of *in silico* OPV sequences all these assays are not suitable for VARV-diagnostic.

One of the great benefits of real-time PCR is the application of additional hybridizing oligonucleotides, called probes, which contribute to a second level of specificity. For diagnostic screening purposes, probe-based assays generate more reliable results than assays that exclusively rely on specific primers. An example for the latter format is an assay described by Carletti *et al.* [18] which relies on staining amplicons with the dye SybrGreen followed by melting curve analysis leading to rather similar melting curves, thereby lacking the general benefits of real-time PCR. Aitichou *et al.* [2] used an LUX primer-based system that needs no probe to specifically detect VARV DNA in the HA gene region. However, this approach also lacks confirmation of the specificity of the amplicon. Therefore, both assays are not recommended for a reliable VARV-specific diagnostic.
7.8.5 Oligonucleotide Microarray Analysis

Many of the previously mentioned problems that arise during species-level detection of the viruses can be solved using hybridization of DNA molecules on oligonucleotide microarrays, frequently called microchips. The first method is based on hybridization of a fluorescently labelled amplified DNA specimen with oligonucleotide DNA probes immobilized on a three-dimensional polyacrylamide-gel microchip. The probes identify species-specific sites within the viral CrmB gene [66]. An alternative oligonucleotide microarray was developed using plain glass slides. The target gene is G3R, which encodes a chemokine binding protein [63]. This microarray-based method simultaneously detects and discriminates between four OPV species pathogenic for humans and distinguishes them from VZV. To ensure redundancy and robustness, the microchip contains several unique oligonucleotide probes specific for each virus species. This new procedure takes only three hours and it can be used for parallel testing of multiple samples. Simultaneous analysis of multiple genes can further increase the reliability of the assay. Another microarray-based method for simultaneous detection and identification of six OPV species has been developed, which also allows the discrimination of OPV species from VZV, HSV-1 and HSV-2 [95].

7.8.6 Genome Sequencing

A total of 45 epidemiologically varied VARV isolates from 1940 to 1977 have been sequenced [30]. A low degree of sequence diversity suggests that there is probably very little difference in the isolates’ functional gene content. This increases the likelihood that sequence-based detection methods will efficiently identify a re-emergent VARV strain. In addition, the low sequence diversity is reassuring and important from a biodefense perspective, because it suggests a high probability of identifying VARV infections if tracking single or multi-source outbreaks. The ability to track the virus might be a deterrent to deliberate use in its own right. Sequencing of various PCR amplicons in a diagnostic setting enables allocation of a sample to known relatives after comparison with the respective database. Sequences of the HA gene of more than 200 OPVs are available and have proven useful for phylogenetic studies. These studies confirmed the current concept of established OPV species, which, historically, was based on the different phenotypes of the respective species. A bio-barcode assay using sequencing technologies has been developed for pathogen detection, including VARV [43]. Progress in sequencing technologies will certainly make them a valuable forensic tool should smallpox re-emerge, since sequencing is one way to verify the virus strain with clarity.

7.8.7 Virus Isolation

Despite the availability of new diagnostic techniques, virus isolation remains the gold standard: virus culture is the only existing method by which to produce a supply of live virus for further examination. The use of chick embryos for poxvirus diagnostics was first described in 1937. The only known poxviruses that produce human infection and pocks on the Chorioallantoic Membrane (CAM) of chicken eggs are four OPVs:

- VARV;
- Monkeypox Virus (MPXV);
- CPXV; and
- VACV.

Differences in the pock morphology were useful in differentiating the OPV species; as a consequence, the CAM assay was widely and successfully used during the smallpox eradication campaign. Although VARV grows satisfactorily in chick embryos, cell culture is generally the simpler option. VARV can be grown in a variety of
established cell culture lines, including Vero, BSC-1 and CV-1 (African green monkey kidney cells), LLC-MK2 (rhesus monkey kidney cells), human embryonic lung fibroblast cells, HeLa (human ovarian cancer cells), and MRC-5 (human diploid fibroblast cells). A cytopathic effect is seen within one or two days; if there is little infectious material, individual plaques may not be visualized for three to four days.

7.8.8 Diagnostic Assays to Detect OPV Antigen

Although a number of laboratories had evaluated various antibody preparations for use in antigen-capture detection of OPVs [22], currently, only one protein-based diagnostic assay, the Orthopox BioThreat® Alert assay is commercially available. This antibody-based lateral-flow assay captures OPV viral agents. The assay might be of interest in the field; however, since concentrations of 10^7 pfu/ml for VACV and MPXV are needed to get a signal, this assay seems not very well suited for onsite diagnostics [107].

7.8.9 Diagnostic Assays to Detect OPV Antibodies

When virus specimens are not available, antibody assessment by the Neutralization Test (NT) or other methods may be the only way to define the disease etiology. Another type of test, frequently requested in bioterrorism response awareness, is for the evaluation of residual protection from previous vaccination. However, there is no single routine immunologic test that defines an individual’s degree of protection. Protection requires a concert of cell-mediated and humoral immune responses. The presence of neutralizing antibodies generally indicates recovery from an infection, not always protracted protection from future infection.

Neutralizing antibodies against VARV, MPXV, CPXV or VACV may be detectable as early as six days after infection or vaccination; NT efficacy using sera of infected animals or humans ranges from 50% to 95%. Neutralizing antibodies have been detected more than 20 years after vaccinia vaccination or natural infection with other human OPVs [90]. In the NT, a four-fold rise in antibody titre between serum samples drawn during the acute and convalescent phases is usually considered diagnostic of poxvirus infection. More recently, the neutralizing effects of antibodies against the two infectious forms of the virus have been better characterized. The two forms of infectious virus (Mature Virus – MV, and Extracellular Virus – EV) have different membrane structures and different surface membrane proteins that are recognized by the immune system. Neutralizing responses to a number of proteins of the MV are characterized; in contrast, only one protein of the EV (B5) is known to be recognized by a neutralizing antibody response.

Serologic methods currently in use for antibody detection include assays of antibodies against human OPVs. These assays have included the virus NT, the haemagglutination inhibition assay, the Enzyme-Linked Immunosorbent Assay (ELISA) and western blots [91]. The recent description of an OPV Immunoglobulin M (IgM) assay could improve investigations of OPV outbreaks, often semiretrospectively ([55]. This technique offers the advantage of measuring recent infection or illness with an OPV. Its sensitivity and specificity when diagnosing recent OPV infection are both approximately 95% when assayed between days 4 and 56 after onset of rash. With appropriate epidemiologic surveillance, these assays may be valuable in evaluating disease incidence; however, antibodies cross-react among members of each poxvirus genus, rendering serology non-specific for a given virus species [109]. Additional tissue culture-based enzyme immunoassays, microplaques reduction assays, and Cellomics-based and Fluorescence-Activated Cell Sorting (FACS)-based analyses are also available for the serologic virus neutralization responses [29], [13], [54].

Although cell-mediated immune responses play an important role in poxvirus infections and are believed to be crucial for long-term immunity, current routine testing for T-cell response is not reliable and reproducible.
A recent real-time PCR assay that assesses CD8+ T-cell response post-VACV vaccination could be an additional approach to measure variola infection. However, at this time, there is no specificity for this assay.

### 7.8.10 The Variola Virus Genome

The complete DNA sequence of two VARVs had been published in the early 1990s. In 2006, the full genomic sequences from 47 geographically distinct VARV isolates were reported [30]. These genomic sequences provide a rich source of new insights into VARV genetics, evolution, relationships with other OPVs and co-evolutionary interactions with the human host [39]. They have also been used to develop diagnostic tools to distinguish VARV from other OPVs [71], [105]. The sequences, together with the known gene sequences and drug sensitivities of other OPVs, provide evidence that all VARV strains should be sensitive to drug therapeutics such as ST-246 and the cidofovir derivative CMX001.

The genomes of all the VARV isolates are closely related to each other, in part because evolutionary drift occurs more slowly in poxviruses than in many other viruses. The sequenced VARV genomes, which are approximately 185 kilobases in length, all contain approximately 200 Open Reading Frames (ORFs). These ORFs express proteins with varying degrees of similarity to proteins expressed by other OPVs, such as Camelpox Virus (CMLV), taterapox virus (GBLV), MPXV, CPXV and VACV. Like all poxviruses, the viral genome is double-stranded DNA, with hairpin termini and Terminal Inverted Repeat (TIR) sequences. In contrast to most other poxviruses, the TIR sequences of VARV do not encode any viral proteins, and so all the VARV ORFs are present in a single copy. The function of the hairpin termini is thought to relate strictly to genomic replication, ensuring complete synthesis of all the viral DNA sequences during the virus life cycle.

Within the central region are clustered approximately 90 highly conserved genes [39] encoding the essential elements for poxvirus replication, gene expression and virion morphogenesis. Genes that encode the unique aspects of VARV biology, such as virulence, anti-immune determinants and disease pathogenesis markers, tend to cluster more towards the ends of the genome. The greatest variation in DNA sequences between VARV strains with different case fatality rates appears in the genomic regions closest to the TIR sequences [30]. Approximately 90% of VARV genes have clear orthologues in other poxvirus genomes, while truncated versions of the remainder can be found in at least one other OPV. Genetically, the two OPVs most closely related to VARV, with about 98% nucleotide identity over the central 110 kilobases of the genomes, are CMLV and the gerbil pathogen GBLV. The genetic distances between VARV and MPXV, CPXV and VACV are considerably greater [100], [39], [79].

Among the 48 VARV genomes individual pairs can differ by as many as 700 Single Nucleotide Polymorphisms (SNPs) and up to 90 insertion/deletions (indels). The full spectrum of VARV genomic variation – more than 1700 SNPs and 4800 indels – has been documented; overall, however, the sequences are most notable for their close similarities.

The VARV isolates selected for genomic sequencing were chosen to represent a broad cross-section of archived strains, with different geographic origins and clinical properties. Further genetic variations would probably be uncovered if the remaining archived VARV isolates were sequenced; this additional information may be of benefit in finer molecular epidemiologic or “forensic” studies.

Further insights into VARV biology have come from study of individually expressed VARV proteins, based on DNA sequence information [26]. For example, proteins interact with serum complement, interleukin-18, interferon-gamma, tumour necrosis factor, chemokines and various cell signalling pathways [93], [32], [3], [122]. Most recently, to gain insights into how many different VARV proteins might physically interact with the...
complete set of human proteins, researchers used a yeast two-hybrid system to systematically screen all the unique VARV proteins that are not found in VACV against the complete human proteome. This study revealed many new interactions between human and VARV proteins, including a new family of VARV inhibitors of an important human inflammatory signalling cascade that is mediated by nuclear factor kappa B [81].

7.9 **VARIOLA VIRUS EVOLUTION**

All poxviruses continue to diverge from each other by genetic mechanisms that include point mutations, insertions, deletions, various recombination events, and the loss or acquisition of entire genes [78], [39], [7]. The available genomic sequencing data show several distinctive groups of VARV. Depending on the method of analysis, these groupings can be considered either as three distinct clades, or as two major clades, one containing two sub-groups [30], [70]. Members of the first group include isolates of variola major from Asia that are associated with high mortality, and isolates from Africa that are associated with varying mortalities. The second group consists of variola minor (alastrim) from South America, which has lower fatality rates. Closely-related isolates from the third group are from western Africa, and are associated with intermediate levels of disease severity. These sequencing studies provide important clues about the spread of smallpox around the world.

It has been estimated that VARV diverged from an ancestral poxvirus, probably from rodents in Africa, at some time between 16,000 and 68,000 years ago [70]. The original ancestral virus that was the evolutionary parent to the current OPVs remains unknown. The best evidence suggests that existing strains of CPXV are more closely related to the original ancestor virus, because CPXV contains the largest number of the variable genes, some of which are closely related to orthologues in VARV [99]. It is also interesting that all other known OPV genomes, including VARV, contain a number of genes that have been fragmented or inactivated compared with the larger CPXV genome.

7.10 **POXVIRUS GENOME TECHNOLOGIES**

Today it is now technically possible to synthesize the entire VARV genome from scratch, using only publicly available sequence information, and to reconstitute an infectious VARV. Gene synthesis has been used to synthesize infectious poliovirus *de novo*, and to resurrect the 1918 pandemic strain of influenza virus [20], [111]. The ability to synthesize any gene raises the concern that anyone could reconstruct a live OPV. This would not be as straightforward a task as it was for poliovirus, since naked poxvirus DNA is not infectious; however, all the necessary technical methods exist. In 2002, infectious VACV was recovered from a full-length viral genome cloned into a bacterial artificial chromosome [24]. Furthermore, it was possible to assemble and reactivate VACVs using mixtures of DNA fragments transfected into cells that had previously been infected with a helper leporipoxvirus [124]. There is no compelling reason to believe that wholly synthetic fragments could not be used to resurrect live VARV. Thus it may no longer be possible to eradicate the threat of re-emergence of live VARV, even if the existing virus stocks at the WHO collaborating centres are destroyed.

*De novo* synthesis of an intact VARV is not the only way VARV or a VARV-like virus could be created. There is no doubt that methods could be used to modify the virulence of any OPV, including VARV. For example, OPV genomes are readily altered using homologous recombination, and drug-resistance markers are easily introduced into normally drug-sensitive strains. Similarly, the insertion of host immunoregulatory genes has the potential to alter poxviral virulence or the sensitivity of the infection to previous vaccination as demonstrated by Jackson *et al.* [50] having inserted the IL-6 gene in the genome of mousepox virus, thereby increasing virulence significantly. No barrier would prevent the substitution of one OPV gene for another, the incorporation of a gene unique to pathogenic OPVs into VACV, the replacement of homologous parts of one genome with synthetic
segments copied from another virus, or the generation of hybrid viruses (for example, a hybrid of MPXV and CMLV) with potentially novel virulence patterns that could mimic those of VARV. Certainly, this is a sobering thought, especially if drug resistance alleles of target genes were to be engineered into a reconstructed virus.

7.11 GUIDELINES FOR WORK WITH VARIOLA VIRUS DNA

The distribution, synthesis and handling of VARV DNA are governed by a series of rules [120]. Other than at the two WHO Collaborating Centres for Smallpox and Other Poxvirus Infections, it is strictly forbidden to hold clones containing more than 20% of the VARV genome at any one time. A request to handle VARV DNA greater than 500 nucleotides in length must be submitted through WHO headquarters, and the receiving laboratory may not distribute VARV DNA to third parties. In addition, VARV DNA cannot be used for insertion into VACV or any other poxvirus, and no other OPV can be handled in laboratory rooms where VARV DNA is present. However, VARV DNA not exceeding 500 base pairs can be used as a positive control in diagnostic PCR kits without prior permission, although notifying WHO is desirable in these instances. Likewise, the production of DNA microarrays, on which oligonucleotides (less than 80 base pairs) are covalently bound and therefore difficult to reassemble through ligations, can be performed without permission of the WHO. These oligonucleotides could – in aggregate – span the entire genome.

The advances in genomic technologies discussed above require a reappraisal and updating of current VARV containment strategies. These strategies were designed in the 1980s, and have been revised frequently since then. The possibility that poxviruses could be recovered from cloned DNA using reactivation methods is why no single laboratory is permitted to retain more than 20% of the VARV genome, and why any manipulation of VARV DNA must be geographically isolated from work with other poxviruses. The existing controls focus sensibly on physical and administrative control of access to live virus or cloned fragments of the VARV genome; they certainly remain relevant and need to be retained, along with prohibitions on performing activities such as the deliberate introduction of VARV genes into other poxviruses. However, when these procedures and guidelines were developed, nobody anticipated that, 25 years later, advances in genome sequencing and gene synthesis would render substantial portions of VARV accessible to anyone with an internet connection and access to a DNA synthesizer.

This problem has been discussed particularly by researchers in the field of “synthetic biology”. In 2007 a proposal was outlined for managing biological security [16]. These proposals have not been adopted by any Member States as official policy; however, they have been adopted as operating principles by some of the commercial companies. For example, BLAST searches are being used to filter all requests for its services against the lists of controlled pathogens – including VARV and MPXV – that have been identified by the Australia Group (http://www.australiagroup.net). Customers requesting synthetic services that match with these lists are required to identify themselves and provide the necessary import and export documents. Even if companies and institutions offering such services adhere to surveillance guidelines, this type of surveillance would not cover scientists carrying out gene synthesis with their own equipment.

7.12 ANIMAL MODELS

This chapter describes briefly animal models for OPV disease that promise to be useful for developing countermeasures for smallpox. Primate models using VARV or MPXV are most relevant for this purpose, however, primate studies are expensive, and use of VARV requires the highest level of biosafety (BSL-4) and biosecurity. MPXV, while less restricted, still requires BSL-3 biocontainment and is a Select Agent. As a result
of these restrictions, the use of small animal models for OPV disease, using Ectromelia Virus (ECTV), CPXV, Rabbitpox Virus (RPXV) and VACV, has a place in efforts to develop countermeasures.

7.12.1 Variola Virus Primate Models

An animal model in which VARV produces a disease similar to human smallpox would be ideal to demonstrate the protective efficacy of vaccines and anti-viral drugs. Because of the species specificity of VARV, it was not surprising that attempts to infect and produce disease with VARV in rodents and rabbits were unsuccessful [75]. Indeed, even in primates, early experiments with VARV resulted in mild but self-limited infections. The historical record thus suggested that there were no suitable models for the pathogenesis of VARV in humans [112]. However, infection of macaques was known to produce skin lesions and evidence of systemic infection, and a primate model was used to license MVA in Germany in the 1960s [45]. It was therefore reasonable to test other VARV strains in higher doses by a variety of routes to seek a model for lethal smallpox. Aerosol exposure to either the Yamada or Lee VARV strains (10^8.5 PFU) resulted in infection but no serious disease [68]; however, when monkeys were exposed to either Harper or India 7124 VARV strains by the IV route, acute lethality resulted [51]. Doses lower than 10^9 PFU caused lower lethality. End-stage lesions closely resembled human pathology [51]. Apoptosis of T-cells occurred in lymphoid tissue and the resultant cytokine storm. “Toxaemia”, described by clinicians as the terminal event in human smallpox, probably results from overstimulation of the innate immune response. Whether MPXV in monkeys is a better model for human smallpox than VARV in monkeys is a focus of intense investigation. It is generally acknowledged that both primate models replicate some, but not all, features of human disease. IV infection leads to a sequence of disease manifestations that is similar to the disease in humans [15], although it is accelerated due to the elimination of a prodromal period.

7.12.2 Monkeypox Animal Models

MPXV is a human pathogen that clinically resembles smallpox, although it has less potential for transmission from person to person [53]. There is evidence that MPXV strains of West African origin are less virulent than those that arise in the Central African basin [77]. The name “monkeypox” is a misnomer, since the virus is maintained in nature in rodent reservoirs, including squirrels [58], [21]. In 2003, MPXV was inadvertently imported into the United States in a shipment of rodents originating in the Republic of Ghana [72]. A number of prairie dogs held in the same facility were infected, and more than 75 human cases were seen. Experimental infection of ground squirrels with MPXV was reported to kill all squirrels by the IP route, or by the intranasal route, within 6 – 9 days [107]. MPXV infection of squirrels might be a useful animal model for testing countermeasures for monkeypox and smallpox and recent advances have been reported [48]. STAT1-deficient C57BL/6 mice were reported to be susceptible to low doses of MPXV via the intranasal route [101] as well as the commercially available inbred mouse strain CAST/EiJ [4].

Primates have been infected with MPXV via the aerosol [125], intramuscular [117], intratracheal, intrabronchial and Intravenous (IV) routes of exposure [104]. Aerosol exposure is most appropriate for modelling primary exposures following a biological warfare attack. Experimental MPXV infection resulted in five of six monkeys dying with a mean time to death of 10.4 days, high titres of virus (> 10^6 PFU/g) were isolated from lungs and spleens [125]. The clinical parameters occur in a sequence similar to that in humans [15]. All monkeys followed this typical pattern of progressing through the stages of lesion development, and those that lived long enough ultimately developed scabs. The IV MPXV challenge model was used to test the efficacy of a candidate vaccine for smallpox, the highly attenuated Modified Vaccinia Virus Ankara (MVA). There has been some reluctance to accept the IV challenge model, on the grounds that the challenge should be via the “natural” route.
7.12.3 Ectromelia, Vaccinia and Cowpox Animal Models

Much of the knowledge about VARV pathogenesis is inferred from studies with ECTV in mice [17]. ECTV is a natural pathogen of laboratory mice and, after it was discovered in the 1930s, Fenner used the model to elucidate the concept of primary and secondary viraemia, which parallels exanthematous disease in humans [34]. ECTV has been extensively used to study viral pathogenesis, cell-mediated immunity, and genetic resistance to infection. The classic inbred strains vary greatly in their susceptibility with lethality occurring with doses of 1 PFU or less in the most sensitive strains [115]. BALB/c and DBA/2 strain mice are, like A/J mice, highly susceptible via both dermal and aerosol routes. Resistance and susceptibility can also vary with the ECTV strain. Following low-dose inoculation of ECTV into the footpad, the disease course is delayed involving successive local and systemic spread mimicking smallpox, though with more rapid severe disease onset and accentuated hepatic involvement [34]. In an intranasal mouse (BALB c and C57BL 6) model, there is a 7-day disease lag period and a 10-day mean time of death [87]. Early virus proliferation in the lungs is followed by spleen and liver infection reaching peak values at 8 days.

VACV also infects mice; the outcome depends on mouse genetics, VACV strains, doses and routes of exposure. Infection of C57BL/6 mice by VACV strain Western Reserve (WR) via the intranasal route using doses greater than 10^5 PFU is lethal [14]. BALB/c mice are somewhat more resistant to VACV and had been used to rank VACV strains for virulence. The New York City Board of Health (NYCBH) strain, with an LD_{50} via the intranasal route of 10^{4.8} PFU, was more virulent than the WR strain, with an LD_{50} of 10^{4.8} PFU.

Rabbits exposed to RPXV via the aerosol route develop a disease syndrome similar to humans with smallpox [64], [118]. Rabbits typically remained healthy for a 4 – 6 day incubation period, followed by fever, weakness, rapid weight loss, and profuse, purulent discharges from the eyes and nose. A bright erythema appeared on the lips and tongue, coinciding with a generalized skin rash, with the number of lesions varying from a few to confluence. The lesions started as red papules, converting to pseudo-pustules with caseous contents. Death usually occurred before true scabs could form. In more recent studies, intradermal inoculation of rabbits resulted in a similar disease pattern [1]. A viral dose of 1 × 10^2 PFU administered intradermally results in systemic infection, but a higher dose (5 × 10^2 PFU) is required for lethality. RPXV infection of the rabbit has parallels to human smallpox. RPXV rabbit model could possibly be developed further and used to test candidate therapeutics and vaccines.

CPXV is endemic in wild rodents in Europe and central Asia and can be transmitted to cats, pet rats, and other animals to humans. In immunocompetent humans with no history of skin disorders, CPXV causes a localized lesion. No human-to-human transmission has been reported. Genetic analysis indicates at least two major CPXV groups and several sub-groups of CPXV. CPXV has many more immune modulators than other OPXV including VARV. In mice, the 50% lethal intranasal dose of the Brighton strain is similar to that of VACV WR. One promising approach is CPXV infection of marmosets (Callithrix jaccus) [60]. Marmosets are lethally infected by CPXV challenges as low as 5 × 10^2 PFU via the intranasal route, and they develop a progression of signs reminiscent of smallpox. Although the marmoset is more distantly related to humans than are macaques, and immunological reagents are not yet readily available, this species holds significant promise for future model development.

7.13 ANTI-VIRAL DRUG DEVELOPMENT

Naturally occurring smallpox disease was being eradicated by mass vaccination at a time when anti-viral therapy was still in its infancy. Drug development for smallpox is a complicated, lengthy and costly process. It begins with discovery of a compound that selectively inhibits viral replication in vitro. This discovery guides organic
chemists to synthesize similar chemical structures and to determine the most active compound in that class. Often, this is followed by a medicinal chemistry approach, in which the structure is systematically modified to produce the most potent compound, now referred to as a “lead compound”. Complex multi-disciplinary studies in animals, conducted in compliance with Good Laboratory Practice (GLP), are then required to understand the compound’s metabolism, pharmacokinetics, distribution in tissues and toxicity. At this time, an extensive series of evaluations, referred to as the “microbiology section”, must be conducted in vivo and in vitro. These evaluations provide information on the compound’s ability to inhibit viral replication, and to reduce morbidity and mortality. The studies suggest the minimum concentration required for drug activity, and, in conjunction with Investigational New Drug (IND) Phase I human studies, provide the initial estimate for the corresponding target dose in humans.

Phase I studies in humans begin with a small number of subjects and a concentration of the compound below the level predicted to cause any toxicity. If the drug candidate is safe at levels predicted to be therapeutic, an expanded safety study is conducted. These studies ultimately involve special populations that are candidates for treatment, including those with complicating medical conditions. Ultimately, safety will need to be evaluated in hundreds of subjects (evaluation in 600 subjects will detect an adverse event at the 1% level). Historically, the efficacy data that the United States Food and Drug Administration (USFDA) has required for drug approval come from “well-controlled”, pivotal human clinical trials. This requirement cannot be met with drugs for smallpox. To address this problem, the USFDA has published what is commonly referred to as the “animal rule” [113]. The animal rule allows demonstration of efficacy using an animal model (or models) that adequately reproduces the critical aspects of disease, and in which a similar reduction in the magnitude of the disease in humans would be expected to reduce morbidity and/or mortality.

### 7.13.1 Intravenous Cidofovir

IV cidofovir (Vistide) is approved by the USFDA for treatment of HCMV retinitis in acquired immunodeficiency syndrome patients. Cidofovir, a small molecule nucleoside analogue that selectively inhibits the viral DNA polymerase, reduced the replication of VARV 1000- to 100 000-fold in vitro [9]. Two primate models of post-exposure prophylaxis were used. Treatment was initiated 24 hours after infection, when replication reached more than 10^4 genomes/g of tissue. To determine whether a lesion model could be treated successfully, the lethal intravenous MPXV model was used to show that cidofovir prophylaxis completely protected the animal. Cidofovir-treated monkeys showed no signs of illness, and viral replication in blood was controlled. Studies with MPXV demonstrated that doubling the cidofovir dose (four times the approved human dose) resulted in better control of viral replication; however, this higher dose is not approved for treatment of HCMV retinitis in human AIDS patients. Cidofovir is associated with potential significant nephrotoxicity – requiring IV pre-hydration, administration of probenecid and post-IV hydration – which would be a serious drain on medical facilities during an outbreak. Cidofovir was then evaluated against VARV in the post-exposure prophylaxis model. Groups of three cynomolgus monkeys were treated beginning on days 0, 1 or 2 and compared with placebos. One of three (33%) placebo-treated monkeys died, and all three were critically ill. None of the cidofovir-treated monkeys died or became seriously ill. Lesion counts and viral load were reduced in all cidofovir-treated groups compared with the placebo group. These results demonstrated that cidofovir given before the onset of rash illness, but not afterwards, can prevent mortality.

### 7.13.2 Oral CMX001 (Brincidofovir)

Brincidofovir, the lipid analogue (HDP-cidofovir, CMX001) of cidofovir, is orally bioavailable, and no nephrotoxicity has been detected [57], [67]. Compared with cidofovir, which is taken up into cells by inefficient processes, the conjugate is designed to act like lysophosphatidylcholine, using natural lipid uptake pathways to
achieve high intracellular concentrations. Once inside target cells, the lipid side chain of CMX001 is cleaved to yield free cidofovir. Conversion of cidofovir to the active anti-viral agent occurs via a two-step phosphorylation process. Cidofovir diphosphate exerts its anti-viral effects intracellularly, by acting as a potent alternative substrate inhibitor of viral DNA synthesis. Brincidofovir is active against the five families of double-stranded DNA viruses, including all OPVs. Brincidofovir is well absorbed in humans, leading to high plasma concentration, it has a high barrier to development of resistance, is stable for several years, and has been administered to more than 800 people. A comprehensive non-clinical safety assessment has been completed. Since 2009 it has been used under Emergency IND regulations and in an open-label study with 215 patients to treat infections caused by cytomegalovirus, adenovirus, multiple other herpes viruses and a case of progressive vaccinia. Following discussions with the USFDA, the development of brincidofovir for an indication of treatment of smallpox will use the intradermal rabbitpox model and the intranasal ectromelia model in mice. Brincidofovir was effective in both models when treatment was initiated 3 – 5 days after infection, and survival correlated with dose. On the basis of the results in animal models it is predicted the concentrations of CMX001 and the active metabolite cidofovir-diphosphate needed for treatment of smallpox can be achieved in humans with doses currently being evaluated clinically for treatment of other diseases. Tablet and liquid formulations are expected to be commercially available. Manufacturing on a commercial scale has been validated.

7.13.3 Oral Arestvyr™ (ST-246)

Arestvyr™ (ST-246) is a smallpox anti-viral drug that is in late-stage development for use as a therapeutic for symptomatic patients, as a prophylactic in infected but non-symptomatic patients, and for concomitant administration with smallpox vaccines to improve their safety profiles.

The drug targets a gene that encodes a major envelope protein, found on the outer membrane, it acts as an egress inhibitor which blocks the formation of enveloped forms of OPVs. Plaque formation and virus-induced cytopathic effects are inhibited, thereby effectively inhibiting dissemination both in vitro and in vivo. Arestvyr™ is OPV-specific and is not cytotoxic at concentrations that are inhibitory for virus. Pre-clinical safety/pharmacology studies in mice and non-human primates indicate that Arestvyr™ is readily absorbed by the oral route and is well tolerated (no observable adverse effect at 20 to 30 times the effective anti-viral concentration for these species). Excellent post-exposure efficacy has been demonstrated in more than 40 different animal challenges including mice, ground squirrels, prairie dogs, rabbits, and non-human primates challenged with a variety of different pathogenic OPVs including VACV, CPXV, RPXV, ECTV, MPXV and including VARV-infection of cynomolgus monkeys, which closely resembles human smallpox [123], [8]. In this experiment the placebo group developed typical disease, with many pox lesions and 33% mortality. Oral treatment with Arestvyr™ began 24 hours after infection, when bone marrow, spleen, some lymph nodes and liver had more than 10⁸ genomes/g and all tissues had 10⁴–10⁶ genomes/g. The treatment eliminated disease [47].

There is significant progress of Arestvyr™ towards regulatory approval; the drug has investigational new drug status. In clinical studies, the drug appears to be safe and well-tolerated. Once a day oral dosing provides blood exposure at or above that which has been shown to be protective in animal studies. Drug substance and drug product processes have been developed and commercial-scale batches have been produced using processes that conform to Good Manufacturing Practice guidelines. The manufacturing company was awarded a contract worth up to US$ 2.8 billion in 2011 to supply the U.S. Strategic National Stockpile; so far 725,000 of the initial 2 million courses ordered have been delivered. Protocols are in place for the compassionate use of Arestvyr™ in case of adverse event following vaccination with smallpox vaccines. The company has worked with the U.S. regulatory authorities and elaborated a feasible approach to approval under the Animal Rule, which included efficacy studies in an intradermal rabbitpox model and non-human primate model. The European Medicines Agency has given orphan designation to Arestvyr™ in May 2012 for the treatment of CPXV infection. Work
continues on the development of additional Arestvyr™ formulations and towards approval of the drug for additional indications.

### 7.13.4 Effect of Administration of ST-246 on Vaccine Protection

The effect of co-administration of cidofovir and Dryvax has been examined in mice and monkeys. In cynomolgus macaques VACV viral loads and Dryvax adverse events were reduced; however, cidofovir also reduced immunity and decreased protection from an MPXV challenge [116]. When Dryvax and cidofovir were co-administered to A/NCR mice, lesion sites were smaller and healed faster, but as with the monkey study, antibody responses were reduced. These data are expected when a drug that inhibits viral replication is combined with a vaccine that requires multiple rounds of replication.

Studies have shown that Dryvax and ACAM2000 vaccine efficacy is not compromised by ST-246 treatment given at the time of vaccination. Normal immunocompetent mice were vaccinated with Dryvax [38] or ACAM2000 [12], using the standard human dose and route, and treated with ST-246 immediately after vaccination. The severity of vaccine lesions and time to resolution were reduced by ST-246 treatment. Furthermore, virus shedding from the lesion site was reduced [11]. Humoral immune responses may have been slightly reduced by ST-246 treatment, but cellular immune responses appeared to be slightly increased. Animals that were vaccinated and treated with ST-246 were equally protected from a subsequent lethal challenge in both short- and long-term experiments, clearly demonstrating that ST-246 does not adversely affect vaccine efficacy. Further experiments demonstrated that, even in a partially immunodeficient setting, ST-246 improves vaccine safety while allowing the induction of robust immune responses that are capable of resisting lethal challenge.

### 7.14 SMALLPOX TODAY?

Since widespread (pre-exposure) vaccination against smallpox is extremely unlikely to occur, an aerosol release of smallpox has the potential for a catastrophic scenario. In the past, the items of the intensified smallpox eradication program were:

i) Early detection;

ii) Isolation of infected individuals;

iii) Surveillance of contacts; and

iv) A focused selective vaccination program [44].

Today – and this is a major achievement – with the availability of anti-virals, cases can be treated before mass vaccination campaigns have time to provide adequate protective immunity. The goal for an effective therapeutic treatment is a reduction in mortality when treatment is begun after onset of lesions, and this is the only practical diagnosis during a large outbreak. A simple, rapid test to identify smallpox in the oropharynx during the prodrome would be of great help in triage of suspected patient, but there is no test available. Vaccination after exposure to smallpox virus infection may be effective in minimizing casualties [82]. Several studies have evaluated this possibility in animal models. Most have concluded that the vaccine has to be administered no later than 1 – 2 days after exposure to protect against death [102], [97], [87]. Interestingly, MVA elicited a more rapid protective response than the NYCBH strain in a monkeypox challenge model [28]. Therefore, it is of great advantage that both, vaccination and anti-viral therapy can be given simultaneously without negative impact.

Prior to its eradication, smallpox as a clinical entity was relatively easy to recognize. Today it is even more important to educate health care professionals about the diagnostic features of smallpox, and to conduct
advanced region-wide planning for isolation and care. Ultimately, success in controlling will depend on the availability of adequate supplies of vaccines, VIG and anti-virals.

7.15 REFERENCES


Chapter 8 – FILOVIRUSES: POTENTIAL VACCINES, THERAPEUTICS, AND DRUG TARGETS

Paula Bryant¹, Brian M. Friedrich², Julia E. Biggins², John C. Trefry², Anna N. Honko³, Lisa E. Hensley³ and Gene G. Olinger³

1: Defense Threat Reduction Agency, Chemical and Biological Technologies Directorate, Fort Belvoir, VA; 2: United States Army Medical Research Institute of Infectious Diseases, Division of Virology, Frederick, MD; 3: National Institutes of Health, National Institutes of Allergy and Infectious Diseases, Integrated Research Facility, Fort Detrick, MD; 4: National Institutes of Health, National Institutes of Allergy and Infectious Diseases, The Office of Biodefense, Research Resources and Translational Research, Rockville, MD

UNITED STATES

Corresponding Author (Bryant): paula.bryant@nih.gov

8.1 INTRODUCTION

Ebolavirus and Marburgvirus, the two genera of the family Filoviridae, cause Viral Hemorrhagic Fever (VHF) in infected humans and Non-Human Primates (NHP). VHF is an acute febrile syndrome characterized by systemic involvement, which includes generalized bleeding in severe infections. Clinical symptoms usually appear after an incubation of 2 – 21 days [1]. Patients often present with fever, malaise, myalgia, and chills. There is evidence of multi-system involvement as the disease progresses, with manifestations including anorexia, vomiting, prostration, nausea, diarrhea, abdominal pain, shortness of breath, confusion, edema, and coma. Filoviruses are considered among the deadliest of human pathogens due to their high morbidity and mortality rates (ranging from 23 – 90 %), and death typically occurs between day 6 and 16 after exposure [1]-[3]. Fatal filovirus infections are usually also associated with high viremia, widespread focal tissue destruction, increased endothelial cell permeability, lymphopenia, and severe coagulation abnormalities [1]. The U.S. Centers for Disease Control and Prevention (CDC) classifies filoviruses as category A bioterrorism agents [4]. Filoviruses have been shown to be highly infectious and lethal to NHPs when delivered by aerosol and are stable as respirable aerosols, thereby posing a significant threat to military personnel and global security [2], [4], [5]. Indeed, the Soviet Union, Russia, and the United States weaponized several hemorrhagic fever viruses, and both the Soviet Union and Russia produced large quantities of Ebola and Marburg viruses until 1992 [4].

Currently, there are no vaccines or anti-viral drugs effective against filoviruses that are licensed and available for human use. Despite increased interest in studying these viruses, many aspects of filovirus pathogenesis – especially in man- remain unknown, leaving researchers to rely on animal models. Moreover, studies of filoviruses are confined to maximum-containment laboratories due to their high lethality rates, which can make new research tedious and slow.

8.1.1 Filoviridae

The Ebolavirus genus consists of five species (each with a single virus) in the new proposed taxonomy:

- Zaire ebolavirus (EBOV);
- Sudan ebolavirus (SUDV);
- Reston ebolavirus (RESTV);
The Marburgvirus genus consists of a single species, Marburg marburgvirus, consisting of two viruses: Marburg virus (MARV) and Ravn Virus (RAVV) [6]. The first known outbreak of filovirus hemorrhagic fever occurred during simultaneous outbreaks in Marburg and Frankfurt, Germany in 1967. The agent responsible was named “Marburg virus” for the German town where illness was initially observed [7]. This new virus would later be reclassified as the first member of the family Filoviridae [8]. Most Marburg virus infections are prevalent in arid woodlands of western, south-central, and eastern Africa, while Ebola virus outbreaks tend to occur in central and western African rain forests [9]. More than 40 years of effort have been focused on the search for the reservoir of these viruses in Africa. Currently, MARV is thought to circulate in bat populations (most likely the fruit bat Rousettus aegyptiacus), and there is suggestive evidence that bats are also carriers of EBOV [6], [10]-[13]. However, it was recently reported that domestic swine in the Philippines are hosts for RESTV, suggesting that other animals may act as carriers for these viruses [14].

Filoviruses possess an approximately 19 kb, single-stranded, linear, non-segmented, negative sense RNA genome encased within a ribonucleoprotein complex [1]. The viral genome contains seven genes that encode a Nucleoprotein (NP), matrix protein (VP40), polymerase cofactor (VP35), transcriptional activator (VP30), a secondary matrix protein (VP24), a Glycoprotein (GP), and a RNA-dependent RNA polymerase (L) [15], [16]. Homotrimers of the viral GP cover the surface of the virion, and this viral GP is believed to be the sole host attachment factor for filoviruses [15]-[17]. Studies have focused on identifying the host receptors required for filovirus entry into the cell, with the aim of developing therapeutics. While transferrin and DC-sign have been proposed as cellular ‘attachment’ receptors for filoviruses, the cellular ‘entry’ receptor has remained elusive as filoviruses exhibit a wide cellular tropism in infected individuals [17], [18]. On epithelial cells, the T-cell immunoglobulin and mucin domain-containing 1 (TIM-1) was recently identified as a cellular receptor for both Ebola and Marburg viruses, but how it promotes entry remains to be determined [19]. In addition, two independent groups demonstrated a requirement for the ubiquitously expressed cholesterol transporter, Niemann-Pick 1 (NPC1), in EBOV entry, although additional studies are necessary to determine its exact role in entry and if additional factors are required [20], [21]. After entry, filoviruses replicate their genomes and viral proteins in the cytoplasm. Particle assembly then occurs at late endosomal surfaces with the help of VP40 and various cellular factors [17]. VP40 contains highly conserved motifs that enable the protein to interact with cellular components during assembly and budding [22].

8.1.2 Pathogenesis and Host Immune Response

Fatal filovirus disease is characterized by a failure of the infected host to clear the infection, excessive host inflammation, activation of coagulation cascades and lymphocyte apoptosis. In a natural infection, filoviruses are thought to enter the host through mucosal surfaces, breaks, and abrasions in the skin, or by parenteral introduction. Most human infections have occurred by direct contact with infected patients, cadavers, re-use of contaminated needles, in butchering chimpanzees for food, and from the handling and consumption of freshly killed bats [1]. The role of aerosol transmission in natural outbreaks – the route of administration of most concern if the virus were to be weaponized is unknown, but is thought to be rare [1]. The route of exposure seems to affect the disease course and outcome, as the disease in NHPs infected with Ebola Zaire by intramuscular or intraperitoneal injection progresses faster than in animals exposed by aerosol droplets [23]. Early during infection, filoviruses target and replicate in monocytes, macrophages and DCs, and these cell types are responsible for disseminating the infection [1]. As the infection progresses, additional cell types become viral antigen positive, with lymphocytes being the only cells believed to be resistant to Ebola infection [1], [24].
The endothelium is thought to play an important part in the pathogenesis of filoviruses, although the molecular mechanisms of endothelial impairment are not well understood. In addition, the liver and the adrenal gland seem to be important targets for filoviruses, and the impairment of adrenocortical function by Ebola virus infection could play an important role in the evolution of shock that typifies late stages of Ebola hemorrhagic fever [1].

Filovirus infections are characterized by lymphocyte depletion and necrosis in primary lymphoid organs of patients and NHPs with fatal disease [1]. Large numbers of lymphocytes undergo apoptosis in man as well as NHPs, partly explaining the progressive lymphopenia and lymphoid depletion at death [1]. The observed apoptosis during infection is thought to be provoked by several different agonists or pathways, to include the TNF-related apoptosis-inducing ligand and Fas death receptor pathways, impairment of dendritic cell function induced by infection, abnormal production of soluble mediators such as nitric oxide, or possibly by direct interaction between lymphocytes and filovirus proteins [1]. Interestingly, an immunosuppressive motif has been identified in the carboxyl-terminal region of the virus’ glycoproteins lending support to the hypothesis that virus particles or proteins might partly contribute to the dysfunction and/or loss of lymphocytes [1].

Much of the disease associated with Ebola or Marburg infections appears to be caused by dysregulation of the innate immune response, although replication of these hemorrhagic fever viruses in target cells and tissues can directly contribute to the pathological manifestations of VHF. One key pathogenic mechanism of filoviruses is the ability to inhibit the Type I Interferon (IFN) response. Filoviruses encode two proteins that block induction Type I IFN. Ebolavirus VP24 directly blocks IFNαβ and IFNγ signaling by preventing STAT1 localization to the nucleus [24]-[26]. The dsRNA-binding protein VP35 inhibits Type I IFN induction by several independent mechanisms. Combined, global inhibition of Type I IFN is achieved, which is strongly associated with increased virulence. In addition, Ebola directly inhibits the anti-viral activities of the RNAi pathway through the actions of VP35, VP30, and VP40. VP35 inhibits the RNAi pathway by both binding dsRNA and, along with VP30, direct interaction with various components of the pathway [24], [26]. Fatal cases of filovirus infection are characterized by an inappropriately stimulated and quickly depleted immune response. Filovirus infections of macrophages trigger an imbalanced or aberrant cytokine profile, to include IL-1β, IL-6, IL-8, IL-15, IL-16, MIP-1α and –β, IP-10 and nitrous oxide with an upregulation of CD95 on lymphocytes [24], [27]. One consequence is an elevation of TF on macrophages, leading to the breakdown of the vasculature and disseminated intravascular coagulation [1], [27].

Despite replicating in Dendritic Cells (DCs), activation of infected DCs appears to be minimal and the absence of DC activation may be one of the primary reasons non-productive immune responses are not observed during filovirus infections [1], [28], [29]. In studies that examined DC function following Zaire ebolavirus infection, the virus induced relatively little cytokine production and little to no IFNαβ [28], [29]. Instead of effectively activating DCs, Ebola Zaire infection induced “aberrant” DC maturation, evidenced by upregulation of cell surface CD40 and CD80, and only small increases in CD86 and Class II MHC (HLA-DR), an absence of CD11c and CD83 upregulation and a failure to decrease CCR5 [28], [29]. Based on these observations, it has been argued that EBOV suppression of DC function prevents initiation of adaptive immune responses and facilitates uncontrolled, system virus replication [28], [29]. Consistent with this hypothesis, APCs from fatally infected individuals do not upregulate Class I and II MHC complexes, preventing T-cell activation [1] and, consequently, despite the significant production of cytokine release from macrophages, it is nonetheless ineffective at stimulating effective adaptive immune responses and production of T-cell-specific cytokines is limited [24], [27]. Survival in humans is correlated with lower levels of pro-inflammatory cytokines, T-cell survival, production of filovirus-specific antibodies, and lower viremia [24], [27]. Consistent with these data, the least pathogenic Ebola strain identified (Bundibugyo) induced 2 – 10 fold lower levels or pro-inflammatory cytokines, reduced macrophage activation and decreased cell death when compared to cells infected with the highly pathogenic Zaire strain [30].
8.1.3 Animal Models

Human clinical trials to determine medical countermeasure efficacy against filoviruses cannot be conducted because there are too few cases, the occurrence is too sporadic, and the severity of the disease is too great. Instead, licensure in the United States of filovirus-specific countermeasures will only be possible using the “Animal Rule” of the U.S. Food and Drug Administration (FDA), which allows for a demonstration of efficacy in one or more well-characterized animal models which are considered relevant to the disease in humans [3], [31]. Several animal models are available for the study of filovirus infections. Multiple species of Non-Human Primates (NHPs), including rhesus monkeys, cynomolgus macaques, African green monkeys, vervet monkeys, and baboons are susceptible to wild-type filovirus infection. However, these distinct NHP models show substantial differences in filovirus pathogenesis and host response to infection [15], [32]-[34]. The field has focused on the use of Rhesus monkeys and Cynomolgus Macaques as NHP models, as albeit the limited data available from human outbreaks suggests these species more accurately depict filovirus disease in humans [15]. Filoviruses must be adapted via serial passage through the rodent to produce lethal infections in guinea pig and mouse models. Nonetheless, due to expense, space and assay limitations of NHP models, the use of rodent models is indispensable for early testing and modification of vaccines and therapeutics, as well as determining correlates of immunity for filovirus studies [15].

Despite the extensive use of in vivo models by researchers to study the disease caused by filoviruses, the lack of understanding of filovirus disease pathogenesis in humans, immune correlates, strain selection, and natural history studies make identifying the appropriate animal model difficult. Moreover, the threat of filoviruses as biological weapons would likely be in the form of an aerosol, and aerosol transmission is not thought to be a major factor in natural filovirus outbreaks. Researchers are actively attempting to fill these basic science gaps with hopes to facilitate the successful advancement of candidate therapeutics and vaccines through the regulatory path towards licensure via the animal rule.

8.1.4 Medical Countermeasure Strategies

As stated, there are no currently available FDA-licensed vaccines or therapeutics effective against filoviruses. As described above, filoviruses have developed several mechanisms to subvert the host immune response [1], [24]. Thus, in order to be successful, medical countermeasures must either prime the immune response before the pathogen has a chance to establish an infection (i.e. vaccine), or reduce and control viral load (i.e. anti-viral therapeutic).

For vaccines, the strategy to date has followed the typical path of beginning with testing conventional inactivated whole virions, and then moving to platforms such as recombinant sub-units, plasmid DNA expressing filovirus genes, virus-like particles, and viral-vector-based approaches (discussed below). Inactivated vaccines – with or without liposome or adjuvant, and independent of route of administration – were not effective in protecting NHPs against EBOV [15]. Due to significant safety concerns coupled with poor results, the inactivated approach has not been aggressively pursued further. However, it should be noted that in a more recent study, mice vaccinated with two doses of a replication-deficient EBOV lacking the VP30 gene and protein product protected mice and guinea pigs from lethal challenge [35].

The majority of vaccine platform approaches pursued against filoviruses are based on generating immune responses against GP, with the aim of eliciting protective neutralizing antibodies. In contrast to MARV vaccines, EBOV vaccines have demonstrated protection only when the GP expressed in the vaccine platform is homologous to the infectious challenge species, aggravating progress toward cross-species protective vaccines [15], [36]. A single study demonstrated that limited but improved cross-protection could be achieved by including EBOV VP40 or NP as a second antigen expressed by the same rVSV vector that encodes the
heterologous GP [37]. An exception to the requirement for homologous GP to confer protection occurred in studies of the newly emerged (2007) Ebola Bundibugyo Virus (BDBV). Both rVSV expressing GP from Ebola Zaire [38] and DNA/AdV vaccine expressing Ebola Zaire GP [39] vaccine constructs provided cross-protection against BDBV. Interestingly, protection in the latter example occurred without the appearance of specific BDBV antibodies, and appeared instead to be mediated by cellular immunity.

Despite identifying GP as the required immunogen some time ago, the advancement of a filovirus vaccine towards licensure has been extremely slow. The primary reason for this lack of progress is that the key to licensing through the animal rule lies in determining correlates of immune protection that are indicative of survival in the vaccinated animal model, and to confirm that these immune responses are indeed observed in humans immunized with the same vaccine. Despite the characterization of at least seven distinct vaccine platforms that confer protection to NHPs from EBOV and or MARV challenge (described below), it is still unclear what types of immune responses need to be induced for successful vaccination against filovirus infection. Evidence to date indicates that each vector platform capable of inducing significant antibody responses is correlated to their efficacy. However, it is unclear whether antibodies alone are sufficient for protection, as ELISA-based IgG levels do not always predict survival outcomes [3], [31] and passive transfer of immune serum to naïve NHPs was not protective [40], [41]. However, it has recently been reported that purified convalescent IgG successfully protected 100% of rhesus macaques from MARV challenge [42], indicating additional studies are required to truly understand the role of antibody in protection. A role for CD8+ Cytotoxic T-cells has also been documented [31], [43]. The broad array of protective vaccine platforms characterized to date suggests that there are multiple ways to abrogate filovirus pathogenesis. Moreover, even with the same platform, mechanisms of immunity may differ based on the filovirus strain being tested.

Antivirals that reduce viral loads, even transiently, may also function as effective medical countermeasure to control filovirus infections. Therapeutic strategies to date have focused on inhibiting the virus directly by targeting mRNA of different viral genes directly through steric hindrance (e.g. Phosphorodiamidate morpholino oligomers, PMOs) [44], or degradation of the target sequence (e.g. siRNA’s targeting EBOV polymerase) [45]. An additional approach to block replication is to target cellular entry by the virus. To this end, research has focused on identifying host cell receptors required for filovirus entry (described above), with the aim of developing Ab’s or small molecule inhibitors to block GP-receptor interaction. A significant challenge with filovirus therapeutics is the window of opportunity for their use.

Whether pursuing vaccine or therapeutic modalities to abrogate filovirus infections, progress depends on a better understanding of filovirus pathogenesis and host response, which in turn relies on the development of animal models that mimic human disease. The confinement of filovirus research to maximum-containment BSL4 laboratories and the lack of financial incentive for adequate industry participation require significant investments and sponsorship by government entities to ensure success.

8.2 SUPPORTIVE CARE

In the absence of a licensed vaccine and approved drug therapy, supportive care is the standard for treating filovirus infection. Supportive care currently consists of oral fluid rehydration, oral medication, blood volume maintenance, nutritional supplementation, and psychosocial support. Oral medication includes drugs that alleviate symptoms such as nausea, vomiting, dyspepsia, anxiety, agitation, confusion, and pain [46]. Additionally, in outbreak settings, supportive care has been expanded to include prevention and treatment of dehydration via Intravenous (IV) fluids, nasogastric delivery of nutritional and vitamin supplementation, and IV administration of medication [46], [47]. Fluid replacement was evaluated briefly in rhesus monkeys, and while less severe renal compromise was observed, there was no apparent benefit to survival observed [48].
supportive care may (or may not) reduce the overall case fatality rate in humans, the infection remains lethal in a high number of cases and the true impact of even simple interventions such as fluid management has yet to be evaluated fully [49].

Severe coagulation disorders are one of the most prominent features of filoviral infection. During activation of the clotting system, the host will make an effort to block the process through inhibitors of the clotting system. In this process, however, the inhibitors are consumed, and, if the rate of consumption exceeds the rate of synthesis by liver parenchymal cells, plasma levels of inhibitors will decline. A number of studies have found positive correlations between plasma levels of inhibitors and the degree of DIC [50], [51]. During DIC, the extrinsic Tissue Factor (TF)-dependent pathway is the dominant route to thrombin generation that leads to fibrin deposition [50], [52]. Over expression of TF, a 47-kDa transmembrane glycoprotein that functions as the primary cellular initiator of the coagulation protease cascades, is a leading cause of DIC and thrombosis-related organ failure [53]. Recombinant nematode Anti-coagulant Protein c2 (rNAPc2), an inhibitor of the TF pathway, provided partial post-exposure protection to rhesus macaques [54]. The anti-thrombotic potential of rNAPc2 has been demonstrated in Phase II trials in the clinical settings of orthopedic surgery [55] and coronary revascularization [56]. Post-infection treatment with rNAPc2 resulted in a 33% survival (3.4-day increase in mean time to death) in EBOV-infected rhesus macaques and a 17% survival (1.7-day increase in mean time to death) in MARV-infected rhesus macaques [54], [57]. While complete protection was not observed, survival and a beneficial effect were achieved in an otherwise uniformly lethal model of virus challenge. In a similar strategy, continuous intravenous drip of recombinant human-activated protein C, a major component of the blood anti-coagulation and currently licensed treatment of sepsis, protected 18% of rhesus monkeys from a fatal challenge [26]. Although these palliative approaches alone are not sufficient to counter a filovirus infection, they do extend the time to death thereby providing a larger window for a potential post-exposure treatment to be effective.

8.3 VACCINES AND THERAPEUTICS IN CLINICAL TRIALS

8.3.1 Vaccines

Although there are quite a few vaccine candidates that have shown significant efficacy in NHP models and several in pre-clinical studies, only one platform has completed first in human, Phase I clinical studies in the United States. This candidate utilizes a replication defective recombinant adenovirus (serotype 5, rAd5) vector to express GP, and was developed at the Vaccine Research Center (VRC) of the National Institutes of Health (NIH) [15], [36]. The goal is to employ a prime-boost strategy utilizing DNA plasmid vaccines expressing GP as the prime, and rAd5-GP as the boost. To this end, two distinct Phase I clinical trials were successfully completed. The first was a safety and efficacy DNA-only vaccine study. Twenty-seven human volunteers were vaccinated three times with either 0, 2, 4, or 8 mg of each of three plasmids, containing Ebola Zaire (ZEBOV) NP, ZEBOV GP and Sudan (SEBOV) GP [58]. The vaccine was well-tolerated and considered safe. Although antibodies were generated, neutralizing antibody titers were not detected. CD4+ T-cell responses were generated in all subjects, with SEBOV-GP responses being strongest. Only about 25% of vaccines generated CD8+ T-cell responses against the antigens. A second Phase I trial vaccinated human subjects with a single dose of two rAd5 vectors: one expressing GP from ZEBOV, and one expressing GP from SEBOV [59]. These vaccines were also found safe and were well-tolerated. CD4+ or CD8+ T-cell responses were generated in less than half of vaccinated individuals. Antibody generation was dependent upon dose. Patients having pre-existing antibodies against AdV had lower response rates and antibody titer magnitude compared to those who did not have pre-existing immunity against the vector. However, pre-existing immunity to AdV did not affect generation of memory T-cells. Additional adenovirus vector-based strategies to circumvent pre-existing immunity will be discussed below.
8.3.2 Therapeutics

Recent success in generating candidate filovirus therapeutics was achieved by exploiting antisense oligonucleotides, specifically, positively charged Phosphorodiamidate Morpholino Oligomers (PMOs) [44]. Phosphorodiamidate Morpholino Oligomers (PMOs) are uncharged antisense agents that are composed of moieties with a morpholino base (versus RNA, whose moieties have a ribose base) linked through methylene phosphorodiamidate [44]. Unlike traditional siRNA, PMOs interfere with the translation of products by sterically blocking mRNA [44]. PMOs are attractive as anti-viral agents due to their favorable base stacking, high degree of duplex stability, high degree of solubility, and lack of hybridization complexities [60]-[63]. PMOs have previously been used to inhibit the replication of flaviviruses and coronaviruses [64], [65]. PMO compounds developed by Sarepta Therapeutics (formerly AVI therapeutics) are PMOs consisting of positively-charged piperazine groups along the molecular backbone to increase potency. Initially, PMOs targeting EBOV VP24 and EBOV VP35 were utilized to highly protect mice and guinea pigs against a lethal challenge with EBOV and MARV [44], [66]. Subsequently, AVI-6002 (a combination of PMOs against both EBOV VP24 and VP35) and AVI-6003 (a combination of PMOs against both MARV VP24 and NP) were developed and tested in NHPs post-exposure. These PMOs, delivered 30 – 60 minutes after infection, protected > 60% of rhesus monkeys against lethal EBOV infection and 100% of cynomolgus monkeys against MARV infection [67]. A recent press release by Sarepta therapeutics announced that their lead therapeutic drug candidate based on their PMOplus™ technology demonstrated 83 – 100 % survival in a NHP study exploring the drugs effect when treatment was delayed for up to 96 hours post-infection with Marburg virus. Phase I Clinical trials of AVI-6002 and AVI-6003 for the treatment of Ebola and Marburg, respectively, were initiated in May 2011. These studies are the first to evaluate the safety and tolerability of Sarepta’s PMOplus™ technology in humans, and are supported by the DoD. The PMOs possess multiple drug properties favorable for further development for use in humans to counter filoviruses or other highly virulent emerging viruses. They are highly stable and can be rapidly synthesized, purified and evaluated for quality. As a result, these PMO agents may be useful for treating these and other highly pathogenic viruses in humans [67].

8.4 VACCINES AND THERAPEUTICS EFFECTIVE IN NON-HUMAN PRIMATES

8.4.1 Vaccines Effective in NHPs

8.4.1.1 Adenovirus Vector Vaccines

Additional variations of the rAd5 vaccine are also in development and have been evaluated in NHP models. Based on the Adenovirus vector platform a pan-filovirus Complex Adenovirus (CAdVax) was developed that substantially increased the genetic payload capacity of the vector, up to 7 kB. Additionally, this strategy involved the blending of four separate vectors expressing the glycoproteins of EBOV, SUDV, and MARV along with the nucleoproteins of EBOV and MARV. When administered in a prime/boost strategy this technology offered 100% protection against EBOV, SUDV, MARV-Musoke, and MARV-Ci67 [68]. Another variation of the CAdVax system designed to express modified EBOV glycoprotein and SUDV glycoprotein was effective in protecting against both parenteral and aerosol challenge when administered in a prime/boost strategy [69]. Both implementations of the CAdVax technology demonstrated significant antibody titers as well.

Further improvement upon the Adenovirus-based EBOV vaccine technology is ongoing. Richardson et al. reformatted the genetic insert for the vector which included the addition of a CMV-chicken β-actin hybrid promoter, optimized codons and a consensus Kozak sequence [70]. These improvements led to 3- to 7-fold increases in EBOV glycoprotein expression. Neutralizing antibody titers were found at doses at low as 10⁴ viral
particles with comparable titers requiring $10^7$ viral particles of the unmodified vaccine in mice. These modifications demonstrated 100% protection of mice at doses two orders of magnitude lower than the unmodified vaccine. Interestingly, at 30 minutes post-challenge, the modified Ad-CMVZGP/Ad-CAGoptZGP offered 100% protection compared to the 22% protection of mice offered from the original vaccine [70].

While this Adenovirus vector vaccine technology is promising, demonstrations that pre-existing immunity to the Adenovirus serotype 5 vector depresses the desired immune response may impede its implementation. In efforts to circumvent issues of pre-existing immunity to Adenovirus serotype 5, Geisbert et al. sought out a less prevalent sero-variation [71]. In their study, a heterologous prime/boost strategy with recombinant Adenovirus serotypes 26 and 35 carrying GP (Z) and GP (S/G) demonstrated complete protection among NHPs. Each of these vectors was capable of stimulating humoral and cell-mediated immune responses in the context of NHPs pre-immunized with rAd5 as evidenced by antibody titers reaching an order of magnitude above those achieved in rAd5 vaccinated subjects, 1:32,000 compared to 1:6,800, and CD8+ intracellular cytokine staining 4.7-fold greater among heterologous prime/boosted subjects, 0.41% compared to 0.09% [71]. As an alternative means of avoiding pre-existing immunity, Ad-based vectors can be delivered by oral or nasal vaccination, which protects against a lethal challenge in the mouse and guinea pig models, but these studies have not been confirmed in NHPs [72].

### 8.4.1.2 Rhabdoviridae Vector Vaccines – rVSV

The Rhabdoviridae family of viruses has recently offered unique vaccine platforms to generate both genus/species-specific immunity as well as potential for cross-protective immunity among the family Filoviridae and even other members of the Rhabdoviridae family, such as Rabies virus. Of this family of viruses, Vesicular Stomatitis Virus (VSV) has shown promise for both genus of the family Filoviridae as protection against both EBOV and MARV has been demonstrated. Based on an attenuated recombinant VSV (rVSV), the replication competent virus expresses the glycoprotein of the target filovirus in place of its wild-type membrane glycoprotein, G. As this virus is primarily an agricultural pathogen, pre-existing immunity interfering with the desired immune response and subsequent protection is unlikely [73]. Additionally, the VSV platform did not cause any observable signs of disease when used to vaccinate immunocompromised NHPs suggesting that this technology may be safe among humans similarly immunocompromised [74]. Further encouragement for the safety of this vaccine came recently from Mire et al. who showed that EBOV and MARV rVSV showed no signs of neurovirulence associated with rVSV [75].

The utility of the VSV-based vaccine for protection against filoviral hemorrhagic fever was highlighted by Geisbert et al [76]. Using a blended vaccine consisting of three different VSV vectors each carrying either EBOV, SUDV or MARV glycoprotein they were able to generate 100% protection of NHPs against challenges with EBOV, SUDV, TAFV, and MARV with no observed ill effects from this replication competent vaccine. Of all vaccinated NHPs only one showed signs of viremia as assayed by qRT-PCR. Each of the vaccinated NHP also demonstrated elevated antibody responses following vaccination with titers ranging from 1:32 to 1:100 for all three glycoprotein components of the blended vaccine [76]. Recent evidence also suggests that emerging Ebolavirus species may be susceptible to cross-protection. NHPs immunized with EBOV glycoprotein rVSV demonstrated 75% protection after heterologous challenge with recently discovered Bundibugyo ebolavirus [38]. Further utility of this vaccine platform was shown by vaccination with either EBOV or MARV rVSV offering complete protection to NHPs from aerosol challenge with each respective virus [23].

In addition to providing such high levels of protection as a vaccine strategy the VSV-based technology has demonstrated post-exposure protection for both EBOV and MARV [77]. When administered i.m. 20 – 30 minutes post-challenge with MARV 100% of NHPs survived. In this study transient viremia was observed on day three post-challenge when assayed by PCR but was unobservable by traditional plaque assay. Clinical
chemistry results demonstrated that these surviving NHP experienced significant rises in aspartate aminotransferase, gamma-glutamyl transferase, total bilirubin and blood urea nitrogen indicating that while protective the post-exposure treatment did not completely prevent typical pathogenic events associated with MARV infection. Interestingly, there were low levels of neutralizing antibodies and no detectable T-cell activation typical of vaccination studies performed with this technology in NHPs [78]. Similar experiments have also been attempted for EBOV demonstrating SUDV glycoprotein incorporated into rVSV delivered 20 – 30 minutes after challenge offered 100% protection [79]. Post-exposure protection for EBOV glycoprotein incorporated into rVSV was less effective at 20 – 30 minutes but still afforded 50% protection to NHPs [80]. As a post-exposure treatment, EBOV glycoprotein rVSV was used recently 48 hours after a suspected human exposure via needle stick in the laboratory. While there is no direct evidence the laboratory worker was indeed exposed, that person survived the experience with no discernible sequelae from the treatment outside of fever occurring 12 hours after injection of $5 \times 10^7$ viral particles [81].

### 8.4.1.3 VEE Replicon Particles (VRPs)

Vaccination against multiple potential bio-threat agents is an ideal scenario when structuring a vaccine technology. Such is the case for the Venezuelan Equine Encephalitis virus (VEE)-based Replicon Particles (VRP) derived to express viral envelope proteins as potential vaccines [82]. For these VRPs the VEE structural proteins are replaced by the GP from EBOV or MARV creating an infectious VEE particle that is replication deficient but carries these trans genes [82], [83]. Virus particles are produced through helper cells containing the VEE structural proteins and subsequent infection of target cells results in high level expression of the trans genes [82], [83]. NHPs exhibited 100% survival after vaccination with $10^7$ focus forming units of VRP in three consecutive doses spaced at 28 day intervals prior to challenge with MARV [84]. This protection was offered when the VRPs were constructed to express GP alone or GP + NP; however, the NHPs vaccinated with NP alone all exhibited clinical symptoms of illness and only two out of three survived the challenge. Substantial antibody titers were found in each of the vaccinated NHPs. Additionally, no conspicuous elevations in clinical chemistries were observed in NHPs throughout the experiment. Experiments performed on mice and guinea pigs supported the ability of VRPs expressing GP, along with other MARV and EBOV genes, to mediate complete protection from lethal MARV challenge [84]. In mice, adoptive transfer of CD8$^+$ cells, but not CD4$^+$ cell or passive antibody transfer, from VRP-NP immunized mice was protective suggesting this vaccine may be most protective by stimulating the host cell mediated immunity [85]. Additionally, adoptive transfer of CD8$^+$ T-cells after specific activation via specific EBOV peptides provided mice complete protection indicating a mechanism for VRP-based immunity [86].

### 8.4.1.4 Paramyxovirus-Based Vaccines

Paramyxovirus-based vectors for vaccination against filoviral threats have recently demonstrated the capacity to protect NHPs from infection and stimulate strong immune responses. These viruses have a natural tropism for the respiratory tract and, as filoviruses are both emerging diseases and potential weaponized threats, the idea of targeting vaccines to this area is ideal. Two candidates for this category of vaccines have been investigated to date, Human Parainfluenza Virus 3 (HPIV-3) and Newcastle Disease Virus (NDV). Of these two systems, the HPIV-3 system has been evaluated in NHP models of EBOV infection. Combinations of EBOV GP alone EBOV GP + NP and EBOV GP + human Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) were inserted into the genome of HPIV-3 and each of these vaccine vectors was used to immunize NHPs both intranasally and intratracheally as initial studies offered complete protection of guinea pigs vaccinated via the respiratory route [87]. For all three vaccine groups, NHPs receiving $4 \times 10^6$ TCID$^{50}$ of their respective vector all
displayed clinical signs of illness during the study. Each group held two NHPs and out of the three groups only one vaccinated animal from the EBOV GP + NP succumbed to the disease. Immune responses from these subjects, prior to challenge, revealed antibody titers in the range of 1:400 to 1:1,600 [88]. By manipulating dose and administration strategies Bukreyev et al. were able to achieve complete protection of NHPs after two successive doses of $2 \times 10^7$ TCID$_{50}$ given at day zero and again at day 28 with challenge occurring on day 67 [88]. The two dose strategy produced IgG titers ranging between 1:1,600 and 1:25,600, much higher than in the single dose paradigm. Each of these experiments highlights the potential of the HPIV-3 platform for EBOV vaccination, but the known prevalence of pre-existing immunity to HPIV-3 in humans could hinder the generation of targeted immunity [89]. To address these concerns, Bukreyev et al. compared the immunogenicity of a EBOV GP expressing HPIV-3 vector among naïve and pre-immune NHPs [90]. In these experiments EBOV-specific IgG levels were substantially decreased among HPIV-3 pre-immune NHPs; however, this hindrance was overcome when the NHPs were vaccinated with two doses of recombinant vector which was previously shown to offer complete protection against EBOV challenge [90].

In efforts to diversify the paramyxovirus-based vectors and avoid issues surrounding the pre-existing immunity found for HPIV-3, a new vector design based on NDV was established. NDV is an avian paramyxovirus that infects the respiratory tract. This virus has been shown to be highly attenuated in NHPs due to natural host restriction processes [91]. Additionally, this vector system has proven successful as a vaccine platform for severe acute respiratory syndrome-associated coronavirus and influenza H5N1 in NHPs [92]. Although this system has yet to be evaluated in the context of NHP models of EBOV infection and disease, it was recently shown to be immunogenic in NHPs. Single immunization with NDV expressing EBOV GP produced lower titers than the HPIV-3 platform demonstrating this vector is less immunogenic; however, in a homologous prime/boost vaccination strategy EBOV-specific mucosal IgA levels reached those similar to the HPIV-3 homologous prime boost vaccination strategy [93]. IgG specific for EBOV did not reach levels comparable to the previous HPIV-3 platform. These reports support the potential of paramyxoviruses as possible vaccine candidates but further examination of immunostimulatory effects and pre-existing immunity will require investigation.

8.4.2 Therapeutics Effective in NHPs

8.4.2.1 Recombinant Human-Activated Protein C

It has been shown that Ebola Hemorrhagic Fever (EHF) and severe sepsis (or septic shock) share many clinical features, including fever, hypotension, increased production of tissue factor, elevated of nitric oxide, and elevated levels of D-dimers [94]-[96]. In addition, the most prominent and consistent finding in severe sepsis is severe protein C deficiency [97], [98]. It was shown that treatment of patients with severe sepsis with recombinant human-Activated Protein C (rhAPC) resulted in improved survival [99]. Later experiments in NHPs demonstrated that ZEBOV infection results in rapid reduction of circulating protein C levels [54], [100]. Therefore it was tested whether treatment with rhAPC could confer protection against lethal ZEBOV infection in rhesus macaques. Fourteen rhesus macaques were infected with a lethal dose of ZEBOV; eleven were then treated with intravenous rhAPC 30 – 60 minutes after challenge, continuing for seven days. All control animals died on Day 8 post-infection; however, two of the eleven rhAPC-treated animals survived (~ 20% survival). Additionally, the mean time to death for rhAPC-treated animals was 12.6 days, which is significant compared to historical controls [95]. This suggests that rhAPC could be used as an effective post-exposure therapeutic.

8.4.2.2 RNA Interference

RNA interference (RNAi) represents a powerful, naturally occurring biological strategy for inhibiting gene expression. RNAi interferes with the translation of mRNA to protein products by either sterically blocking
mRNA or by triggering RNase H-mediated cleavage of the DNA/RNA duplex, resulting in inhibition of gene expression [101]. RNAi has been used in cell-culture systems to inhibit the replication of a number of viruses that cause disease in humans, including HIV, Hepatitis B Virus (HBV), hepatitis C virus, influenza virus, herpesviruses, poliovirus, human papillomavirus, respiratory syncytial virus, coxsackievirus, lymphocytic choriomeningitis virus, and severe acute respiratory syndrome coronavirus [102]-[105]. Fowler et al. demonstrated that siRNA downregulation of various Marburg mRNA transcripts was able to significantly decrease viral protein production and subsequent viral release in cell culture [106]. However, clinical use of siRNA has been hampered by the lack of delivery of these molecules to target cell populations in vivo due to their instability, inefficient cell entry, and poor pharmacokinetic profile. To overcome this problem, various delivery vectors, including liposomes, polymers, and nanoparticles, have been developed [107].

8.4.2.3 Stable Nucleic Acid-Lipid Particles

Geisbert et al. utilized lipid encapsulation as a means of improving the pharmacology of siRNA targeting the Ebola RNA polymerase L protein [45], [108]. These Stable Nucleic Acid-Lipid Particles (SNALP) consist of a lipid bilayer containing a mixture of cationic and fusogenic lipids that enables the cellular uptake and endosomal release of the particle’s nucleic acid payload. SNALPs are also coated with a diffusible Polyethylene Glycol-lipid (PEG-lipid) conjugate that provides a neutral, hydrophilic exterior and stabilizes the particle during formulation, and this encapsulation was initially demonstrated to significantly increase the stability, half-life, and effectiveness of siRNA directed against HBV [109]. This SNALP-encapsulation of siRNA targeting the Ebola L protein was initially shown to completely protect guinea pigs when administered shortly after a lethal EBOV challenge [108]. This treatment was then assessed for efficacy in rhesus macaques. SNALP-encapsulated siRNAs targeting EBOV L polymerase, VP24, and VP35 were given to rhesus monkeys either four or seven times post-exposure to a lethal challenge of EBOV. Two of the three monkeys given four doses survived lethal infection, while all four monkeys given seven doses survived infection [45]. The treatment regimen in this study was well tolerated with minor changes in liver enzymes that might have been related to viral infection. This shows the potential of RNA interference as an effective post-exposure treatment strategy for people infected with Ebola virus [45].

8.4.2.4 Immunotherapy

Passive transfer of either polyclonal or monoclonal antibodies remains an attractive solution to preventing and treating filovirus infections. The history of immunotherapy for other infections, such as respiratory syncytial virus, offers a direct scientific and regulatory pathway to human-use licensure [110]. Passive transfer of polyclonal antibody via hyperimmune serum or convalescent serum has been reported in human filovirus infections [111]-[114]. However, the overall success of these therapies has been controversial and difficult to ascertain due to the study conditions (lack of adequate experimental controls, appropriate medical equipment, etc.), and the outbreak was already well-contained [115]. These results have also been tempered by conflicting results from studies in laboratory animal models as discussed above.
8.5 VACCINES AND THERAPEUTICS EFFECTIVE IN SMALL ANIMAL MODELS AND IN VITRO

8.5.1 Vaccines Effective in Small Animal Models

8.5.1.1 Rhabdoviridae Vectored – Rabies Virus (RABV) Vaccines

Also of note for Rhabdoviridae family of filoviral vaccines was the more recent characterization of inactivated and live rabies vaccines expressing Ebola GP, which generated dual immunity for both EBOV and Rabies virus infection [116]-[118]. EBOV GP was efficiently expressed from an attenuated vaccine used for wildlife against Rabies virus in the place of the wild-type rabies envelope glycoprotein, G [116]. This vaccine vector was capable of inducing protective immunity to both EBOV infection as well as Rabies virus infection in both live-attenuated format and β-propiolactone inactivated virus. Neurovirulence of the recombinant vector was unobserved in suckling mice when compared to the unaltered vaccine [116]. Both live and killed vaccines induced primary EBOV GP-specific T-cells and a robust humoral and recall response [118]. In addition, humoral immunity to GP could be induced by immunization of mice with inactivated RV-GP in the presence of pre-existing immunity to RABV. This versatility offers increased storage options with an inactivated vaccine as well as the opportunity to vaccinate for both diseases where they are both endemic.

8.5.1.2 Ebola Immune Complex (EIC) Produced in Nicotiana

The production of recombinant proteins for use as sub-unit vaccines relies on fermentation using bacterial suspensions or cultures of insect, yeast, or mammalian cells. Recently, there has been substantial progress in the use of green plants as a platform for the production of biologics. Advantages include rapid time frames for the production of gram quantities of protein, the ability of plants to produce large and complex proteins with correct processing and assembly into multi-sub-unit complexes [119], and recent advances in plant biotechnology allowing the production of glycoproteins with human-like glycosylation patterns [120]. DNA viruses such as the geminiviruses can be used as plant viral vectors to express recombinant protein. For recombinant protein expression, the geminiviral coat protein and movement protein can be replaced with the gene of interest. After the expression of the Rep protein, the replica containing the gene of interest is amplified, and the double stranded DNA intermediates act as transcription templates, leading to high amounts of mRNA and subsequent recombinant protein [121].

Ebola GP consists of two sub-units, the extracellular GP1 and membrane anchored GP2, linked by disulfide bonds. GP1 is responsible for receptor binding, and GP2 mediates membrane fusion. Transient expression of GP in cultured cells causes cytopathic effects. Ebola GP or segments thereof have been expressed in recombinant systems such as E. coli, insect cells, and mammalian cells, and in the latter, GP1 expression was regulated by an ecdysone-inducible system to reduce its cytotoxicity. Recombinant immune complexes were originally expressed in tobacco plants via fusion of Tetanus Toxin Fragment C (TTFC) to the heavy chain of TTFC-binding IgG and co-expression with its light chain [122]. Mice immunized with the recombinant TTFC immune complexes showed much higher antibody titers than those immunized with TTFC alone, thus demonstrating the recombinant immune complex as a strong vaccine candidate. The Geminiviral replicon system derived from bean yellow dwarf virus was used to produce Ebola Immune Complexes (EIC) in N. benthamiana [123], [124]. Ebola GP1 was fused at the C-terminus of the Heavy chain of humanized 6D8 mAb [125], which specifically binds to the 6D8 linear epitope on GP1. When the fusion protein was co-expressed with the light chain of 6D8 mAb, IgG molecules assembled, leading to immune complex formation. EIC were expressed, purified, and used to immunize mice. Mice were immunized s.c. on days 0, 21, 42, and 63 with purified EIC. The VRP-GP vaccine
was used as a positive control. The plant made EIC was immunogenic in mice to the same extent as VRP-GP vaccine [123].

In a subsequent study, vaccination with EICs in combination with PIC (a TLR3 agonist) resulted in a robust Ab response [124]. PIC shifted the TH2 dominant response observed with EIC alone toward a more TH1 phenotype. EIC co-delivered with PIC protected 80% of mice from a lethal amount of live Ebola Zaire. Protection from challenge corresponded not only to the amplitude of the antibody response, but also with the IgG sub-type profile (associated with a Th1 response), and in particular with the amount of IgG2a. Whereas IgG1 was produced in all groups that received the h-EIC antigen, IgG2a was only produced in the mice vaccinated with h-EIC co-delivered with PIC, and this corresponded to greater protection [124].

8.5.1.3 ZEBOVGP-Fc Fusion Protein

The majority of filovirus vaccine strategies exploit viral vectored platforms rather than recombinant sub-unit vaccines. Initial studies using baculovirus-expressed filovirus GP only induced partial protection in mice, which could be attributed to the glycosylation and process of GP in insect cells. In an alternative strategy, a GP-Fc fusion protein was generated and tested as a potential vaccine candidate [126]. The investigators hypothesized that the Fc tag in the vaccine would simplify purification of the fusion protein through protein A columns using mild conditions, increase protein stability, and confer an adjuvant effect in NHPs and humans due to the interactions with Fcγ receptors on APCs. The extracellular domain of the Zaire Ebola (ZEBOV) GP fused to the Fc fragment of human IgG1 (ZEBOVGP-Fc) in mammalian cells showed that GP undergoes the complex furin cleavage and processing observed in the native membrane-bound GP [126]. Mice were vaccinated i.p. with 100 µg of ZEBOVGP-Fc in complete Freund's adjuvant followed by three boosts with 25 µg protein in incomplete Freund's adjuvant. Mice immunized with the GP-Fc fusion protein developed T-cell immunity and neutralizing antibodies, and were protected against challenge with a lethal dose of ZEBOV [126]. Whether or not a sub-unit vaccine based on filovirus GP-Fc fusion proteins is truly feasible will require further studies utilizing an adjuvant licensed for human use, i.m. delivery, and NHP models of infection.

8.5.2 Therapeutics Effective in Mouse Models

8.5.2.1 PMOs Targeting VPS4

There is increasing evidence that many different viruses, including the filoviruses and retroviruses, use host proteins normally involved in the vacuolar protein sorting (VPS) pathway for the final steps of budding [89], [127]. This requires movement of the vps proteins from multi-vesicular bodies to the plasma membrane, an event mediated or performed by viral proteins. In the final step of the vps pathway, the ATPase activity of VPS4 generates the energy needed for dissociation of the protein complex, allowing for subsequent rounds of sorting [128]. A dominant-negative VPS4 mutant, lacking ATPase activity, can inhibit release of both HIV and EBOV [129], [130]. Recruitment of VPS4, VPS28, and VPS37B to the plasma membrane was not dependent on VP40-TSG101 interaction. A lack of VPS4 ATPase activity did not affect its recruitment to the plasma membrane, but it did inhibit VLP release. Administration of PMOs to knock down VPS4 gene expression protected mice from lethal EBOV infection [128].

8.5.2.2 Mannose-Binding Lectin

Circulating Mannose-Binding Lectin (MBL) is a first-line host defence against a wide range of viral and other pathogens. MBL is a C-type lectin that recognizes hexose sugars including mannose, glucose, fucose, and N-acetylglucosamine on the surface of many pathogens. It does not recognize the terminal carbohydrates
galactose and sialic acid on normal host cells [131]. As a result of common genetic variants, MBL serum levels in humans range from 0 to 10,000 ng/mL. Thirty percent of the human population has levels < 500 ng/mL, which are associated with increased susceptibility to infections in children and immunocompromised individuals [132]. MBL-knockout mice are highly susceptible to several pathogens, and Recombinant human MBL (RhMBL) improved survival in MBL-null mice to approximate survival among infected wild-type mice at doses that reconstituted the complement-activating capacity of MBL-knockout serum to a level comparable to that of wild-type mouse serum [133]. Filovirus surface Glycoproteins (GPs) are heavily glycosylated and contain high-mannose. As a result, MBL binds to Ebola and Marburg viruses and mediates complement-dependent virus neutralization [134]. Recent experiments showed that supraphysiological doses of MBL rescued ~ 40% of mice from lethal challenges when administered pre- or post-Ebola virus exposure [131].

8.5.2.3 Ebola-Specific Monoclonal Antibodies

Monoclonal Antibodies (mAbs) that can be used as anti-virals have been identified as a priority by the U.S. Department of Health and Human Services for protection from EBOV used in acts of bioterrorism or war. These ebola-specific antibodies have been generated in a number of ways. In one study, an antibody (13F6) was derived against the EBOV GP mucin-like domain and was shown to provide 100% prophylactic and therapeutic protection in a mouse challenge model [135]. This antibody was then modified, deimmunized, and chimerized with human constant regions to generate h-13F6, a human recombinant antibody. This human recombinant antibody also significantly protected mice against a lethal challenge of EBOV [136]. In another method, a recombinant VSVΔG/ZEBOVGp was used to generate a total of eight mAb’s which were subsequently characterized. All 8 mAb’s improved survival rate of mice (33% – 100%) against a high dose lethal challenge by mouse adapted EBOV [137]. Another antibody, KZ52, was isolated from the bone marrow of a human survivor of EBOV infection and is specific for the complex of GP1 and GP2 [138]. KZ52 neutralized EBOV in vitro and offered protection from lethal EBOV challenge in a rodent model, but was non-protective in NHPs [40].

8.5.2.4 Small Molecule Inhibitors

High-Throughput Screening (HTS) is a significant tool for novel drug discovery. HTS involves screening libraries consisting of thousands to hundreds of thousands of unique molecules against specific targets. Available libraries used in HTS have included natural compounds [139], [140], peptides [141], drugs [142], and synthetic compounds [140], [143]. In one screen, multiple compound libraries were obtained from the National Cancer Institute (NCI, Frederick, MD). Compound FGI-103 was initially identified during a screen with an EBOV-GFP pseudotyped virus. Subsequently, FGI-103 was shown to exhibit strong anti-viral activity in vitro against high doses of EBOV and MARV. Finally, FGI-103 protected mice against lethal challenges of both EBOV and MARV [144].

A second screen was done using a collection of 1990 small molecule compounds obtained from the NCI. This screen, utilizing EBOV-GFP resulted in the identification of NSC 62914 [145]. Subsequently, this compound showed high anti-viral activity against EBOV, MARV, Lassa virus, Rift Valley Fever Virus (RVFV), and Venezuelan Equine Encephalitis virus. Compound NSC 62914 was then tested for in vivo efficacy. This compound protected mice from a lethal challenge of EBOV and MARV when given either pre- or post-infection [146].

A third screen was done utilizing a library of small molecule compounds developed at USAMRIID (Frederick, MD). Compound FGI-106 was initially identified during a screen with an EBOV-GFP pseudotyped virus. FGI-106 was shown to then exhibit strong anti-viral activity in vitro against EBOV, RVFV, Dengue Fever Virus (DENV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV). FGI-106 was then shown to
protect mice against a lethal challenge of EBOV when given post-infection [147]. Taken together, this suggests that FGI-106 probably acts on a conserved pathway common to these four viruses, and potentially makes for a very intriguing anti-viral.

8.5.3 Therapeutics Effective In Vitro

8.5.3.1 Niemann-Pick C1

Niemann-Pick C1 (NPC1) was identified as being required for EBOV replication during a gene trap screen in HAP1 cells using a Replication-competent Vesicular Stomatitis Virus bearing the Ebola virus Glycoprotein (rVSV-GP-EboV). The Niemann–Pick disease locus NPC1 encodes an endo/lysosomal cholesterol transporter, which affects endosome/lysosome fusion and fission, calcium homeostasis, and HIV-1 release [148]-[151]. Loss of NPC1 causes Niemann–Pick disease, a neurovisceral disorder characterized by cholesterol and sphingolipid accumulation in lysosomes [148]. NPC1-mutant cells were infected poorly or not at all by rVSV-GP-EBOV and VSV pseudotyped with filovirus GP proteins and infection was restored by expression of wild-type NPC1 [20]. Certain small molecules such as U18666A cause a cellular phenotype similar to NPC1 deficiency possibly by targeting NPC1 [152]. NPC1-mutant cells and Vero cells treated with U18666A showed marked virus yield compared to controls. Also, Heterozygous Npc1 (Npc1+/−) knockout mice were largely protected against a lethal challenge of mouse adapted EBOV and MARV [20]. In agreement, small molecule compounds directed against NPC1 were shown to block infection of EBOV in vitro [21].

8.5.3.2 HSP-90 Inhibitors

Heat-shock protein 90 (Hsp90) is a molecular chaperone that guides the folding, intracellular disposition, and proteolytic turnover of many key regulators of cell growth and differentiation [153]. Hsp90 has a specific set of client proteins in vivo such as steroid receptors, transcription factors, protein kinases, and oncogenes [154]. Inhibitors of Hsp90 have proven effective at driving cancer cells into apoptosis by preventing the proper folding of oncogenes required for promoting cancer cell growth. Because of this, several Hsp90 inhibitors are now in Phase I and II clinical trials [155], [156]. Hsp90 was shown to be an important host factor for the replication of negative-strand viruses [157]. The effects of several Hsp90 inhibitors on EBOV replication were tested in vitro. Some were natural product inhibitors while others were synthetic inhibitors. Results of this study showed that Hsp90 inhibitors significantly inhibited the replication of EBOV in Vero cells and primary human monocytes, suggesting their use as a potential therapeutic [153].

8.5.3.3 Δ-peptide Immunoadhesins

In addition to GP1,2, Ebola viruses express two other secreted glycoproteins, soluble GP (sGP) and small soluble GP (ssGP) [158]. All three glycoproteins have identical N termini that include the Receptor-Binding Region (RBR) but differ in their C termini. Δ-Peptide is a highly post-translationally modified peptide (predicted mass of ∼ 4.7 kDa; actual mass of ∼ 10 to 14 kDa) that is rapidly and efficiently cleaved from the sGP precursor expressed from plasmids in vitro. However, in contrast to mature sGP, Δ-peptide is retained in producer cells for extended periods of time before being released into the cell culture supernatant, and the overall amount of secreted peptide is less than that of sGP [159]. Recombinant Fc-tagged Δ-peptides of three ebolaviruses (EBOV, SUDV, and TAFV) specifically inhibited cell entry of retroviruses pseudotyped with MARV-GP. Subsequently, these Δ-peptides significantly inhibited both EBOV and MARV, in a dose-dependent manner and at low molarity [158]. These data indicate that Δ-peptides are functional components of ebolavirus proteomes, and might play an important role in pathogenesis, and could be exploited for the synthesis of powerful new anti-virals [158].
8.5.3.4 C-peptides

Filovirus fusion is thought to involve conformational rearrangements of the transmembrane sub-unit (GP2) of the envelope spike that ultimately result in formation of a six-helix bundle by the N- and C-terminal Heptad Repeat (NHR and CHR, respectively) regions of GP2 [160]. Infection by other viruses employing similar viral entry mechanisms (such as HIV-1 and severe acute respiratory syndrome coronavirus) can be inhibited with synthetic peptides corresponding to the native CHR sequence (“C-peptides”) [161], [162]. C-peptides are synthetic peptides corresponding to the CHR sequence that inhibit membrane fusion and inhibit viral infection by competing with the endogenous CHR for binding to the NHR core [160], [163], [164]. Because membrane fusion by filoviruses is thought to occur deep in the endocytic pathway [165]-[167], previous attempts to develop C-peptides to inhibit EBOV yielded only modest activity [168], [169]. The weak activity of EBOV C-peptides may be due to inaccessibility to the endocytic pathway, so the C-peptides were then conjugated to the arginine-rich segment from HIV-1 Tat protein, which is known to target to endosomes [170], [171]. This conjugated C-peptide exhibited marked anti-viral effects, up to 99% inhibition of EBOV and MARV in vitro [160]. Concentrations required for inhibition are currently too high to be used clinically, but this is the first evidence that intracellular targeting of a C-peptide can improve activity against a virus that enters via endosomes.

8.5.3.5 Alkylated Porphyrins

A high-throughput screen of approximately 2,200 compounds was tested for the ability to reduce the amount of enveloped viral DNA in the culture medium of HBV-replicating cells. This led to the discovery of chlorophyllide, which non-cytotoxically reduced the level of secreted HBV virion DNA by more than 16-fold at micromolar concentrations [172]. Chlorophyllide is an alkylated porphyrin that contains copper and is charged at neutral pH and thus appears green in solution. During these screens, the chlorin e6 compound, a metal-free chlorophyllide-like molecule, was found the most potent and was subsequently tested against other viruses, including MARV. During testing, the chlorine e6 compound showed significant anti-viral activity in vitro against MARV. This compound also inhibited Junin virus, DENV, HCV, and HIV [172].

8.5.3.6 Benzodiazepine Small Molecule Compounds

A chemically diverse small-molecule library (52,500 compounds) was screened for ≥90% inhibition of HIV/EBOV-GP infection to identify viral entry inhibitors [173]. To assess the target specificity of the HTS hits, these hits were counter-screened against a second pseudotype virus (HIV/VSV-G) having the same HIV backbone but expressing unrelated Vesicular Stomatitis Virus envelope Glycoprotein (VSV-G). Based on this counter-screen, only 57 compounds were demonstrated to block EBOV-GP-mediated infection specifically with little or no effect on infectivity of HIV/VSV-G. Compound 7 was selected as a suitable starting point for further viral specificity studies, Mode-Of-Action (MOA) evaluation, and medicinal chemistry optimization. Compound 7 displays filovirus-inhibitory activity, inhibiting both infectious EBOV and MARV with similar potencies in vitro. It is thought that compound 7 acts at an early stage of viral entry, apparently by binding to a hydrophobic pocket (S2) in the pre-fusion conformation of EBOV-GP and preventing infection by an unknown mechanism [173].

8.6 POTENTIAL FUTURE THERAPEUTIC TARGETS

8.6.1 Targeting Pathogenesis

Reversing or targeting the pathogenesis of disease represents a potential fruitful source of therapeutics. Both activation of the coagulation systems and a profound inflammatory response are critical components of the
filovirus induced disease. As such, targeting of coagulation abnormalities is an obvious first approach [115]. In fact, the first therapeutic identified for filoviruses was a drug that targeted the development of coagulation abnormalities [54]. Depending on the stimuli, either the intrinsic or extrinsic arm of the coagulation cascade may be activated, and this activation can be triggered by a variety of factors. Uncontrolled activation may lead to Disseminated Intravascular Coagulation (DIC) [115]. DIC is neither a disease nor a symptom, but rather is a syndrome with both bleeding and thrombotic abnormalities characterized in part by the presence of histologically visible microthrombi in the microvasculature [174]-[176]. These microthrombi may hamper tissue perfusion and thereby contribute to multiple organ dysfunction and high mortality rates [115].

During activation of the clotting system, the host regulates the process through the production and activation of a variety of inhibitors. In this process, however, the inhibitors are consumed, and if the rate of consumption exceeds the rate synthesized by the liver, plasma levels of inhibitors will decline [115]. A number of studies have found positive correlations between plasma levels of inhibitors and the degree of DIC during sepsis [175], [177]. Human protein C is a serine protease that is secreted as a zymogen. Cleavage of the pro-enzyme yields an active enzyme (activated protein C; APC) that reduces the production of thrombin by catalytically cleaving factors Va and VIII. Thus, APC acts as an anti-inflammatory, anti-coagulant, and fibrinolytic agent [115]. As discussed earlier, rhAPC is a potential therapeutic that was shown to protect 20% of animals and significantly increased mean time to death in NHPs [95].

Cytokines are key mediators of inflammation and vascular dysfunction. They can induce changes in endothelial cell structure that affect permeability, and they can also play a role in regulating the inflammatory response [178]. Tumor Necrosis Factor (TNF)-α has been shown in a number of studies to induce endothelial cell-surface changes [179], [180]. Furthermore, several studies have shown that anti-TNF-α treatment of diseases, such as rheumatoid arthritis, improves endothelial function and endothelium-dependent vasomotor responses [180], [181]. Other cytokines of chemokines may also be involved in modulating endothelial cell function during filovirus infections either directly or indirectly [115]. For example, Interferon (IFN)-α, IFN-γ, IL-6, IL-8, and MCP-1 are upregulated during EBOV infection of humans and/or NHPs and may have indirect effects on endothelial function or permeability [182]-[187].

8.6.2 Targeting of the Host Immune Response

Another primary theme for treatment of filovirus infections has been modulation of the host immune response. This area has mainly involved efforts to boost innate immunity, but more recently has turned to evaluating reversing the subversion of the host immune response [115].

8.6.2.1 Interferons

Treatment with exogenous Type 1 IFNs has been evaluated by several groups. A combination of ridostin (an IFN inducer) and reaferon (IFN-α2a) prolonged the mean time to death of EBOV-infected guinea pigs [188]. However, studies in cynomolgus macaques treated with high doses of recombinant human IFN-α2a immediately after exposure failed to produce any significant reductions in morbidity and mortality [189]. Despite the failure, there exist a number of other potential IFN products and combinations that have yet to be evaluated.

8.6.2.2 Innate Immune Response Interference

As discussed above, subversion of the innate immune system is among the many factors contributing to the pathology of filovirus infection. Data from both in vitro and in vivo experiments show that EBOV initially infects Dendritic Cells (DC) and Macrophages (Mφ) [183], [190]. Infection of Mφ are thought to act as key
triggers for the uncontrolled and rapid secretion of pro-inflammatory mediators. However, despite the systemic release of inflammatory mediators after infection with filoviruses, fatal or severe disease is often linked to a generalized suppression of adaptive immunity. Filovirus infection of DC hinders activation of these cells and limits the ability to initiate an adaptive immune response [29]. Transcriptional profiling of filovirus infected human DC will facilitate identification of genes differentially expressed upon infection and lead to new potential targets [115].

8.6.2.3 Inhibition of Apoptosis

It is likely that the marked apoptosis of Natural Killer (NK) and T-cells seen early in filovirus infections contributes to the observed immunosuppression. In addition, it is likely that the microparticles, which are formed as a natural part of the programmed death cycle, exacerbate the coagulation abnormalities [115]. Studies have shown that shed microparticles from T lymphocytes impair endothelial function and regulated endothelial protein expression [191]. As such, targeting apoptosis may have multiple beneficial impacts.

8.6.3 Targeting Virus-Host Interactions

While the use of therapeutics targeted at the virus itself may prove to be a very effective way to clear or prevent virus infection, an inherent flaw in this method does exist for several viruses. As the HIV literature recounts, there are numerous examples of viral escape mutants which have evolved resistance against not only HIV-specific antibodies, but also anti-retroviral drugs targeted at various aspects of the viral replication cycle [128], [192]. As such, an alternate therapeutic approach for filoviruses could target important molecules or pathways within the host cell itself that the virus appears to require for efficient replication [115].

8.6.3.1 Assembly and Budding

The cellular components required for filovirus assembly and release are becoming well-characterized. One such component is Tsg101, an ubiquitin-conjugating E2 enzyme variant, which is part of the Endosomal Sorting Complexes Required for Transport (ESCRTs) within the vacuolar protein sorting (vps) pathway. The ESCRT complexes are used to sort various cellular proteins into internal vesicles that bud into the lumen of the endosome [115]. The filovirus VP40 contains two overlapping late domain motifs and actively recruits Tsg101 and other ESCRT components to the site of viral budding [193]. While mutation of these motifs reveals that they are not essential for viral budding, they are an important component of the budding machinery [194]. This study also demonstrated that only a small conserved peptide motif is required for viral interactions with Tsg101. Small molecule inhibitors that mimic these sequences could prove to be very effective therapeutic agents [115].

It has also been recently demonstrated that filovirus budding can occur independently of interactions with Tsg101. The ability of a mutated VP40 to redirect proteins of the vps pathway from endosomes to sites of particle budding has been characterized [195]. While these mutant VP40 proteins could no longer recruit Tsg101 to the plasma membrane, several vps proteins (VPS4, VPS28, VPS37B) could still be redirected to the plasma membrane. A mutant VPS4 that lacks ATPase activity can still traffic to the plasma membrane, but now inhibits filovirus budding. As discussed earlier, PMOs targeting VPS4 protected mice from a lethal EBOV infection [128].

8.6.3.2 Ubiquitination

It is clear that ubiquitinated proteins are important for cellular endocytosis and exocytosis processes (reviewed in [196] and [197]). During normal cellular activities, monoubiquitination is a signal for delivery and internalization into vesicles of the multi-vesicular body complex. The proteins are then available for sorting by
the vps pathway for ultimate delivery to the lysosome for degradation. The PPxY late domain motif of VP40 has been shown to interact with WW motifs of ubiquitin ligase enzymes, such as Nedd4, which play a role in the cellular ubiquitin enzyme cascade as E3 ubiquitin ligases. It has been shown that mutations in the active site of Nedd4 not only abolish ligase activity but also reduces the ability of Nedd4 to enhance filovirus budding [198]. Importantly, it was shown that the IFN-inducible ubiquitin-like protein ISG15 can inhibit filovirus budding via interactions with Nedd4 to inhibit ubiquitination of VP40 [198]. Although it remains unclear whether VP40 is ubiquitinated in infected cells, it has been shown that for several other viruses, ubiquitination is important for assembly and budding [199]. Nedd4 likely provides VP40 with the ubiquitination signal for delivery to the ESCRT complex via the vps pathway. Here, VP40 has the opportunity to interact with Tsg101 and recruit the cellular protein to the plasma membrane for viral assembly [115]. Thus, Nedd4 could be a potential drug target candidate to combat filovirus infection.

8.6.3.3 Protein Transport

During filovirus replication, viral components must be shuttled to and from several locations within the infected cell. While it remains unclear for filoviruses, it is well known that many viruses utilize host cytoskeleton scaffolding during replication [200]. Both EBOV and MARV appear to interact with microtubules and actin filaments, respectively. In fact, EBOV has been shown to directly interact with microtubules via VP40 [201]. Furthermore, EBOV VLP release is dependent on interactions with microtubules [202]. However, release of MARV VLPs is dependent on interactions with actin [201]. As EBOV VLP budding also appears to require interactions with actin, the mechanism by which each virus interacts with its respective host cell cytoskeleton component remains unclear [115].

Another set of proteins involved in cellular transport is the Rab family of proteins. These small GTP binding proteins regulate vesicular transport by tethering donor vesicles to their respective target membranes [115]. Specifically, Rab9 is involved with transport between late endosomes and the trans-Golgi network. The use of Rab9 siRNA resulted in decreased filovirus replication as demonstrated by immunofluorescence and ELISA [203]. However, whether there is direct interaction between any viral proteins and Rab9 remains to be elucidated. However, several therapeutic targets exist during filovirus assembly alone. Further examination of the viral replication cycle will likely reveal many more promising targets to combat filovirus infection [115].

8.6.3.4 Entry Inhibitors

The viral entry process offers several potential targets for intervention. The Glycoproteins (GP) of enveloped viruses facilitate entry into the host cell by interacting with specific cellular receptors. Despite extensive study, a cellular receptor for the filoviruses had yet to be identified and characterized. However, recently it was shown that T-cell Immunoglobulin and Mucin domain 1 (TIM-1) interacts with EBOV GP and enhances EBOV infection by 10- to 30-fold upon expression [19]. As macrophages and dendritic cells are major targets of filoviruses, but do not express TIM-1 [204], additional functional receptors for filoviruses remain to be identified [19]. Identification of cellular receptors could lead to new therapeutic targets for filovirus infection.

Another particular area of interest is the development of fusion inhibitors. In large part, this was due to the approval of the novel fusion inhibitor T-20, or Fuzeon, for use in retroviral therapy. Fuzeon blocks the structural rearrangements necessary for successful fusion of the virus with the cell [205]. Recently there have been tremendous advances in our understanding of how filoviruses enter cells. Specifically, a model for EBOV GP2-mediated membrane fusion with pseudotyped viruses has been developed [169]. Using this model, it has been demonstrated that an oligopeptide corresponding to the coiled-coil structure of GP2 competitively inhibited EBOV entry. These breakthroughs will facilitate the identification of novel small molecules that target filovirus entry.
Finally, it has been well-demonstrated that the cellular endosomal cysteine proteases Cathepsin B (CatB) and Cathepsin L (CatL) may play an important role in preparing the viral GP for interactions with the target host cells by generating a truncated form of GP, which allows for more efficient attachment to host cells [206]-[208]. Moreover, complimentary studies have demonstrated that inhibition of these cathepsins by drug treatment or siRNA knockdown resulted in significant decrease in viral infection [209]. This suggests that CatB and CatL may be prime targets for inhibiting viral entry through use of small molecule inhibitors [115].

8.7 ACKNOWLEDGEMENTS

BMF is a National Research Council (NRC) post-doctoral fellow and is funded by JSTO-NRC CBD postdoctoral funds from DTRA. GGO is funded by the DTRA grant CBM.THRV.01.RD.011.

8.8 CONFLICT OF INTEREST

The authors declare no conflict of interest.

8.9 REFERENCES


Bradfute SB, Dye JM, Jr and Bavari S. Filovirus vaccines. *Hum Vaccin* 2011, 7 (6), 701-711.

Richardson JS, Dekker JD, Croyle MA and Kobinger GP. Recent advances in Ebolavirus vaccine development. *Hum Vaccin* 2010, 6 (6), 439-449.


**Disclaimer:** Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army, the Defense Threat Reduction Agency, or the Department of Defense.
Chapter 9 – ALPHAVIRUSES: REVIEW OF MEDICAL COUNTERMEASURES

Les P. Nagata and Josh Q. Wu  
Defence Research and Development Canada – Suffield, Alberta  
CANADA  
Corresponding Author (Nagata): lnagata@ualberta.ca

9.1 INTRODUCTION

The subject of this article is a review of Medical Countermeasures (MCMs) to the encephalitic alphaviruses which include Eastern Equine Encephalitis Virus (EEEV), Western Equine Encephalitis Virus (WEEV) and Venezuelan Equine Encephalitis Virus (VEEV). Chikungunya, although an important emerging disease, will not be discussed. The encephalitic alphaviruses are endemic within regions in North and South America. Humans are normally infected after being bitten by infectious mosquitoes; however, these viruses are also highly infectious via aerosol exposure and cause encephalitis in equines and humans with severe morbidity and mortality rates. Due to their high titres attained in cell culture and their ease of transmission via aerosol, these three alphaviruses are potential weaponized BW agents. As further support to this assertion, VEEV was produced in multi-ton quantities and stockpiled in the former USSR and the USA as an incapacitating agent [65].

Alphaviruses comprise a group of about 28 enveloped viruses with a positive sense, non-segmented single-stranded RNA genome [7], [68], [74]. They are enveloped virions with icosahedral symmetry, are 60 – 70 nm in diameter and containing a genome of around 11 – 12 kb. All alphaviruses share a number of structural, sequence, and functional similarities, including a genome with two polyprotein gene clusters [68], [36]. The Non-Structural Proteins (NSPs) are translated directly from the 5’ two-thirds of the genomic RNA as a polyprotein which is processed into the four nsPs by a protease encoded by the nsP2 gene. Two versions of the non-structural polyprotein are synthesized in alphavirus-infected cells, due to frequent read through of an opal termination codon between the nsP3 and nsP4 genes in several alphaviruses [69]. The nsPs function in a complex with host factors to replicate the genome and transcribe the subgenomic mRNA. A subgenomic positive-stranded RNA (the 26S RNA), transcribed from a negative strand RNA, is identical to the 3’ one-third of the genomic RNA and serves as the messenger RNA for the structural proteins (capsid, E3, E2, 6K and E1). New viral particles are formed when the capsid complexes with the genomic RNA and is enveloped by a lipid bilayer containing E1 and E2 glycoprotein hetero-dimers.

9.2 THE ENCEPHALITIC ALPHAVIRUSES

EEEV was first recognized as disease of horses in 1831, with epizootics recorded between 1845 and 1912 [27]. EEEEV Group 1 (North American strains) is more virulent than the other alphaviruses and EEEEV Groups, with a case mortality rate around 50%. Groups IIA, IIB and III are endemic in Central and South America and result in mild or sub-clinical disease. The North American EEEEV strains are highly conserved. They are endemic in the Eastern United States and Canada. EEEEV is transmitted by the mosquito Culiseta melanura to birds, serving as its reservoir. Both equines and humans can develop severe encephalitis after being bitten by infectious mosquitoes Aedes, Coquillettidia and Culex [79]. Young children are more susceptible than adults to developing encephalitis, and have higher case mortality rates [17], [7].
WEEV is a member of the WEE antigenic complex and is serologically related to Sindbis (SIN), Highlands J, Fort Morgan, Buggy Creek, and Aura viruses [8]. Of these viruses, only WEEV is associated with human disease. WEEV was later determined to be a natural recombinant virus, formed from a SIN-like virus and EEEV [25]. WEEV is endemic in western North America and strains/varieties have been isolated from South America [71], [22]. In nature, WEEV is transmitted from its amplifying hosts or reservoir in wild birds to human and horses, by mosquitoes with *Culex tarsalis* being the principal vector. While the endemic cycle has resulted in only a limited number of human infections in recent years, major epidemics of WEEV have been recorded in the past. The most extensive epidemic, including 3,336 recognized human cases and 300,000 cases of encephalitis in horses and mules, occurred in the western United States and Canada in 1941 [60]. The case fatality rate for WEEV has been estimated to be 10% for humans and 20% for equines, and similar to EEEV, severe disease is more likely in young children.

VEEV is found in Central and South America, and in the Southern United States. It forms an antigenic complex composed of 6 sub-types, some of which have been further subdivided into serological groups [72]. Enzootic VEEV is primarily transmitted by *Culex (Melanoconion)* spp mosquitoes, while epizootic VEEV (IAB, IC and IE sub-types) are transmitted by a number of different mosquitoes. Epizootic strains are associated with significant disease in horses which are important amplifying hosts. Both enzootic and epizootic strains cause human disease. Enzootic strains normally cause mild febrile illness, while epizootic strains can cause more severe illness with morbidity rates varying from 13 to 93% of the population in a community. A flu-like illness is observed in adults and older children, with an incidence of encephalitis less than 5% in younger children and a case mortality rate of less than 1% [61]. VEEV is highly infectious and a frequent cause of laboratory acquired infections [9], [36], with as little as a few viral particles able to cause an infection.

9.3 CLINICAL DISEASE AND PATHOLOGY

In humans, EEEV and WEEV are neurotropic viruses with limited viremia, followed by CNS infection across the cerebral vascular endothelium or the olfactory epithelium. On the other hand, VEEV causes a systemic illness with pathogenesis in the lungs and lymphoid tissue [22]. For EEE, the prodrome disease course consists of 1 – 2 weeks of sudden onset of fever, chills and malaise. This can be followed by recovery or progress to severe headache, vomiting, restlessness, respiratory symptoms, seizures and coma. Death can occur in 2 – 10 days following onset of encephalitis. Recovery is more likely in patients who have a long prodrome (5 – 7 days) and do not develop coma, however, 35 – 80% of survivors have significant long-term neural impairment including paralysis, seizures and mental retardation [16], [11]. Viral infection is localized to the neurons, with widespread destruction of in cortex, basal ganglia, and brainstem [21].

WEEV produces similar signs and symptoms to EEEV. A 3 – 5 day prodrome of sudden headache, fever, dizziness, vomiting and malaise may progress to restlessness, tremor, irritability, rigidity, altered mental state, paralysis, seizures [33], [19]. In infants under the age of one, up to 60% can have brain damage. Sequelae is 5% in older individuals, with rapid recovery in 5 – 10 days. Lesions occur in the basal ganglia, brainstem, cerebellum, cerebral cortex and spinal cord [44]. Laboratory acquired infections have been reported with fatal outcomes [20], [58].

VEEV enzootic strains produce mild febrile disease in humans. The epizootic strains of VEEV cause flu-like, incapacitating illness in adults and older children which is characterized by fever, malaise, headache, myalgia, sore throat and vomiting. Lymphopenia and elevated hepatic enzymes are clinical indicators [65]. Neurological symptoms typically appear 4 – 10 days after onset of illness, with disorientation, ataxia, mental depression and convulsions detected in up to 14% of individuals, especially children. Approximately 1 – 4% of cases with younger children develop fulminant disease with reticuloendothelial involvement, lymphoid depletion and
encephalitis. Other symptoms include seizures, behavioral changes stupor and coma with a case fatality rate of less than 1% [7], [61]. Accidental laboratory aerosol infections of young adults cause a febrile illness with abrupt onset of chills, headache, myalgia, somnolence, vomiting, diarrhea and pharyngitis, with symptoms appearing 1 – 5 days after exposure [9], [36], [34].

9.4 ANIMAL MODELS

The alphaviruses can be readily grown to high titers in primary chicken embryo fibroblast, monkey kidney (Vero) and baby hamster kidney cells, to name a few [22]. Reverse transcription polymerase chain reaction has been developed for identification of the viruses [52]. Animal models have been developed for determination of the pathogenesis of alphavirus infectivity and have proven to be useful for medical countermeasures development.

Rodent and Non-Human Primate (NHP) models have been developed for the encephalitic alphaviruses, mimicking human disease in general. Mouse infectivity studies have provided much knowledge on alphavirus pathogenesis [22], [65]. Mice are susceptible to infection by all three alphaviruses and develop lethal severe encephalitis when administered through a number of routes of infection. As is the case for other alphaviruses, resistance to infection increases with age of the host for EEEV. Calculated LD₅₀ values for EEEV are 500 pfu for aerosol and 1250 pfu for subcutaneous inoculation in mice [55]. Similar to VEEV in mice, EEEV follows a biphasic viremia, where the virus first replicates in the peripheral tissue, followed by CNS invasion and encephalitis. VEEV differs from both WEEV and EEEV in that the virus replicates well in the lymphoid tissue and lymphocytopenia is normally observed. In consideration of the three alphaviruses as biothreat agents, aerosol or intranasal routes of infection must be considered. For EEEV and VEEV, the infection follows the olfactory neurons into the olfactory bulbs and neuroepithelium of the brain, by-passing the need for viremia.

Hamster and guinea pig models have been developed for VEEV which showed massive necrosis of lymphoid tissue with accompanying fulminant disease, unlike infections seen in humans, no CNS involvement observed in these models [22], [65]. However, hamster and guinea pig models for EEEV develop severe encephalitis [47], [62], which is similar to mouse models for EEEV. WEEV hamster model is more sensitive than mouse models, with less virulent strains able to cause lethal infections [78].

Most studies for VEEV infection in NHPs use cynomolgus macaques or rhesus macaques. VEEV infections in macaques involve fever, lymphopenia and viremia within 1 – 3 days post-infection, and some show sign of encephalitis several days later [65]. Studies with adult cynomolgus macaque infected with aerosolized EEEV developed fever, elevated liver enzymes, and the majority of macaques (8 of 12 cohorts) succumbed to encephalitis after 6 – 9 days [57]. Similarly, a WEEV aerosolized infectivity model in cynomolgus macaque has been developed, but required high doses of the virus to obtain similar clinical results. In this model, 3 of 12 cohorts succumbed with fever, leukocytosis and encephalitis consistent with those reported for humans [58]. A maroset model has recently been developed for EEEV using intranasal challenge and is a good NHP model for assessment of alphaviral pathogenesis. Animals died around 4 – 5 days post-infection due to neurological signs or anorexia. No viremia was detectable, but the virus was detected in the brain, liver and muscles, similar to those found in human disease [1].

9.5 HOST IMMUNE RESPONSE

The innate immune response for the alphaviruses is through Induction of Interferon (INF) which is an important part of the initial host response to infection [30]. The replication of the virus and formation of a double-stranded RNA is a critical step for INF induction. INF offers a measure of protection early in infection and may serve to
limit virus replication while the adaptive immune system responds [22]. Excess cytokines induced by interferons may cause damage due to cytokine-storm effects, as mice deficient in Interleukin 1β have reduced mortality when infected with neurovirulent strains of SINV [37]. Virus-specific adaptive immunity (cellular and humoral) is induced within 3 – 4 days upon infection [23], [24], [22]. The humoral antibody response is detected with 3 – 4 days post-infection in the form of IgM and IgA, with IgG appearing at 7 – 14 days post-infection and is maintained at relatively high levels for years. The antibody response is important for viral clearance and recovery [23]. A virus-specific T-cell response also facilitates recovery from alphavirus infection by directly killing infected cells, producing anti-viral cytokines and enhancing the humoral response [22]. CD8 T-cells remove virus-infected cells and CD4 T-cells produce INF-γ, which have been demonstrated to clear virus from the CNS in a SINV infectivity model in mice [6]. Additionally, mucosal immunity and T-cells may play a role in the protection from aerosol challenge of alphaviruses [15].

9.6 THERAPEUTICS

We are currently lacking licensed vaccines and therapeutics against VEEV, WEEV and EEEV. The following review of MCMs to the alphaviruses covers promising potential candidates in which proof-of-principle studies have been conducted or the products are in pre-clinical or clinical evaluation.

9.6.1 Antibodies

The importance of antibodies has been well documented in the host response to alphaviruses [23]. Neutralizing and non-neutralizing antibodies to E1 and E2 administered to mice, before or after infection with virus, were protected against Sindbis virus [24]. Epitopes of protective antibodies for VEEV were identified through passive transfer of monoclonal antibodies [41]. Humanized monoclonal antibodies to VEEV have been demonstrated to protect mice and can be used therapeutically to treat mice 24 hour post-infection [29], [28]. Furthermore, antibodies can be used synergistically with interferon alpha for treatment of alphavirus infections [22].

9.6.2 Interferons

Interferons are a key component of the innate immune response. Interferon alpha (INF-α) has been well characterized as an anti-viral drug and is approved for the treatment of hepatitis C virus. INF-α mechanism of action is through four major pathways to induce an anti-viral state.

Current costs per dose of INF-α are prohibitive in making it a widespread anti-viral treatment. A human adenovirus vector expressing INF-α (Ad5-mIFNα) was evaluated for its efficacy for the pre- and post-exposure treatment of WEEV infection in mice [76]. In these murine studies, Ad5-mIFN-α provided 100% protection against a number of WEEV strains after a single intramuscular inoculation given at 24 h, 48 h or 1 week prior to virus challenge. When given a 6-hour post-virus challenge, a single IM inoculation significantly delayed the onset of disease progression and provided 60% protection. These proof-of-concept studies demonstrate that the human adenovirus vector expressing INF-α could provide broad-spectrum protection against various strains of WEEV and possibly provide cross-protection against other alphaviruses.

Activation of Toll-Like Receptors (TLR) is another therapeutic strategy to induce interferons [63], [5]. INF inducers can also protect against VEEV before or within 24 hours of exposure [39], [31]. Poly ICLC is a synthetic, double-stranded polyriboinosinic-polyribocytidylidc acid stabilized with poly-L-lysine carboxymethyl cellulose. It elicits broad anti-viral immune responses through recognition and interaction with TLR-3, and induces the production of interferons-α, -β and -γ in vivo [73]. Poly ICLC is an experimental drug in pre-
clinical development with broad-spectrum anti-viral activity against VEEV [66]. Liposomal Encapsulated (LE) Poly ICLC has been developed with reduced toxicity and longer therapeutic efficacy [73]. In mouse studies, LE Poly ICLC has been shown to be promising in protecting experimental animals against WEEV with 100% protection obtained [74].

Other therapeutic approaches that may show promise are siRNA and antisense oligonucleotides which were recently shown to protect mice in a VEEV model [45], [50].

9.7 PROPHYLAXIS

9.7.1 Immune Modulators

Immune modulators are a class of compounds that are able to provide broad spectrum protection to a wide range of viruses, through non-specific stimulation of the immune system. A broad range of compounds have been identified such as CpG and polysaccharides. In a WEEV mouse infectivity model, cationic liposome–DNA complexes (cationic lipids mixed with CpG oligomers) given to mice 24 hour pre-exposure provided 80% protection after subcutaneous challenge; however, no protection of intranasal or aerosol challenged animals was obtained [38].

9.7.2 Vaccines

Although a commercial, trivalent inactivated vaccine is available for horse immunization against VEEV, EEEV, and WEEV [4], human vaccines against these viruses are non-existent. The most advanced vaccine candidates for humans against VEEV are live, attenuated vaccine (TC-83) and formalin-inactivated TC-83 vaccine known as C-84. Laboratory workers at risk of exposure to VEEV are currently vaccinated with these candidates under Investigational New Drug (IND) status [27]. Similar formalin-inactivated EEEV (known as TSI-GSD 104) and WEEV vaccines (known as CM 4884) under IND were also available [27]. The major drawback of the formalin-inactivated alphavirus vaccines is low immunogenicity, requiring three doses for immunization and annual boosters to maintain protection. On the other hand, the live, attenuated VEEV vaccine TC83 is highly immunogenic; however, the possibility of reversion to virulence could pose a risk for vaccinees.

Several approaches have been used to overcome the problems facing live attenuated and inactivated vaccines against VEEV [46]. The first is reverse genetics, which introduces mutations, deletions, additions, or other changes to the viral sequence through molecular engineering. Through this approach, V3526, a live-attenuated VEEV vaccine candidate, was developed from a full-length cDNA clone of the IAB Trinidad donkey strain of VEEV [10]. The attenuation was made by deleting a furin cleavage site from the PE2 glycoprotein and inserting a single amino acid mutation in the E1 glycoprotein. Pre-clinical animal studies showed V3526 is protective against various sub-types of VEEV in different animal models such as mice [54], horses [18] and non-human primates [59]. Unfortunately, a significant number of vaccinees demonstrated mild to moderate adverse effects of headache, fever, malaise and sore throat in a Phase 1 clinical trial [40]. Moreover, the vaccinees with sore throat and fevers had viral shedding of V3526. These clinical findings prompted the discontinuation of the clinical trial. Currently, studies are underway to test whether inactivated V3526 could be used to replace currently used, formalin-inactivated VEE vaccine C-84 [40].

A second approach for overcoming the problems of the traditional VEEV vaccines is through the construction of recombinant, chimeric Sindbis/VEEV [48]. To construct the chimeric virus, the genes of SINV (one of the least human-pathogenic alphaviruses) encoded the replicative enzymes and the cis-acting RNA elements were ligated
with the genes of VEEV TC-83 encoding the structural proteins. Animal studies showed the chimeric SINV/VEEV virus is highly attenuated and immunogenic [49].

A third approach is a DNA vaccine, which is safe and easy to manufacture, but suffers low efficacy. For instance, a DNA plasmid expressing the structural proteins of VEEV sub-type IAB was tested against VEEV infection in non-human primates. The results showed that the vaccine administered by Particle-Mediated Epidermal Delivery (PMED) only partially protected the animals against an aerosol VEEV challenge [13]. Directed molecular evolution was used to improve the cross-reactivity and immunogenicity of DNA vaccines to VEEV [12]. Delivery by intramuscular electroporation elicits high levels of protective antibodies, 100% protection in mice, and reduction of febrile symptoms in non-human primates [14].

Finally, recombinant viral vectors have been used to make DNA vaccination more efficient. Viruses, such as vaccinia viruses and adenoviruses, can be modified into molecular vehicles to deliver genes encoding VEEV antigens. A recombinant VEEV vaccine was first made based on a vaccinia virus vector, which expresses the structural proteins of the IAB Trinidad donkey strain of VEEV [32]. The vaccine provides protection of mice against peripheral challenge of various sub-types of VEEV. However, only partial protection was achieved against aerosol challenge. Later on, an adenovirus vector expressing VEEV envelop proteins was used for vaccine development. It appears that intranasal immunization of the adenovirus-vectored VEEV vaccine protects mice against aerosol challenge [53]. Additionally, boosting with the adenovirus-vectored VEEV vaccine improves the efficacy of a DNA vaccine against VEEV [51]. Lastly, a VEEV replicon system was developed to express the structural proteins of homologous or heterologous alphaviruses [56]. In this system, replicon genome is made by combining the non-structural genes and cis-acting RNA sequences of VEEV with the genes encoding the structural proteins of the alphaviruses. The replicon genome is then packaged into virus-like particles by capsid and envelope proteins provided in trans from packaging cell lines [46]. By doing so, the structural proteins can be expressed from the replicon without the safety concerns of generating a live VEEV virus. In addition, studies in animals demonstrated that the VEEV replicon system possess intrinsic systemic and mucosal adjuvant activity, suggesting that the system could be useful for making an effective VEEV vaccine [69].

Strategies for making improved EEEV vaccines are mainly focused on chimeric Sindbis/EEEV and DNA vaccination. The chimeric EEEV vaccine candidate, which contains structural protein genes from the North American strain of EEEV strain, is highly attenuated in mice and confers complete protection from disease [70]. For DNA vaccination, a library of chimeric genes expressing variant envelope proteins of EEEV was generated to improve the immunogenicity and efficacy of DNA vaccine [12].

Efforts have also been made to improve safety and efficacy of WEEV vaccine. To construct a DNA vaccine, the 26S cDNA was place behind a CMV promoter. DNA vaccination against WEEV using PMED with the level of protection reached from 50 to 100 % amongst different strains of WEEV [43] after intranasal challenge in mice. In addition, an adenovirus-vectored WEEV vaccine encoding the envelop proteins of the virus confers rapid and complete protection of mice against a lethal intranasal challenge of WEEV [3], [75]. Chimeric Sindbis/WEEV vaccine was investigated for their potential as live attenuated vaccine and mice vaccinated with the vaccine showed no infection after intranasal challenge with high dose of virulent WEEV strains [2].

In conclusion, development of alphavirus vaccines that are safe and provide rapid protection for warfighters should remain a priority. Currently, studies in animals demonstrated that adenovirus-vectored and chimeric Sindbis/alphavirus vaccines are promising candidates. Because of sporadic outbreaks of VEEV, EEEV, and WEEV, it is not feasible to conduct Phase II and III clinical trials for the vaccine candidates. Therefore, the major challenge to bring these vaccine candidates to clinical trials will be the development of suitable animal
models which are closely related to the human infection of the alphaviruses. Without these models, the vaccines will have difficulties to obtain approval by regulatory agencies under the United States Food and Drug Administration’s “animal rule” and “Emergency Use New Drug” regulatory approval in Canada.

9.8 SUMMARY

The encephalitic alphaviruses, EEEV, WEEV and VEEV are potential biothreat agents and can infect humans via an aerosol route of exposure with severe consequences as demonstrated by the number of laboratory acquired infections. The host’s immune system initially activates an innate immune response mainly through the production of INFs. Within 4 – 5 days of infection, an adaptive immune response is stimulated, in which both cellular and humoral immunity contribute to virus clearance. A recent study with neurovirulent SINV in mice indicates that virus in the CNS is initially rapidly cleared (by day 8 post-infection) by CD8 T-cells and IgM antibody-secreting cells, followed by gradual clearing of the virus over a period of several weeks. CD4 T-cells (INF-γ production and helper function), and cells secreting IgG and IgA accumulate and remain in the CNS. Lastly, B memory and plasma cells maintain low levels of viral RNA and prevent establishment of persistent viral infection [42]. Taken together, these properties are what we may want a vaccine, interferon inducer or immune modulator to mimic for effective pre- or post-exposure prophylaxis/therapy of alphaviruses.

9.9 REFERENCES


[39] Lukaszewski RA and Brooks TJ. Pegylated alpha interferon is an effective treatment for virulent Venezuelan equine encephalitis virus and has a profound effect on the host immune response to infection. *J. Virol.* 2000; 74:5006-5015.


Chapter 10 – ANTHRAX AND BACILLUS ANTHRACIS

Christopher K. Cote, Stephen F. Little, Joel A. Bozue and Susan L. Welkos
Division of Bacteriology, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD
UNITED STATES

Corresponding Author (Welkos): susan.l.welkos.civ@mail.mil

ABSTRACT

Anthrax is primarily a zoonotic disease of animals, predominantly herbivores, and humans are incidental hosts which typically become infected by contact with infected animals or contaminated animal products. Human disease is manifested in one of three major forms:

• Cutaneous;
• Gastrointestinal; and
• Inhalational.

Although the large majority of human cases reported world-wide are cutaneous infections which are often self-limited and with low mortality rates, the oropharyngeal/ gastrointestinal and inhalational forms have higher fatality rates. Additionally, a new type of injectable septicemic anthrax has been observed among intravenous drug users. The most dangerous form, inhalational anthrax, is almost 100% fatal if left untreated. Due to its non-specific symptoms, early diagnosis of this disease is challenging and it can progress to a stage that is no longer curable by antibiotic treatment since anti-microbials do not ameliorate the effects of the anthrax toxins.

The major impetus for research on anthrax pathogenesis and development of improved vaccines and therapeutics is the threat of the use of Bacillus anthracis spores as a biologic weapon. The threat is primarily associated with a potential aerosol release, which has been substantiated by various accidental and intentional airborne releases of anthrax spores such as the 2001 letters attack. Therefore the primary focus of this review is on inhalational anthrax.

Inhalational anthrax does not initiate as a primary pneumonia but instead the lungs serve as the portal of entry for the spore, the infectious form of B. anthracis. The spores are thought to be transported to regional lymphoid tissues where they germinate and outgrow into the vegetative bacilli. The latter are then released, begin to rapidly spread systemically, and produce large amounts of the anthrax toxins.

Although much is known about the pathogenesis of infection by B. anthracis, the mechanism of cell uptake of the anthrax toxins, and the roles of these toxins in promulgating infection, much remains to be elucidated about the definitive site(s) of spore entry and germination and the exact nature of the complex spore-toxin-host innate immune cell interaction. Furthermore the ultimate cause(s) of death of an infected host have yet to be established. However it is clear that B. anthracis is both a toxigenic and invasive pathogen, and one of the major sets of bacterial virulence factors consists of the two anthrax toxins, Lethal Toxin (LT) and Edema Toxin (ET). These toxins each consist of a pairwise combination of three toxin components, Protective Antigen (PA), Lethal Factor (LF), and Edema Factor (EF). PA in combination with LF forms LT, and ET is composed of PA together with EF. These toxins play major roles in impeding the host defences against anthrax, and since PA is a highly
immunogenic and protective protein, it has also had a major role in development of vaccines which are effective against anthrax, as described in detail in this review.

In addition to the toxins, B. anthracis produces a second major virulence factor, the capsule, which confers resistance to host cell phagocytosis. Also fragments of capsule can be released from the bacilli due to the action of the CapD capsule depolymerase. Although these fragments could conceivably divert the host immune system and protect the bacterium, administering CapD exogenously appears to increase host phagocytic killing of B. anthracis and has been proposed as an effective adjunct therapy for anthrax. In addition, vaccines consisting of a capsule conjugate or containing capsule antigen have been shown to be protective. Additional effective countermeasures consisting of antibiotics alone or combination regimens given pre- or post-exposure are being explored as viable treatment strategies. For instance, pre- or post-exposure vaccination when combined with antibiotics has been shown to be effective, as has been post-exposure prophylaxis or treatment with a combination of anti-toxin antibodies with antibiotics. These strategies often target both the toxin and the organism and their roles in driving the outcome of an anthrax infection.

Research on the many facets of anthrax pathogenesis and of countermeasure development continues to be of critical importance as will be described in this review.

10.1 INTRODUCTION

Anthrax is an acute infection caused by the Gram-positive spore-forming bacterium *Bacillus anthracis*. In its natural setting, anthrax is primarily a zoonotic disease which occurs in domesticated and wild animals, predominantly herbivores. *B. anthracis* is found throughout the world and exists in the soil in a dormant and highly resistant spore form. Animals become infected when they ingest spores while grazing on contaminated land or ingesting spore-laden feed, although spread mechanically by flies or vultures from an infected animal could be possible [1]-[3]. Humans are incidental and relatively rare hosts which typically become infected by contact with infected animals or contaminated animal products [3]-[8]. These infections occur most often in agricultural settings and developing countries, i.e. exposures to contaminated carcasses and meat. Before the development of effective vaccines and disinfection practices, industrial exposures were also common, in Europe and North America. The processing of animal materials (hides, hair, wool, and bones), was associated with an illness known as Woolsorter’s disease. This disease occurred after inhalation of spore-laden dust and aerosols in wool and textile mills in England and the industrialized regions of Northeast USA. [4], [7], [9].

Human disease is manifested in one of three major forms, cutaneous, gastrointestinal, and inhalational; secondary meningitis can occur in all three forms. More than 95% of human cases reported world-wide are cutaneous infections. Although all three forms are potentially fatal if untreated, cutaneous anthrax is often self-limited, with mortality rates in untreated cases of about 20% (10 – 40 %) [3], [4], [8], [10]. The pathognomonic characteristic of cutaneous anthrax is a painless black lesion referred to as an eschar [3], [10], [11]. The second most common form of human anthrax is gastrointestinal, which includes an oropharyngeal form. Gastrointestinal anthrax is usually caused by consuming contaminated and inadequately cooked meat; and has been associated with 25 – 60 % fatality rates if left untreated [3], [8], [10], [12], [13]. Lesions can be observed in the oral cavity, stomach, or intestinal tract and can result in swelling, perforation, or hemorrhage. Oropharyngeal anthrax is often characterized by lesions in the oral cavity (i.e. tongue, tonsils, or pharynx) which, if left untreated, can result in massive swelling and eventual airway blockage. Finally, inhalation of *B. anthracis* spores results in the most severe form of anthrax which is almost 100% fatal if left untreated. Due to its non-specific symptoms, early diagnosis of anthrax can be very challenging. Unless there is a high index of suspicion, the disease can progress to a stage which is no longer amenable to antibiotic treatment since anti-microbials do not counter the effects of
the anthrax toxins. A dramatic widening of the mediastinum as observed radiographically, as well as pleural effusions, are frequently seen and are the result of hemorrhagic lymphadenitis attributable to bacterial replication and toxin production in the mediastinal lymph nodes. The onset of severe respiratory distress and hypotension can lead to death within 24 hours. B. anthracis bacilli can typically be recovered from blood cultures of patients not treated with antibiotics. Approximately 50% of infected individuals develop meningitis which is often hemorrhagic [4], [8], [14]. Inhalational anthrax does not present as a true primary pneumonia. The lungs serve as the portal of spore entry, but germination and outgrowth of the vegetative bacilli are thought to occur mostly in lymphoid tissue, leading to systemic bacteremia.

Although the incidence of naturally acquired human anthrax in developed countries is very low [3], [4], [8], occasional large outbreaks and, more frequently, smaller clusters of disease continue to be reported worldwide. An outbreak in Zimbabwe from 1979 – 1985 involved thousands of human cases and was linked to infected cattle [1]. Outbreaks of primarily cutaneous anthrax linked to the handling and consuming of “black market” meat from infected animals have been and continue to be reported in regions such as countries of the former Soviet Union [15]. Also, in recent years, unusual and often more lethal cases of anthrax presenting new sources of exposure or routes of infection have been described. Inhalational and gastrointestinal anthrax was observed on different occasions in patients residing in the USA, (New York, New Hampshire) and Scotland and was linked to the production or use of “bongo” drums made from spore-contaminated hides from Africa [16], [17]. A relatively new form of injectable septicemic anthrax was observed among intravenous drug users in Western Europe [18], [19]; the most recent outbreak was postulated to originate from B. anthracis spore-contaminated heroin from Pakistan, Afghanistan, or Iran [18], [19].

The major impetus for research on anthrax pathogenesis and development of improved countermeasures is the threat of B. anthracis spores being used as a biologic weapon. The threat is primarily associated with a potential aerosol release. This scenario was given credence by the accidental release of spores from a military microbiology facility in Sverdlovsk, Russia in 1979 which led to approximately 77 cases of inhalational disease and at least 66 deaths among people exposed to a spore-contaminated aerosol [20], [21]. The revelations after the 1991 Persian Gulf war of Iraq’s capability and intentions for producing biological weapons heightened concerns about the use of B. anthracis as a biologic weapons [22], as did the release of B. anthracis (an attenuated Sterne strain) spores in a Tokyo subway by the Aum Shinrikyo cult [23]. This fear was confirmed with the 2001 letters attack which proved the catastrophic effects on civilian populations of an intentional aerosol release of virulent spores [4], [24]-[28]. For these reasons, although aspects of non-pulmonary routes of infection will be discussed, the major focus of this report will be on inhalational anthrax.

10.2 BACTERIAL VIRULENCE AND ANTHRAX PATHOGENESIS

The infectious form of B. anthracis is typically the spore. The spore is an inherently highly stable particle which can withstand extreme conditions, ensuring survival of the bacterium and the infection of subsequent hosts [29], [30]. The properties that result in such unusual stability include a large number of spore coat proteins that form a thick shell which ensures dehydration and at the same time protects genomic material. Spores of all species of Bacillus are built as a set of concentric shells (Figure 10-1). The inner-most spore compartment is the core, which contains the chromosome [31]. The cortex is a thick layer of peptidoglycan that surrounds the core [32]. The cortex, in turn, is surrounded by the coat, a proteinaceous layer composed of many proteins. Many spore coat proteins are conserved between Bacillus subtilis and B. anthracis, however a number of spore proteins found in B. anthracis and Bacillus cereus, but absent in B. subtilis, are also important to spore formation [33], [34]. For some Bacillus species, including B. anthracis, there is an additional layer that envelops the coat, called the exosporium (Figure 10-1), consisting of a basal membrane and a series of fine hair-like structures.
which project from the membrane and are described as a nap [35]. This nap is primarily composed of the immunodominant protein Bacillus collagen-like protein A (BclA) [36]. Although lacking a clear role in virulence [36], [37], distinct roles for BclA in increasing spores hydrophobicity, reducing non-specific interactions with host cells, and facilitating spore targeting of macrophages have been demonstrated [37]-[41].

Figure 10-1: A Schematic Diagram Paired with a Transmission Electron Micrograph Illustrating the Layers of a \textit{B. anthracis} Spore. It is this complex and sturdy structure that results in the resistance properties associated with \textit{B. anthracis} spores. The spore coat is composed of dozens of proteins that ensure dehydration of the core. The exosporium structure is the outermost layer of the spore and contains “hair-like” structures composed of the immunodominant BclA protein.

When the dormant spores are inhaled, they are deposited in different locations ranging from deep lung to areas within the naso-oropharynx, based in part on particle size of the spore inoculum. Once the spore reaches a suitable location where appropriate nutrients are present, germination initiates. This transition from a spore to the vegetative cell is essential for virulence. The small molecules which induce germination are referred to as germinants. Germinants and cogerminants include amino acids and nucleosides such as L-alanine and inosine [42]. Germination occurs rapidly and begins with the rehydration of the spore interior and degradation of the outer spore layers and interior proteins. These processes occur in the absence of any detectable metabolic activity [43]. Once germination has been initiated it is irreversible; and as the spores germinate into the replicating vegetative forms, they become susceptible to host defences.

Although the sites of actual spore entry and germination in the host remain topics of debate, the generally accepted sequence of events in inhalational anthrax involves the inhalation of ungerminated spores and their uptake by phagocytic cells which transport the intracellular spores to regional lymph nodes. The subsequent germination of the spores into vegetative bacilli occurs in the lymphatic system, where the organisms replicate.
and then spread systemically [44]. Recent work has suggested that spores may be able to utilize other points of pulmonary entry. Glomski and colleagues demonstrated that the nasal passage and the surrounding Nasal-Associated Lymphoid Tissue (NALT) of mice may serve as a portal by which spores can initiate an infection [45], [46]. Russell et al. also demonstrated that spores may be able to exit the alveolar spaces via internalization by lung epithelial cells [47], although this appears to be a rare event. These observations regarding alternate routes for host invasion by spores deserve further investigation. Similar debate continues regarding the exact location of germination after the spores have been inhaled. Numerous reports have documented that, except under rare or specific circumstances, the lungs are not a favorable location for germination of spores into the vegetative bacilli; and retention of dormant spores in the lungs, sometimes for extended periods, has been well documented [44], [46], [48]. However, it has been shown that spores are capable of germinating within the lungs after being phagocytosed by alveolar macrophages [44], [45], [49]-[53].

It is interesting to note that, in contrast to most pulmonary infections, cutaneous infections in at least one animal model appear to lead to germination and vegetative growth of \textit{B. anthracis} in the absence of cellular uptake. A model for cutaneous anthrax was developed in mice in which Sterne strain spores are inoculated onto intact or abraded skin [54]. The primary innate host defences involve the infiltration of neutrophils into the epicutaneous region of infected skin. These inflammatory cells appear to destroy the germinated bacilli and prevent their further invasion; complete resolution of infection may depend on the extent of the cellular response and other host factors [55].

While the site of germination in inhalational anthrax is not entirely clear, especially in human disease, recent studies have highlighted the critical nature of the germination state of the spore in pathogenesis and subsequent disease progression. Although mutant spores unable to germinate \textit{in vitro} can better survive the anti-bacterial environment in macrophages, they are severely attenuated in mouse models [56]-[58]. However, spores that have been chemically germinated before delivery are also significantly less infectious, probably due to their increased susceptibility to host immune responses [59], [60]. Thus germination \textit{in vivo} appears to be a finely orchestrated event and one that is not readily manipulated as a therapeutic strategy. Using a murine aerosol model involving challenge with spores of the fully virulent Ames strain of \textit{B. anthracis}, Cote et al. demonstrated that germination-altering strategies are likely not beneficial to the infected host or useful as potential therapeutic options.

The pathogenesis of infection by \textit{B. anthracis}, especially the early stages, appears to be largely dependent on spore and host phagocytic cell interactions. In an early study, a peritoneal model of infection was used to assess the phagocytic response of different strains of mice to infection by the peritoneal route with attenuated Sterne strain spores. The infection resulted in a rapid recruitment of neutrophils which quickly waned in numbers [61]. Shortly thereafter the recruitment of a more sustained population of macrophages was observed. In a later intranasal model employing the unencapsulated toxigenic Sterne strain, Cleret et al. and others showed that spores are initially phagocytosed primarily by alveolar macrophages; but within a short time, other monocytic cells, predominantly lung dendritic cells, take up spores present in the alveoli and appear to be the primary cell population that transports the spores out of the lung [62]-[65]. However, although intranasal infection models are used extensively, they may not necessarily accurately depict the events occurring during inhalation of aerosolized spores. In a small particle (i.e. \( \leq 5 \) \( \mu \)m) inhalation model, aerosolized spores are deposited more deeply in the lungs near the alveoli, intranasal instillation delivers the inocula as liquid droplets and deposited in the nasal region and upper airway, potentially resulting in a different immune response.

Professional phagocytic cells appear to be the major targets of host cell contact and uptake by spores in the lungs [53], [64], [66], [67]. Bozue et al. showed that in addition to macrophages, \textit{B. anthracis} can bind to epithelial cells, fibroblasts, and human endothelial cells [37], [38]. However the presence of the outermost protein BeIA on the spore surface acts to reduce non-specific interactions between \textit{B. anthracis} spores and non-professional
phagocytic cells in favor of a more directed uptake by phagocytic cells. Spores of a BclA-deficient mutant bound non-professional phagocytic cells to a much greater extent than the wild-type (BclA-expressing) spores, even though spores of the wild type and bclA deletion mutant adhered to and were phagocytosed by macrophages to an identical extent. Oliva et al. [41] extended these findings by identifying the integrin Mac-1 (CR3) as the specific receptor on professional phagocytic cells involved in binding wild-type BclA-expressing B. anthracis spores and directing their internalization into professional phagocytes. Mac-1 is a non-covalently bound association between CD11b and CD18 found on polymorphonuclear cells, natural killer cells, and mononuclear phagocytes. CD14 acts as co-receptor for spores in the Mac-1 pathway which binds to rhamnose residues within BclA and enhances the internalization of spores via the Mac-1 dependent mechanism as alluded to previously [68].

Spore phagocytosis has been documented in vivo and in vitro by macrophage-like cell lines [58], [68]-[71] and primary cells from mice, NHP, and humans [70], [72]. Kang and co-workers observed that germination of Sterne spores was greatly enhanced in murine macrophage-like cells, and that specific germinant receptors of B. anthracis were required for this enhancement [50], [58], [73]. In animal models [44], [74]-[76], spores were thought to be engulfed by alveolar macrophages which partially served as a “Trojan horse” used by the spores merely as a vehicle for trafficking out of the alveolar spaces to tissues more favorable for spore germination and disease progression [75]. Thus alveolar macrophages are important for spore translocation out of the lungs. However, macrophages also appear to be important for host survival. For example, the reduction of spores reaching regional lymph nodes in a guinea pig aerosol model was ascribed to germination and subsequent killing of spores by a portion of infected alveolar macrophages [44]. Also, mice that have undergone macrophage depletion procedures are significantly more susceptible to the ensuing infection [67]. In contrast, mice supplemented with additional quantities of macrophages are able to better survive parenteral spore challenges [67], [71].

Less is known regarding the interactions of B. anthracis spores with neutrophils and dendritic cells. Both neutrophils [61], [77] and dendritic cells have been shown to phagocytose spores in vitro [62], [64]. Data collected through neutrophil depletion studies indicated that these cells are essential for optimal resistance to infection with the fully virulent Ames strain of B. anthracis but appear to be less important than macrophages [67]. Mouse survival was not associated with increased neutrophil populations but was associated with increased numbers of macrophages, confirming earlier observations with the Sterne strain and various inbred strains of mice [61]. Recent studies have demonstrated that dendritic cells likely play a crucial role in host cell interactions and transit of spores [64]. Cleret and colleagues observed significant lung dendritic cell recruitment about 6 hours after spore exposure and the initial macrophage response [64].

The actual process and extent of translocation out of the alveolar spaces has been a long-standing topic of research. In a seminal paper, Joan Ross elegantly described the translocation of B. anthracis spores from the alveolar spaces of guinea pigs that had been infected by either intratracheal administration or by exposure to aerosolized spores [44]. This paper confirmed previous findings that indicated that inhalational anthrax was not truly a pneumatic disease, but rather the lungs served mainly as a port of entry [44], [78]. Recent implications of Nasal-Associated Lymphoid Tissue (NALT) in the pathogenesis of inhalational anthrax, discussed above, do not conflict with these data but rather detail other mechanisms of disease initiation. As observed later by Cleret et al. [64], Ross found that spores were phagocytosed within minutes after exposure and were only observed to germinate after they had been phagocytosed. Tracheal damaged caused by the intubation may have altered the pathogenesis and allowed for more rapid spore germination and bacillary outgrowth, due to the introduction of host-derived germinants into the lung. It was also observed that polymorphonuclear cells did not play a major role in the translocation process and that phagocytes could contain organisms simultaneously in various stages of germination and outgrowth. These early data have been confirmed by many recent studies.
In addition to facilitating germination and transport of spores, host phagocytic cells have been shown to be sporicidal, unless overwhelmed by a large multiplicity of infection, once germination is initiated [48], [70], [77], [79]. B. anthracis spores are thought to rely mainly on the professional phagocytes for translocation out of the lungs; however if germination is initiated early during this trafficking, the germinated spore (or pre-bacillus) may be rendered susceptible to the innate anti-microbial environment within these host cells. Cote et al. hypothesized that various scenarios can be observed concurrently. For example, the battle between spores and host-phagocytes can be radically influenced based upon spore burden and possibly activation state of the macrophage. A macrophage with a low spore burden may be more likely to be sporicidal compared to an alveolar macrophage with a much higher spore burden [71].

The anthrax toxins appear to play a role in spore-host cell interactions very soon after germination. The characteristics and specific functions of the toxins will be detailed below. Anthrax toxin components have been reported to be produced and secreted very rapidly after germination in vitro [80] or after phagocytosis by alveolar macrophages [53]. The anthrax lethal toxin is well known to be toxic to macrophages and to adversely affect other cell types, such as neutrophils and dendritic cells [63], [81]-[86]. Banks et al. showed that after uptake and initial germination of a large multiplicity of spores, toxin production follows soon thereafter. It was hypothesized that the ubiquitous toxin receptor CMG2 was expressed on the inside of the phagosomal membrane [69]. Thus, as the toxin components are secreted from the germinating spores within the phagolysosome, the toxin binding component, Protective Antigen (PA), can interact with the anthrax toxin receptor resulting in toxin translocation into the host cytosol. The toxin effector molecules can then incapacitate the cell resulting in eventual cell death and release of vegetative organisms. It should be noted at this point that pulmonary infections with B. anthracis are innately asynchronous. Ungerminated spores can be found in the alveolar area for an extended period post-infection [37], [44], [48], [67], [87] at the same time other spore germination and vegetative outgrowth is occurring in areas outside of the lung. Spore burden appears to influence the fate of B. anthracis spores [71] and likely the extent of toxin production early in germination [69]. Furthermore, since the toxins can impair a number of innate immune functions, it can be surmised that the pathogenetic process is far from being uniform in nature and that the spore-toxin-phagocyte interactions are quite complex. For example, while germinating spore within the macrophage phagolysosome could be destroyed by various anti-microbial mechanisms, there may be several ungerminated spores within the same cell that survive the cellular insult. These surviving spores can then germinate and be released from the infected macrophage upon its toxin-induced lysis.

Despite the evidence for toxin production early in spore-phagocyte interactions, toxin production is dramatically upregulated in the vegetative bacilli that spread systemically. It is at this stage of pathogenesis that the major virulence factors, anthrax toxins and an anti-phagocytic capsule are produced at significant quantities. These two virulence factors are considered to be the major virulence factor of B. anthracis and both are essential for maximal virulence of B. anthracis in most animals. There are two anthrax toxins, each of which contains the 83 kilodalton (kD) cell binding PA component in association with a catalytically active moiety [88]. PA associates non-covalently with Edema Factor (EF), a calcium and calmodulin-dependent adenylate cyclase, to form Edema Toxin (ET); and with Lethal Factor (LF), a zinc-dependent metalloprotease, to form Lethal Toxin (LT). The genes encoding all three toxin components are located on the native pXO1 plasmid of B. anthracis.

The activities of the toxins are manifested after translocation into host cells, which involves binding of PA to cell surface receptors. There are at least two different cellular receptors for PA, ANTXR1, identified as Tumor Endothelial Marker 8 (TEM8), and the more ubiquitous ANTXR2, also known as Capillary Morphogenesis factor 2 (CMG2) [89], [90]. PA binds to an integrin-like domain of both receptors and is then cleaved to a 63 kD form (PA63) by a cell surface furin-like protease. This PA fragment assembles into either heptameric or octameric prepore complexes [91]-[94] to which LF and/or EF bind. The toxin complexes are then taken up into cells by receptor-mediated endocytosis [95]. Acidification of the endosome stimulates the PA to form a
membrane pore through which the LF and EF components translocate into the cytosol leading to toxicity [88], [92]. The production of cAMP by EF leads to edema formation and the LF protease cleaves and inactivates Mitogen-Activated Protein (MAP) kinase kinase, resulting in disruption of cell signaling [82], [96]-[99]. There is evidence that the 83 kDa PA can alternately be cleaved by a serum protease within the bloodstream of infected animals and form oligomeric PA complexes which are taken up into cell [100], [101]. Both anthrax toxins can be individually lethal after injection in small animal models of toxemia [102] [103]; however, they lack the extreme potency of toxins such as botulinal toxin.

Both anthrax toxins have been extensively studied by numerous investigators using in vitro and in vivo models. Friedlander first established that anthrax LT is cytotoxic for macrophages [104]. This report also suggested that anthrax LT may preferentially target phagocytes, as macrophages were lysed, but other cell types were not. These initial observations were followed by several reports detailing the immunosuppressive interactions of anthrax toxins with the host immune response, manifested by alterations in cytokine expression, inhibition of neutrophil chemotaxis, and induction of apoptosis of activated macrophages [63], [69], [86], [102], [103], [105]-[107]. Recently Hu and Leppla reported that macrophages, dendritic cells, and B cells are more sensitive to LT than are T-cells [108] and may possibly be related to the observation that germinating spores must be able to kill phagocytes to escape the anti-microbial environment and initiate infection. Additionally, the toxin sensitivity of dendritic cells was shown to impair subsequent antigen presentation and to further cripple the host innate immune response against B. anthracis [108], [109]. It was also established that anthrax toxin receptor expression on different host cell types did not correlate with anthrax toxin sensitivity in inbred mouse models. For instance, dendritic cells and macrophages displayed the greatest sensitivity to anthrax lethal toxin but had significantly reduced levels of toxin receptor CMG2 compared to T-cells [108]. Mouse susceptibility to LT is influenced by additional factors besides phagocytic cell sensitivity. In contrast, in a recent study employing recombinant inbred strains of rats, an absolute correlation was demonstrated between in vitro macrophage sensitivity to LT-induced lysis and animal susceptibility to the toxin [110]. The relationship of these animal results to the cellular interactions of toxin and the role of these interactions in the pathogenesis of anthrax in humans is unknown.

There have been several reports documenting an inverse relationship between toxin sensitivity and resistance to infection [111]-[114]. The rat model exemplifies these observations. Rats are exquisitely sensitive to anthrax lethal toxin (i.e. rats succumb to toxemia within minutes to hours post-intravenous administration) but are considerably resistant to infection with B. anthracis [115]. In contrast, animals such as mice and guinea pigs exhibited greater susceptibility to spore infection but less sensitivity than rats to the toxins [114]. This inverse relationship has seemed perplexing for years; however, research in inbred mouse models is beginning to elucidate the basis of this phenomenon.

Using CMG2-deficient mice, Liu et al. showed that the targeting of myeloid cells as a group (including macrophages and neutrophils) by anthrax toxins was not responsible for anthrax toxin-induced end stage disease and lethality [116]; but that anthrax toxin uptake via the CMG2 toxin receptor and consequent impairment of myeloid cells was essential to establish anthrax. Their findings on the targeting of macrophages by LT confirm and extend those observed previously [69], [71]. Terra et al. established a role for the host Nalp1b/Nlrp1b inflammasome [117]. Inflammasomes are NOD-Like Receptor (NLR)-containing multi-protein complexes that control activation of caspase-1 and the processing and secretion of the pro-inflammatory cytokines Interleukin (IL)-1β and IL-18 [118]. In inbred mice harboring the LT sensitivity allele on chromosome 10, the Nalp1b/Nlrp1b inflammasome is activated by and can mediate macrophage and dendritic cell sensitivity to LT [117]. Their data support a model in which the activation of Nlrp1b and subsequent killing of macrophages by LT is not a mechanism of virulence for B. anthracis, but rather these events are the basis of a protective host-mediated innate immune response to infection by stimulating pro-inflammatory cytokine production and inflammation at the site of LT production. Additional studies are required to fully understand the relationship between anthrax
LT sensitivity of the host and resistance to infection, particularly in context with fully virulent strains of *B. anthracis*. In addition, a better understanding of the *in vivo* actions of ET and their significance is needed. Results of recent studies are revealing a potentially major role for ET in anthrax pathogenesis [102], [119], [120].

In addition to the toxins, *B. anthracis* produces a second major virulence factor, the capsule. While capsule formation by *B. anthracis* has been reported to initiate soon after spore germination, the characteristic robust anti-phagocytic capsule is not observed until vegetative growth. The *B. anthracis* capsule is a polypeptide composed entirely of gamma-linked D-glutamic acid residues [121]. Its role in pathogenesis and as a potential vaccine antigen is an active area of research [122], [123]. The capsule confers resistance to host cell phagocytosis, yet it has also been shown to be sloughed from the bacilli at fairly high levels. This phenomenon has been theorized to serve as an immunological decoy for the bacterium. Although the native polyglutamate capsule is weakly immunogenic [124], antibodies directed against the capsule and vaccines consisting of a capsule conjugate or containing capsule antigen have protected small animals to a significant extent against anthrax [122]. While certain immunodeficient strains of mice are susceptible to killing by strains of *B. anthracis* that are unable to produce capsule [125], other mammals are not readily affected by these strains. Thus, toxigenic but non-encapsulated strains, such as the Sterne strain, are effective veterinary vaccines while the STI-1 strain is used as a live human vaccine in some areas of Eastern Europe [3].

The genes encoding the capsule biosynthetic proteins are located on the pXO2 virulence plasmid in the *cap* operon [126]. The capsule depolymerase protein encoded by the *capD* gene is a γ-Glutamyl Transpeptidase (GGT) capable of cleaving the γ-linked peptide bond of the capsule to facilitate attachment to the peptidoglycan and causing the release of small capsule fragments. The capsule-degrading property of the CapD protein has been exploited recently to enzymatically remove the capsule from the surface of bacilli, resulting in increased phagocytic killing of *B. anthracis* by human and murine phagocytes [127]. Scorpio *et al*. administered Cap D to mice and demonstrated protection against *B. anthracis* strain Ames; and thus CapD has been proposed as a potentially effective adjunct therapy for anthrax [128].

In addition to the two major virulence factors, a number of extracellular proteins and enzymes produced by *B. anthracis* have been characterized recently and their roles in virulence in small animals studied. These are reviewed in detail elsewhere [3], [129], [130].

In conclusion, although much is now known about the pathogenetic mechanisms of anthrax, many aspects of pathogenesis as well as the ultimate cause(s) of death of an infected host have yet to be established. It is clear that *B. anthracis* is both a toxigenic and invasive pathogen. Evidence from the pathology described for post-mortem cases of human inhalational anthrax and from experimental infections of animals reveals that rapid and irreversibly terminal disease is associated with both a massive systemic infection as well as toxin-mediated effects on a large range of immune and somatic cell types [14], [81], [107], [131]. The spore lifecycle may partially explain this. Although *B. anthracis* can exist for decades in the environment as dormant spores, the spores generally must infect a host, multiply rapidly and kill the host, and be released into the environment. Only those bacilli which survive and sporulate will persist and contribute to maintaining an infectious level of spores.

10.3 VACCINES

10.3.1 Vaccine Immunity

Vaccines which include PA as the major immunogen provide protection against anthrax. Serological correlates of immunity have been described in mice [132], guinea pigs [133], rabbits [134]-[136], and Non-Human
Primates (NHPs) [137] by a PA-based ELISA or a Toxin-Neutralizing Antibody (TNA) assay. This latter assay measures the functional ability of PA (and LF) antibodies to interfere with formation of \textit{B. anthracis} LT \textit{in vitro}. Additionally, passive protection studies suggest that antibodies to PA correlate with immunity [138]-[142]. Several studies also suggest the importance of CD4\(^+\) T-cell Th1 and Th2 responses after vaccination [143]-[145] and after cutaneous anthrax [146] and that a CD4\(^+\) T-cell response may mediate protection of mice against non-toxigenic encapsulated (tox\(^-\) cap\(^+\)) bacteria [147]. Although the efficacy of innovative approaches to anthrax vaccine development has been evaluated by measuring the immune response or protection against a toxin challenge, our review will be confined to discussing the efficacy of vaccines examined against infection.

### 10.3.2 Filtered Cell-Free Culture Supernatant Vaccines

The current U.S. anthrax vaccine, Anthrax Vaccine Adsorbed (AVA), currently produced as BioThrax\textsuperscript{™} (Emergent Biosolutions), was developed from modifications of the Wright \textit{et al.} alum-precipitated vaccine [148]-[151] which was more than 90\% effective in protecting woolen mill workers against cutaneous anthrax [152]. AVA BioThrax\textsuperscript{™} is prepared by adsorbing filtered, microaerophilic culture supernatant fluids from the toxigenic unencapsulated (tox\(^-\) cap\(^+\)) \textit{B. anthracis} V770-NP1-R strain onto aluminum hydroxide gel (1.2 mg/ml of aluminum). Benzethonium chloride (25 µg/ml) is added as a preservative and formaldehyde (100 µg/ml) is added as a stabilizer in the final product. The recent re-licensure of AVA BioThrax\textsuperscript{™} prescribes 0.5 ml Intramuscular (IM) injections at 0 and 4 weeks followed by injections at 6, 12, and 18 months and yearly boosters thereafter [153]. A somewhat similar vaccine licensed in the U.K., Anthrax Vaccine Precipitated (AVP), is prepared by precipitating aerobic culture filtrates of the \textit{B. anthracis} tox\(^+\) cap\(^-\) Weybridge (Sterne) strain with alum and adding thiomersal as a preservative [154]-[156]. AVP is administered in 0.5 ml doses at 0, 3, and 6 weeks and at 6 months, also with yearly boosters thereafter. In addition to antibodies to PA, lesser amounts of LF and EF, as well as other spore-associated proteins, are also present in AVA BioThrax\textsuperscript{™} and AVP [157]-[161]. Both AVA BioThrax\textsuperscript{™} and AVP have been found to be safe and well tolerated [162], [163] and efficacious in rabbits and NHP against diverse \textit{B. anthracis} challenge isolates [164]. Several reports have noted improvement in the efficacy and/or antibody response after AVA BioThrax\textsuperscript{™} vaccination in the presence of CpG Oligodeoxynucleotides (ODN) [165]-[167]. In fact, Rynkiewicz \textit{et al.} incorporated CpG 7909 with BioThrax\textsuperscript{™} in a Phase 1 clinical trial and reported a 6.3-fold increase in peak anti-PA IgG ELISA titer and an 8.8-fold increase in peak TNA antibody response approximately 3 weeks sooner than BioThrax\textsuperscript{™} alone [168]. A second clinical trial, Phase 1b, has also been reported [169]. AVP supplemented with the \textit{Bordetella pertussis} vaccine has also been examined in guinea pigs [170] and administered to military personnel [171] for a short time period. Mice were reported to be protected from a Subcutaneous (SC) challenge with Sterne spores after Intranasal (IN) vaccination with AVA BioThrax\textsuperscript{™}, but a control group of mice vaccinated SC or IM was not included for comparison [172].

### 10.3.3 Recombinant PA-Based Vaccines

The rationale in developing a recombinant PA (rPA) vaccine was to address the inherent disparity between different fermentor lots and the undefined composition of cell-free supernatant-based vaccines. While the gene encoding PA has been cloned in numerous expression systems [173]-[175] including plants [176], clinical trials for the next-generation anthrax vaccine have used rPA expressed from \textit{B. anthracis} [177]-[180] and \textit{E. coli} [181]-[183] combined with aluminum-based adjuvants, aluminum hydroxide gel (aluminum oxyhydroxide; Alhydrogel, Rehydrogel) and potassium aluminum sulfate (alum). In studies with rabbits, IM or Intradermal (ID) injection of rPA formulated with or without aluminum hydroxide gel adjuvant elicited similar antibody responses and protection against an aerosol challenge with \textit{B. anthracis} Ames spores [184], [185]. Formaldehyde, which is present in AVA BioThrax\textsuperscript{™}, enhanced protection of rabbits with a single dose of rPA-Alhydrogel vaccine against a parenteral spore challenge [184]. Numerous experimental adjuvants have been compared with
Alhydrogel in mice, guinea pigs [186]-[188] and NHP [189] to improve the efficacy of parenterally-administered rPA. Addition of CpG to rPA did not significantly improve the antibody response and efficacy against spore challenge compared to rPA adsorbed to aluminum hydroxide gel or rPA alone in rabbits, possibly reflecting the absence of aluminum hydroxide gel adjuvant in the rPA-CpG ODN vaccine preparation [185]. Increased efficacy of PA was also reported in guinea pigs against infection when adsorbed to Alhydrogel and supplemented with the B. pertussis vaccine compared to rPA adsorbed to Alhydrogel [170]. Vaccination with the cleaved cell-bound form of PA, PA63 (which was expressed by Saccharomyces cerevisiae), as would be anticipated, protected rabbits against an aerosol challenge with a fully virulent tox+ cap+ strain at a lower but not statistically significant different rate than AVA BioThrax™, and both PA63 and AVA BioThrax™ gave similar protection in a small number of NHP against aerosol challenge [190].

Vaccines have been prepared using biologically inactive proteins to eliminate the possibility of anthrax toxin formation. A mutant PA containing a deletion of the furin cleavage site (PA163-168), which rendered PA unable to bind LF or EF, and a PA mutant with a deletion of the chymotrypsin cleavage site, which disabled translocation, both provided similar protection of guinea pigs as native PA against a parenteral spore challenge [191], [192]. These regions of PA are illustrated in Figure 10-2.

![Diagram of PA domains and functions](image)

**Figure 10-2: Functional Domains of Anthrax Toxin Components, Protective Antigen (PA), Lethal Factor (LF), and Edema Factor (EF), as Determined by Petosa, C et al. Nature 1997;385(6619):833-8; Pannifer, AD et al.; Nature 2001;414:229-33; Drum, CL et al.; Nature 2002;415:396-02.)**
Alternatively, functionally important antigenic regions of PA involved in toxin formation have also been evaluated for protection. Domain 4 of PA, which binds to the cellular receptor (Figure 10-2), was reported to protect mice against a spore challenge with a tox+ cap- strain [193]-[196]. While Yin et al. [197] observed partial protection of guinea pigs against a SC spore challenge by using a fusion protein between the C-terminally truncated Hepatitis B virus core (HBc-N144) particle and the 2β2 – 2β3 loop of PA which contains the chymotrypsin cleavage site of PA (HBc-N144-PA-loop2, see Figure 10-2, Oscherwitz et al. [198] protected rabbits against an aerosol B. anthracis strain Ames spore challenge by using a multiple antigenic peptide within the 2β2 – 2β3 loop of Domain 2 colinearly synthesized with a heterologous T-cell epitope from Plasmodium falciparum.

Vaccination strategies that target mucosal immunity have been proposed to be less invasive, easier to administer compared to IM or SC injections, potentially more efficacious against inhalational anthrax, and better able to elicit both mucosal and systemic antibodies than the current regimen. Although PA encapsulated within poly-L-lactide microspheres or loosely bound to the microspheres by lyophilization elicited lower titers when two vaccine doses were administered by the IN route compared with IM, similar antibody titers were measured after an IN booster vaccination following a primary IM vaccination. Also, full protection in mice was observed against Intrapерitoneal (IP) or aerosol challenge with the tox+ cap- B. anthracis strain STI-1 whether the vaccine was administered IN and/or IM [193], [194]. In administration of two doses of PA in a water-in-oil nanoemulsion to guinea pigs fully protected against an ID challenge with B. anthracis strain Ames at 6 months and protected 40 – 70 % of animals against an IN challenge at 7 weeks; however, a test group consisting of PA adsorbed to Alhydrogel was not reported [199]. Although the serological titers were slightly lower than those associated with IM vaccinations, IN delivery to rabbits of three doses of dry powder formulations of PA/CpG/trehalose [185] or PA/CpG/trehalose/chitosan [200] were as protective as PA formulated with Alhydrogel.

Early oral vaccination strategies using PA cloned into Salmonella resulted in limited colonization, low anti-PA antibody production, and only partial protection of mice [201], [202]. Enhanced antibody responses and complete protection against an IN challenge with a tox+ cap- strain were observed in mice by fusing PA with E. coli alpha-hemolysis protein in S. enterica Serovar typhi [203]. Targeting delivery of PA to mucosal dendritic cells, accomplished by oral vaccination (5 doses) using a recombinant Lactobacillus acidophilus which expressed PA fused to a dendritic cell peptide, resulted in 75% protection of mice against a Sterne challenge compared to 80% survival for PA adsorbed to the aluminum hydroxide gel Alhydrogel [204], but complete protection was accomplished by oral vaccination using a high-copy vector in L. gasseri [205]. Partial protection has also been reported after oral vaccination with spores of an attenuated B. anthracis tox+ cap- mutant strain expressing high levels of a variant of PA with deletions of the chymotrypsin site (F313F314; mPA313) [206]. Oral vaccination of mice with a recombinant strain of S. enterica serovar Typhimurium expressing PA fused with ClyA protected better than PA Domains 1 and 4 or PA Domain 4 (Figure 10-2) alone against an aerosol challenge with STI-1 spores, but was not as efficacious as IM vaccination [195]. Gorantala et al. [196] improved protection after oral gavage with Domain 4 when administered with cholera toxin against IP challenge with vegetative bacilli of the Sterne strain.

Transcutaneous immunization also has been investigated as an alternative route and method of vaccination. Compared to PA formulated with aluminum hydroxide (Rehydrogel), PA mixed with E. coli heat-labile enterotoxin and exposed to abraded skin of mice by a gauze patch resulted in similar anti-PA titers, higher toxin neutralization titers, and similarly fully protected against a tox+ cap- SC challenge [207], [208]. Similar serological responses were reported by Peachman et al. [132] but they observed only partial protection (20 – 60 %) after transcutaneous vaccination against IN challenge with spores of the fully virulent tox+ cap- Ames strain compared with 10% survival in mice receiving IM vaccination with PA precipitated with alum. Mikszta et al. [185] also compared in rabbits ID vaccination using a microcannula, transcutaneous vaccination...
using a Microenhancer Array (MEA) device and IM vaccination of PA. The vaccines were formulated with Alhydrogel, CpG, or without adjuvant. While transcutaneous vaccination with Alhydrogel- or CpG-adjuvanted rPA resulted in lower serological responses and only partial protection (33%) against an aerosol Ames strain spore challenge, ID and IM vaccinations with rPA formulated with Alhydrogel or CpG elicited comparable antibody responses in rabbits and > 80% protection against challenge [185].

10.3.4 PA Combined with Other Bacillus Components

Several studies have been performed to determine the extent to which LF or EF contributes to protection [209]-[212]; the three components are shown in Figure 10-2. The results are inconclusive, probably reflecting differences in the biological interaction of toxin components, purity of the proteins, and in experimental design. Non-functional PA, LF, and EF proteins have been used to address concerns regarding the possible cytotoxicity resulting from the interaction of biologically active proteins, although this possibility may not be likely when administered with an adjuvant. IN vaccination of mice with PA63 and LF which had an amino acid substitution E687C that abolished its enzyme activity enhanced the antibody response and fully protected against a SC B. anthracis Sterne spore challenge; mice vaccinated with either component alone were not protected [213]. Mice vaccinated with PA Domain 4 (amino acids 552-735; PAD4) fused with LF Domain 1 (amino acids 1-254; LFD1) were fully protected against IP challenge with spores of strain STI-1, as were mice administered individual PAD4, LFD1, and PA [214]. Rabbits inoculated with either the N-terminal fragment of LF (amino acids 1-262; LFn) fused with full length wild-type PA (LFn-PA) or PA mutated at the furin cleavage site to prevent proteolytic processing (PA_{CM}; LFn-PA_{CM}) and adsorbed to Alhydrogel were fully protected against exposure to B. anthracis Ames spores, as were rabbits vaccinated with PA [215]. Although comparable anti-PA titers were observed in the rabbits among the test groups, as well as similar anti-LF titers when LFn fusion proteins were included, interestingly, the TNA ED_{50} titers from rabbits vaccinated with LFn-PA or LFn-PA_{CM} were appreciably lower than rabbits inoculated with only PA [215]. Not unexpectedly, a similar activity with LF has been reported for EF. Expression of the N-terminal fragment of EF (EFn) by a replication-incompetent adenovirus vector elicited anti-EF antibodies that were cross-reactive with LF and partially protected mice against a Sterne spore challenge [216].

Recombinant Bacillus collagen-like protein of B. anthracis (rBclA), an immunodominant exosporium glycoprotein noted above, was reported to elicit a strong anti-BclA antibody response in mice, but while significantly delaying the time-to-death compared to challenge control animals, failed to protect against a SC Sterne spore challenge [217]. However, when rBclA was administered 2 weeks after a sub-optimal vaccination of rPA complete protection was observed against a SC Sterne challenge [217]. Additional protective spore surface proteins were identified by the reactivity of anti-spore serum against recombinant forms of surface-exposed proteins [218]. Strain A/J mice inoculated with two spore surface proteins, p5303 or BxpB (BxpB is also referred to as ExsF), also enhanced protection of a sub-optimal concentration of PA against SC challenge of these immunodeficient mice with spores of the Sterne strain [218]. As with BclA, neither p5303 nor BxpB spore proteins were efficacious alone. The potential of spore proteins to enhance protection was reported by Cote et al. [219] who observed enhanced survival of mice when BclA, ExsF, and p5303 were included with rPA against a challenge with the Ames strain of B. anthracis.

Chabot et al. [122] reported that capsule, co-administered with PA and Ribi adjuvant (monophosphoryl Lipid A and trehalose dicorynomycolate) provided 82% protection of mice against a SC injection of a fully virulent B. anthracis strain compared to PA or capsule alone. Also of note, capsule co-administered with PA conjugated to itself was also 92% protective while the PA-PA conjugate was 58% protective [122]. Vaccination with rPA conjugated to synthetic 10 mer, 15 mer, and 20 mer capsule peptides, as well as a 30 kD capsule from Bacillus licheniformis, and adsorbed to Alhydrogel were also protective in guinea pigs challenged with spores of a fully...
virulent \textit{B. anthracis} strain [220]. Coupling of capsule to the outer membrane protein complex of \textit{Neisseria meningitides} serotype B, an immune-stimulating carrier protein, and precipitating with alum in the absence of added PA resulted in a T-dependent antigen IgG response which protected mice against a challenge with either \textit{B. anthracis} Ames vegetative bacilli or spores [221]. Two IN inoculations of a dry powder vaccine formulated with rPA, MPL, and chitosan and either a 10-mer capsule peptide or PA-peptide conjugate fully protected rabbits against an aerosol Ames challenge but there was no distinction between groups to show that the free peptide was of added benefit [222]. When a single IN vaccination was evaluated, anti-PA ELISA titers, TNA ED$_{50}$ titers, and survival results of animals vaccinated with rPA and capsule peptide conjugate was not as immunogenic or protective in rabbits as a vaccine formulated without the peptide [223].

10.3.5 Microbial Vaccine Strategies

10.3.5.1 Bacterial Expression Systems

Live \textit{B. anthracis} spore vaccines, initially developed for use in livestock [224], may provide increased protection based upon the presence of not only all three toxin components, but of additional bacterial antigens present in the spore or vegetative cell and by stimulating both a humoral and cell-mediated immune responses. Two live tox$^+$ cap$^-$. B. anthracis strains, STI-1 and A16R, are used as spore vaccines in Russia and countries of the former Soviet Union [225], and in China [226], respectively. Several approaches have been pursued to take advantage of the properties of spore- and protein-based vaccines and reduce the risk of an adverse event caused by a vaccine-induced infection. Enhanced protection of animals was reported by combining filtered culture supernatant fluids [227] or PA [228] with a live tox$^+$ cap$^+$ spore vaccine. PA combined with Formaldehyde-Inactivated Spores (FIS), which were prepared from a Sterne strain carrying point mutations in the catalytic sites of LF and EF (RPLC2), along with aluminum hydroxide conferred complete protection of guinea pigs and mice against a tox$^+$ cap$^+$ SC challenge [229]. However, protection of guinea pigs against an IN or aerosol challenge with the highly virulent \textit{B. anthracis} 9602 strain, required an initial SC and IN booster vaccination [230]. Safety concerns may be addressed by expressing PA with mutant LF (PA, EF, LF$_{H686A}$) or EF (PA, ΔEF, LF$_{H686A}$) in attenuated live \textit{B. anthracis} Sterne spores which were shown to protect guinea pigs against challenge with a virulent encapsulated strain [231] although quantitative comparisons with Sterne were not reported.

Limiting the growth of the spore vaccine after vaccination by using aromatic amino acid-deficient (Aro-) \textit{B. anthracis} Sterne mutants [232] or psoralen-Killed But Metabolically Active (KBMA) \textit{B. anthracis} Sterne vaccines which were also asporogenic (ΔspoIIE), deficient in nucleotide excision repair (ΔuvrAB), and had point mutations in the genes for LF and EF [233] were found to be safe and effective alternative strategies. While FIS without added rPA conferred complete protection of guinea pigs and 50% protection of mice against a tox$^+$ cap$^+$ strain (9602P) SC challenge, only 25% of guinea pigs and none of the mice were protected against SC challenge with the tox$^+$ cap$^+$ 9602 strain [229]. Datta \textit{et al.} [234] protected A/J mice against an IN exposure to \textit{B. anthracis} Sterne spores by IN vaccination with irradiated Sterne spores or Sterne spores cured of the toxin plasmid (pXO1) only when co-administered with cholera toxin which the authors showed induced production of IL-17 by CD4 Th17 cells.

The expression levels of rPA in attenuated \textit{B. anthracis} PA-recombinant strains were reported to have a direct bearing on the protective efficacy against infection challenge [235], [236]. Vaccination with spores of \textit{B. anthracis} strains with mutations in either the PA furin cleavage site (mrPA164) or a deletion of the chymotrypsin site (mfPA313) fully protected guinea pigs against a Vollum spore challenge whereas a LF mutant strain in which metalloprotease activity was reduced by mutation of the zinc binding region (H$_{686}$EFGH substituted with A$_{686}$EFGA), protected only 50% of the animals [237]. Alternatively, asporogenic \textit{Bacillus subtilis} transformants [232] or \textit{B. subtilis} spores or vegetative cells expressing PA [238] have also been shown to
be efficacious. Also, as tox cap spore vaccines provide some level of protection [211], [236], spore antigens appear to contribute to protection [217], [239], [240].

10.3.5.2 Viral Expression Systems

Recombinant viral vectors have also been proposed as potential vaccine candidates. Partial protection of mice and guinea pigs from challenge with \( B.\) \( anthracis\) Ames spores was reported by using a vaccinia virus WR PA recombinant strain [241]. Better protection was observed in mice vaccinated with PA expressed in Venezuelan equine encephalitis virus propagation-deficient replicon particles but the challenge was with \( B.\) \( anthracis\) Sterne spores [242]. Two recombinant Sindbis virus vectors expressing PA Domain 4 either from a duplicated 26S promoter or fused to the 2A protease of foot-and-mouth disease virus, elicited anti-PA ELISA titers similar to those of AVA BioThrax-vaccinated mice but failed to elicit TNA titers and were poorly protective against an IN Ames spore challenge [243]. Two doses of a recombinant Wyeth smallpox vaccine strain expressing the PA gene from \( B.\) \( anthracis\) and IL-15 (Wyeth/IL-15/PA) was reported to confer protection of rabbits and mice against an IN challenge with \( B.\) \( anthracis\) Ames spores similar to vaccination with AVA BioThrax [244]. However, Wyeth/IL-15/PA was able to provide slightly greater protection against infection at 3 days and 6 days after a single vaccine dose compared to AVA BioThrax [244].

10.3.6 DNA Vaccines

An early report described using a gene gun to protect rabbits against a \( B.\) \( anthracis\) Ames SC challenge with plasmid DNA encoding the PA gene expressed with a tissue plasminogen activator signal sequence [245]. Mice were also protected against a tox cap \( B.\) \( anthracis\) IP STI-1 spore challenge with plasmid DNA encoding the PA gene on an expression vector with a signal sequence to either permit secretion of PA or retention within the endoplasmic reticulum [246]. In a study using a small number of rabbits per group, protection against an aerosol challenge with \( B.\) \( anthracis\) Ames spores was only observed after an IM protein booster with PA and a mutant LF that contained a point mutation in the active site (E687C; LF7) after IM biojector vaccination with PA63 and LF4 (amino acids 9-254) DNA or an IM booster with PA after IM biojector vaccination with PA63 DNA [247], [248]. Formulation of DNA vaccines expressing PA with the furin cleavage site deleted (PA83Δfurin) or PA83Δfurin and either LF domain I (amino acids 34-295) or LF domains I-III (amino acids 34-583) with cationic lipid A eliminated the need of a protein booster to protect rabbits against an aerosol challenge with Ames spores [249]. Although the added benefit of LF I and LF I-III to PA83Δfurin were not determined, LF I-III alone did provide partial protection to rabbits [249]. When the PA83Δfurin and LF I-III DNA vaccine was tested in monkeys, only modest anti-PA and anti-LF ELISA titers were measured and, in spite of the absence of TNA titers, 3 of 4 NHPs survived an aerosol challenge with Ames spores [250]. However, in a Phase 1 clinical trial, less than half of the subjects had a measurable anti-PA or anti-LF ELISA titer after vaccination with the PA83Δfurin and LF I-III DNA vaccine and there was a dose-limiting reactogenicity associated with the highest dose administered, and an absence of TNA titers [250]. Livingston et al. [251] reported dose-dependent anti-PA ELISA and TNA responses and survival of NHPs against an Ames aerosol challenge using electroporation-mediated delivery of a DNA PA vaccine compared to IM vaccinated animals. Although a synergistic effect was not observed with a combination DNA vaccines expressing PA (secreted and targeted to the endoplasmic reticulum) and the exosporium antigen BclA (secreted), survival of mice against an Ames challenge was better (50%) compared to DNA vaccines expressing either protein alone (20 – 30 %) [252]. The importance of Domain 4 of PA was demonstrated in mice that were protected against a Sterne spore challenge after being primed with a DNA vaccine followed by a booster with a replication-incompetent adenovirus vector each expressing Domain 4 of PA [253].
10.4 DIAGNOSIS

The diagnosis of cutaneous anthrax, the most common form of anthrax, is straightforward using basic microbiological tests for patients exhibiting the hallmark black eschar and for whom a potential source of exposure has been identified (as described below). However, the diagnosis and treatment of inhalational and gastrointestinal anthrax, which are frequently life threatening, is often more difficult. Inhalational anthrax in particular is nearly uniformly fatal if not treated early. It is thought to be the form of anthrax targeted in bioterrorism-related exposures [28], [254] and for which rapid and definitive identification of the infection is needed.

Culture-based conventional microbiological techniques and microscopic examination of smears are the basis of diagnosis of anthrax [255]. *B. anthracis* bacilli are Gram-positive and non-motile organisms. Its colonies on blood or nutrient agar have a matte appearance, are fairly flat, markedly tacky, white or grey-white and are non-β-hemolytic on blood agar. The organism is sensitive to penicillin and lysis by the gamma-phage or its PlyG lysin. Colonies of virulent organisms are mucoid in appearance on blood or nutrient agar containing 0.7% bicarbonate and incubated at 5 – 20 % CO₂, reflecting the production of a capsule which can be observed by using McFadyean’s polychrome methylene blue or India ink staining. Culture methods should be attempted before treatment with anti-microbials. A history of exposure risk factors may also support diagnosis.

Because these culture methods may take 1 – 2 days, there is a need to develop rapid diagnostic methods, especially with patients suspected with inhalational or gastrointestinal anthrax (see below). Several immunodetection-based assays for PA, LF, and capsule in serum include ELISA [256]-[258], Europium Nanoparticle-based Immunoassay (ENIA) [259], and Electrochemiluminescence (ECL) immunoassay [260]. Bacterial smears can be visualized by direct immunofluorescence antibody using fluorescein-labeled mAbs specific for *B. anthracis* cell wall polysaccharide antigen and capsule antigen [261]. Other biomarkers that may be useful include the chaperone and protease HtrA and NlpC/P60 endopeptidase [262]. Physical diagnostic methods include mass spectrometry to identify the products of endopeptidase cleavage products of LF [256], [263], [264] and polymerase chain reaction [265]-[269]. A new confirmatory test utilizes mass spectrometry to quantify LF toxemia even after initiating antibiotic therapy or anthrax immune globulin intravenous treatment [256], [270], [271].

Cutaneous anthrax [272], which accounts for the vast majority of naturally-occurring *B. anthracis* infections, occurs after exposure to contaminated animal products in epithelial tissues and can be readily diagnosed in mild cases by the characteristic black eschar ulceration surrounded by erythema and the presence of a low-grade fever. A rare and more severe form of cutaneous anthrax is displayed with fever and hemorrhagic bullous lesions surrounded by extensive erythema and edema. A complication with the severe form is toxemic shock characterized by lethargy, hypothermia, extensive cutaneous inflammatory reaction and edema, and hypotension. While untreated cutaneous anthrax has a mortality rate of 10 – 40 %, treated cutaneous anthrax has a mortality rate of < 1%. As mentioned above, a new clinical picture of cutaneous has been described recently in intravenous drug users [273].

The rapid severity of gastrointestinal and inhalational anthrax requires an accurate and rapid diagnosis as the infection presents itself with non-distinctive symptoms. Gastrointestinal anthrax [12] symptoms, which occur 1 – 6 days after ingesting *B. anthracis* organisms in undercooked meat, involve the epithelia of the oropharynx or stomach and intestine. Oropharyngeal anthrax is characterized by a fever ≥ 39°C, posterior oropharynx ulcers, severe sore throat, and regional lymphadenopathy. In some cases, the soft tissue swelling and lymph node enlargement in the neck may compromise respiration. After the first week, the early congested edematous lesions undergo central necrosis seen as a whitish patch covering the lesion which later develops into a
ANTHRAX AND BACILLUS ANTHRACIS

pseudomembrane covering. Gastrointestinal anthrax of the stomach or intestine presents itself with a fever ≥ 39°C, ulceration, and gastroenteritis that progressively gets worse. Gastrointestinal anthrax has a propensity to progress to sepsis and symptoms include non-ulcerative, hemorrhagic lesions, nausea, vomiting blood, severe abdominal pain and diarrhea, bowel perforation, toxemia and death. Early intervention is necessary as mortality is ≤ 40% with antibiotic treatment.

Symptoms of inhalational anthrax [28], [274], [275], which develop over 1 – 6 days, initially include a fever ≥ 37.8°C, chills, general malaise, fatigue, cough, nausea, dyspnea, and chest discomfort. Subsequent symptoms include respiratory distress, cyanosis, and diaphoresis which precede death by 24 – 36 h. Clinical findings consist of tachycardia and abnormal chest radiography to include mediastinal widening and pleural effusion and infiltrates. Early intervention (antibiotics, immunotherapy) and supportive measures (pleural fluid drainage) appeared to be important in the lower mortality rate of inhalational cases associated with the anthrax letter attacks (5/11 cases; 45%) compared to earlier cases from 1900 – 2000 (65/71 cases; 92%) [28]. Meningoencephalitis is a complication of anthrax from any primary focus [276]. Patients present with fever > 38°C, malaise, headache, nausea, vomiting, and in later stages of the infection, confusion, delirium, stupor, coma, and seizures. Survival is higher if meningoencephalitis develops from cutaneous anthrax but when the patient is in extremis, the survival rate is quite low (4 – 6 %).

10.5 THERAPY

10.5.1 Antibiotics

Cutaneous anthrax without systemic symptoms in some case may be self-limiting but to avoid further complications may be treated with oral penicillin if the infection did not originate with a potential aerosol exposure. However, if an inhalational exposure is also suspected in conjunction with the cutaneous presentation, ciprofloxacin or doxycycline is recommended as first-line therapy [8], [26], [277]. Effective therapy reduces edema and systemic symptoms but does not change development of the skin lesion. Treatment should be implemented for 7 to 10 days; however, if inhalational exposure is suspected, then treatment should be continued for 60 days. Amoxicillin is recommended for patients who cannot take fluoroquinolones or tetracycline-class drugs; however, significant evidence exists demonstrating that B. anthracis possesses genes encoding proteins with β-lactamase activity that may reduce the efficacy of this treatment [278]-[283]. Tetracycline, erythromycin, and chloramphenicol have been used successfully for treating rare cases of anthrax caused by naturally occurring penicillin-resistant organisms [284]. Additional antibiotics shown to be active in vitro include gentamicin, cefazolin, cephalothin, vancomycin, clindamycin, and imipenem [8], [285]-[287]. These drugs are expected to be effective in vivo, but there is no documented efficacy in clinical cases. However, experimental infections using an inhalational mouse model have demonstrated significant efficacy using the recommended and alternate antibiotics [288]-[291], as described below. It is recommended that inhalational, oropharyngeal, gastrointestinal, or systemic anthrax should be treated with intravenous therapy using at least two antibiotics. The antibiotic therapy should initially include a fluoroquinolone or doxycycline with one or more of the following antibiotics: clindamycin, rifampin, penicillin, ampicillin, vancomycin, aminoglycosides, chloramphenicol, imipenem, clarithromycin, or linezolid [8], [18], [25], [26], [277]. In addition, if a bioterrorism event occurs, the bacterial strains used may be geographically diverse with uncharacterized antibiotic sensitivities or genetically modified to confer resistance to one or more antibiotics, thus further supporting the concept of a multi-antibiotic therapeutic strategy.

In addition to anti-microbial treatment, patients often require intensive support, including appropriate vasopressors, oxygen, and other supportive therapy, because of the disease’s severity and rapid onset. This is
particularly true in patients diagnosed during the later stages of disease progression. Recommendations for treatment during pregnancy and for pediatric populations follow similar guidelines [277], [278].

Because anthrax is a relatively rare disease in humans, much of the data accumulated regarding efficacious treatments have been collected using laboratory animal models. In addition to rodent models such as those described above, non-human primate models have been especially valuable. They have been used to evaluate the efficacy of specific antibiotics in treating anthrax, establish dose and pharmacokinetic parameters, and estimate the latest point in the course of disease when treatment can be initiated and still influence outcome [292]-[297]. Early studies demonstrated the efficacy of antibiotics, such as penicillin, in treating anthrax and also showed that antibiotics are active only after spores have germinated [78], [293], [298]. Later groups demonstrated efficacy attributable to antibiotics such as the tetracyclines and fluoroquinolones [292], [295], [297], [299]. However, it was apparent that residual spores can persist after antibiotics are discontinued and may subsequently germinate and cause disease. Animal experiments to include those with non-human primates have confirmed the prolonged persistence of spores after aerosol exposure and a potential extended period before germination [292], [294]. In one study, rhesus macaques were protected during a 30-day course of antibiotics after aerosol challenge. However, some animals developed fatal infection after the antibiotics were discontinued [292]. Thus complete protection against high doses of aerosolized spores required a prolonged period of treatment [289], [292], [294], [300], [301].

10.5.2 Non-Antibiotic and Combination Therapeutics

Antibiotics remain the primary treatment of individuals with inhalational anthrax; however new countermeasures, alone or in combination with antibiotics, are being explored. These modalities are being developed to reduce the duration of antibiotic treatment, to treat disease due to antibiotic-resistant strains, and to better counter the toxin-associated later stages of infection against which anti-microbials are ineffective. Since prolonged antibiotic treatment can lead to increases in adverse side-effects and a decline in adherence rates among humans, modifications of this treatment scheme, or alternate non-antibiotic treatment strategies, have been investigated in. For instance, Vietri et al. determined that a short course of ciprofloxacin treatment alone, when given after development of symptoms (i.e. therapeutically), instead of prophylactically, could effectively treat inhalational anthrax and prevent disease caused by the germination of spores after discontinuation of antibiotics. In a symptomatic individual, bacterial replication will probably have occurred before the beginning of antibiotic treatment and the immune response generated should protect against disease resulting from subsequent spore germination. However, this approach can be risky since it is dependent on the ability to detect and treat an active infection in time to insure patient recovery. Several groups are evaluating the protective efficacy of monoclonal antibodies specific for the anthrax toxin components given pre- or post-exposure. For example, Vitale et al. reported that a fully human monoclonal anti-PA antibody was effective prophylactically, and partially protective when given post-symptomatically (≤ 48 h), to rabbits exposed to aerosolized B. anthracis Ames spores. One dose given 1 h after exposure also protected cynomolgus monkeys challenged with aerosolized spores [302]. Additional studies are required to determine the extent and kinetics of post-symptomatic protection and the duration of protection with this antibody. Migone and co-workers evaluated the efficacy of a human anti-PA monoclonal as a prophylactic agent and therapeutic in monkeys. A single dose of the anti-PA increased the survival compared to untreated controls when given either prophylactically or post-symptomatically (i.e. after PA and bacteria were detected in blood) to cynomolgus monkeys exposed by aerosol to Ames strain spores; survival beyond the 28 d period of the study was not determined [303]; similar protective results were reported by Pitt et al. using a different human anti-PA monoclonal and the African Green monkey model [304]. Enhanced efficacy and broader protection has been reported by using combinations of PA and LF monoclonal antibodies [305]-[307]. Additionally, passive protection directed against the capsule [123], [308], [309] or spore [310] might also be useful as supplemental therapeutics. Alternatively, interfering with the binding of PA to its cell
A unique approach by Rivera et al. utilized radiolabeled monoclonal antibodies as a radioimmunotherapeutic method [312].

Passive protection strategies have resulted in the development of Anthrax Immune Globulin (AIG) by Cangene, Corp. AIG is an investigational product derived from the plasma obtained from human donors previously immunized with the licensed Anthrax Vaccine (AVA). This product contains antibodies specific for B. anthracis. The majority of these antibodies have been shown to be anti-PA antibodies. Thus, administration of this product would passively protect patients from toxemia associated with anthrax. Under a contract to the U.S. government, Cangene Corp. has supplied this product to the U.S. Strategic National Stockpile (www.cangen.com/biodefense_products.shtml).

Combination regimens given pre- or post-exposure are being explored as viable treatment strategies. Pre- or post-exposure vaccination when combined with antibiotics improved efficacy [292], [294], [313], and, as shown in later studies, potentially enabled a shorter course of antibiotic treatment [314]. Using the rhesus macaque model of inhalational anthrax, Vietri et al. demonstrated that NHP administered post-exposure ciprofloxacin for 14 days had a high mortality rate after antibiotics were discontinued. Adding the AVA vaccine to post-exposure antibiotic prophylaxis enhanced the protection afforded by just 14 days of ciprofloxacin prophylaxis alone and completely protected animals against inhalational anthrax [314]. Klinman and Tross combined antibiotic treatment with Dalbavancin, a long-acting antibiotic, with vaccination with AVA BioThrax™ supplemented with CpG to more rapidly elicit an immune response in mice [315]. An additional approach to post-exposure prophylaxis involves combining anti-toxin antibodies with antibiotics to target both the toxin and the organism [316]. Similar studies evaluating such combination strategies in non-human primate models are currently being evaluated. Dyer et al. developed a therapeutic African Green monkey model for inhalational anthrax for use in demonstrating specifically the value of combination therapies. In this model, African Green monkeys were treated with IV ciprofloxacin at various times after PA was detected in the blood; a delay in treatment resulted in reduced survival and thus permitted the demonstration of added benefit of adjunctive therapies such as antibodies [317]. Using a similar scheme, Henning et al. assessed the efficacy of a monoclonal anti-PA antibody given in combination with ciprofloxacin in a cynomolgus macaque therapeutic model [318]. Antibiotic alone or in combination with the monoclonal anti-PA was given 24 h after confirmed bacteremia and toxemia and, whereas 62% of the animals given the combination therapy survived, only 15% of those given antibiotic alone lived. However, notwithstanding these positive results, the addition of anti-toxin antibodies to an antibiotic regimen might not be optimal for the prevention of anthrax resulting from the residual spores that may germinate after discontinuation of the antibiotic, and repeated doses of antibodies may be required. Finally, innovative approaches including the use of fragments of PA as competitive inhibitors [319], dominant-negative PA mutants that interfere with oligomerization [320], soluble receptors to act as decoys for PA [321], and identification of peptide analogs that inhibit the enzymatic activity of LF [322], [323] are being investigated.

10.6 ACKNOWLEDGEMENTS

This work was supported by the Defense Threat Reduction Agency, Joint Science and Technology Office for Chemical–Biological Defense.

10.7 REFERENCES


ANTHRAX AND BACILLUS ANTHRACIS


ANTHRAX AND BACILLUS ANTHRACIS

[38] Bozue J, et al. Bacillus anthracis spores of the bclA mutant exhibit increased adherence to epithelial cells, fibroblasts, and endothelial cells but not to macrophages. Infect Immun 2007;75(9):4498-505.


ANTHRAX AND BACILLUS ANTHRACIS


ANTHRAX AND BACILLUS ANTHRACIS


ANTHRAX AND BACILLUS ANTHRACIS


[183] Dyson EH, et al. An ascending dose study to assess the safety and tolerability of an rPA anthrax vaccine, and compare its immunogenicity with that of Anthrax Vaccine Adsorbed (AVA), in 46th Annual ICAAC meeting. 2006: San Francisco, CA, USA.


ANTHRAX AND BACILLUS ANTHRACIS


ANTHRAX AND BACILLUS ANTHRACIS


ANTHRAX AND BACILLUS ANTHRACIS


[304] Pitt ML, et al. Therapeutic efficacy of Valortim, an anti-toxin monoclonal antibody, in the African green monkey model of inhalational anthrax, in Bacillus-ACT The international conference on Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis. 2009: Santa Fe, NM, USA.


Disclaimer: Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. Research on human subjects was conducted in compliance with U.S. Department of Defense, federal, and state statutes and regulations relating to the protection of human subjects and adheres to principles identified in the Belmont Report (1979). All data and human subjects research were gathered and conducted for this publication under an IRB-approved protocol, number FY09-03.
Chapter 11 – YERSINIA PESTIS

Patricia L. Worsham
Division of Bacteriology
U.S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, MD
UNITED STATES
Corresponding author (Worsham): patricia.l.worsham.civ@mail.mil

11.1 INTRODUCTION

Plague, a severe febrile illness caused by the Gram-negative bacterium *Yersinia pestis*, is a zoonosis usually transmitted by fleabites. It is foremost a disease of rodents; over 200 species are reservoirs of *Y. pestis* [119], [104]. When fleas feed on a bacteremic animal, the organism is taken with the blood meal into the midgut of the flea where it multiplies, eventually forming a mass of aggregated bacteria that blocks the proventriculus, a valve-like structure leading to the midgut. This blockage starves the flea, which then makes repeated, desperate attempts to feed. Because of the blockage, blood carrying *Y. pestis* is regurgitated into the bite wounds, thus spreading the disease to new hosts. The blocked flea, also a victim of the disease, eventually starves to death [61]. Most often, humans become infected by fleabite during an epizootic event. Less frequently, human disease is a result of contact with blood or tissues of infected animals (including ingestion of raw or undercooked meat), or exposure to aerosol droplets containing the organism [104], [126]. Infectious aerosols can be generated by humans or animals with plague pneumonia, particularly cats. The most commonly used virulent strain of *Y. pestis* in the U.S., CO92, was isolated from a patient who developed fatal pneumonic disease after being exposed to an infected domestic cat [41].

In humans, plague is generally classified as bubonic, septicemic, or pneumonic. *Y. pestis* is a lymphotrophic pathogen. Thus, in bubonic plague, it migrates from the site of entry to the regional lymph nodes, where it multiplies and forms a bubo, the exquisitely painful enlarged node that is the hallmark of the disease. The bubo is packed with bacilli and is often accompanied by an overlaying edema. At times, bubonic plague leads to bacteremia and hematogenous spread to other organs including the liver, spleen, lungs, and, less commonly, the meninges [104], [150]. Cases of plague bacteremia without obvious lymphadenopathy are termed septicemic plague [24], [126], [150]. A small percentage of plague patients develop pneumonic plague secondary to bubonic or septicemic plague and these individuals are capable of spreading the disease directly to other humans. Primary pneumonic plague, acquired by inhaling infectious aerosols generated by the coughing of a plague-infected person or animal, is rare but rapidly fatal [24], [150]. It appears that pharyngeal plague can be acquired by ingesting or inhaling the organism. In some cases, this form of the disease appears to be asymptomatic [103], [24], [126], [104].

*Yersinia pestis* is a Gram-negative coccobacillus belonging to the family *Enterobacteriaceae*. The genus was named in honor of Alexandre Yersin, the scientist who originally isolated *Y. pestis* during a plague outbreak in Hong Kong in 1894; the species name *pestis* is derived from the Latin for plague or pestilence. Previous designations for this species have included *Bacterium pestis*, *Bacillus pestis*, *Pasteurella pestis*, and *Pesticella pestis* [16]. This species is closely related to two other pathogens of the genus *Yersinia*, *Y. pseudotuberculosis* and *Y. enterocolitica*. The extensive genetic similarity (> 90%) between *Y. pseudotuberculosis* and *Y. pestis* led to a recommendation that *Y. pestis* be reclassified as a sub-species of *Y. pseudotuberculosis* [17]. This proposal was not well received, primarily due to fear that this change in nomenclature would increase the potential for
laboratory-acquired infections. Molecular fingerprinting analysis of *Y. pestis* suggests that this pathogen arose from *Y. pseudotuberculosis* through microevolution over millennia [1], [2].

*Y. pestis* is a facultative anaerobe, fermenting glucose with the production of acid. An obligate pathogen, it is incapable of a long-term saprophytic existence, due in part to complex nutritional requirements, including a number of amino acids and vitamins. It also lacks certain enzymes of intermediary metabolism that are functional in the closely related but more rapidly growing species such as *Y. enterocolitica* or *Y. pseudotuberculosis*. *Y. pestis* strains have traditionally been separated into three biovars, based on the ability to reduce nitrate and ferment glycerol [99]. Some molecular methods of typing, such as ribotyping and restriction fragment length polymorphisms of insertion sequence locations, support this division of strains [59], [2]. Biovar orientalis (Gly+, Nit+) is distributed worldwide and is responsible for the third (Modern) plague pandemic. It is the only biovar present in North and South America. Biovar antiqua (Gly−, Nit+) is found in Central Asia and Africa and may represent the most ancient of the biovars [99], [2]. Biovar mediaevalis (Gly+/Nit−) is geographically limited to the region surrounding the Caspian Sea. There are no apparent differences in pathogenicity among the biovars [99], [152].

### 11.2 GENOMICS AND MOLECULAR FINGERPRINTING

The microevolution of *Y. pestis* was investigated by three different multilocus molecular methods. Eight populations were recognized by the three methods and an evolutionary tree for these populations, rooted on *Y. pseudotuberculosis*, was proposed. The eight population groups do not correspond directly to the biovars; thus, it was suggested that future strain groupings be rooted in molecular typing. Four of the groups were made up of transitional strains of *Y. pestis*, “Pestoides,” that exhibit biochemical characteristics of both *Y. pestis* and *Y. pseudotuberculosis* [151]. These isolates represent the most ancient of the *Y. pestis* strains characterized to date [2]. The isolation of *Y. pestis* Pestoides from both Africa and Asia suggests that *Y. pestis* spread globally long before the first documented plague of Justinian in 784 AD. Recently, a large and diverse *Y. pestis* collection was assayed for the presence of 933 distinct Single Nucleotide Polymorphisms (SNPs); this study suggests that the *Y. pestis* strains in the United States and Madagascar each arose from a single radiation from China, while there appear to have been multiple radiations from China to Europe, South America, Africa, and Southeast Asia [92]. Comparative genomics is a tool used with increasing frequency to evaluate plague outbreaks and determine the evolutionary trajectory of the strains [56]. Sequences of several strains of *Y. pestis* have been reported; these data confirm the clonal nature of the organism and the ancient derivation of the Pestoides isolates. A draft genome of the *Y. pestis* responsible for the Black Death was derived from victim remains dated to the London outbreak in 1348 – 1350; they concluded that there was no evidence of greater virulence of that strain (as has been speculated). Rather, it appears that other factors such as the environment, host susceptibility and role of the vector itself may have contributed to the apparent high infectivity and mortality [18].

#### 11.2.1 Use as a Bioweapon

The first attempt at what we now call “biological warfare” is purported to have occurred at the Crimean port city of Caffa on the Black Sea during the years 1346 – 1347 [86]. During the conflict between Genoese sailors and Tatars, the Tatar army was struck with plague. The Tatar leader catapulted corpses of plague victims at the Genoese. Subsequently, the Genoese became infected with plague and fled to Italy. However, the disease was most likely spread by the local population of infected rats, not by the corpses, since an infected flea leaves its host as soon as the corpse cools.

During World War II, the Japanese army established a secret biological warfare research unit (Unit 731) in Manchuria. Early experiments with the plague bacillus demonstrated that dropping bacteria within aerial bombs
was ineffective because the air pressure and high temperatures that were created by the exploding bombs killed nearly all of the bacteria [142]. However, they were successful in using the human flea, *Pulex irritans*, as a stratagem to simultaneously protect the bacteria and target humans. The fleas targeted humans, and could also infect a local rat population to prolong an epidemic. Using clay bombs resulted in an 80% survival rate of fleas when dropped from aircraft. Other items were dropped from the air by Japanese planes in 1940 and 1941; these included wheat and rice grains, pieces of paper, cotton wadding, and other unidentified particles. The release of these items from the planes was associated with subsequent outbreaks of plague that began in the human population. Unusually, rodent mortality was not noted until months later or was not observed at all. These outbreaks occurred in Changteh and Chuhsien, which were not plague endemic areas.

In 1999, Dr. Ken Alibek (Kanatjan Alibekov), a former Soviet army colonel and scientist, published a book entitled *Biohazard* that described the extensive biological weapons program of the former Soviet Union [4]. He explained the weaponization of *Y. pestis* (including a powdered form) and the development of genetically engineered organisms, one of which was a *Yersinia* strain producing “myelin toxin” that induced both disease and paralysis in animal models. Alibek states that “In the city of Kirov, we maintained a quota of twenty tons of plague in our arsenal every year”. Although the accuracy of details presented in the memoir has been a topic of debate in some circles, the former Soviet Union is well known to have had entire institutes devoted to the study of *Y. pestis*. Other state-sponsored or extremist group efforts to obtain biological weapons will likely consider plague as well.

### 11.2.2 Host-Parasite Relationships

The persistence of plague in endemic areas requires cyclic transmission between rodents and fleas; thus, *Y. pestis* has evolved to survive and replicate in two very different hosts. To maintain the transmission cycle, *Y. pestis* must multiply within the flea sufficiently to cause blockage and promote the infection of a new mammalian host. Equally critical is the ability to establish an infection and induce a sufficient bacteremia in the mammal to infect fleas during the blood meal. The milieu of the mammalian host is radically different from that of the midgut of the flea, yet, clearly, the organism successfully adapts to each host to complete its life cycle. The adaptation takes place through environmental regulation of virulence factors. For example, gene products necessary for growth in the flea are expressed most efficiently at the body temperature of this host; presumably additional factors also cue the organism to recognize this environment and respond appropriately. Likewise, genes required for replication in the mammalian host are expressed at highest levels at 37°C, and the synthesis of some proteins, thought to be induced in the phagolysosome, is also regulated by pH. In the laboratory, the synthesis and secretion of certain essential virulence factors are controlled by both growth temperature and Ca++ concentration; the induction of these proteins has been termed the low calcium response [61], [99], [73], [123]. Interestingly, recent evidence suggests that *Y. pestis* passaged through fleas is better able to overcome mammalian innate immunity than *Y. pestis* cultured *in vitro* [131].

There is increasing evidence that there are two phases in *Y. pestis* infection of the mammalian host. It appears that *Y. pestis* is unusual in its ability to create a localized anti-inflammatory state that allows for survival and growth of the organism in the lung early in infection. Co-infection experiments using wild-type *Y. pestis* and mutants of low virulence suggest that this environment is also conducive to the growth of organisms that are normally attenuated. When the host does respond following this early unrestricted growth of *Y. pestis*, the resulting massive inflammatory response is characterized by high levels of pro-inflammatory cytokines and acute septic shock. This response is ultimately detrimental to the host [105]. Similarly, in a bubonic model, transcription of cytokines is low in the developing bubo even with a large bacterial burden in the lymph node and bacteria entering the bloodstream. The Type Three Secretion System (TTSS) is partially responsible for the anti-inflammatory activity, likely by impacting PMN recruitment and/or function, but other *Y. pestis* virulence
factors appear to be involved [30]. One of these may be the Ail protein, which is involved in protection of the bacterium from complement-mediated lysis and also appears to inhibit PMN recruitment in a bubonic plague model [63].

Cytokines, particularly Tumor Necrosis Factor α (TNFα) and Interferon γ (IFNγ), contribute to innate immunity to plague. Injection of TNFα and IFNγ after initial growth of the organism in the spleen to 10^3 CFU diminished bacterial tissue burden and promoted survival of mice [95]. Both of these cytokines are known to upregulate activity of phagocytic cells. The importance of Type 1 cytokines in protection against plague was also demonstrated in the STAT 4 (-/-) mouse model where Interleukin 12 (IL-12) and IFNγ-mediated immune mechanisms are inactive. This mouse strain was not protected from challenge even after passive transfer of CD4(+) cells from immunized BALB/c donors [44]. In several studies, TNFα and IFNγ appeared to contribute to immune defence in vaccinated animals, as well as passively protected animals [95], [98], [76], [81], [157]. The contribution to immunity of these cytokines and other components of the innate response may explain, in part, the difficulty in correlating antibody titers with protection across animal models [81].

The institute Pasteur has identified a strain of mouse with significant resistance to wild-type Y. pestis. SEG mice have early systemic dissemination of the organism and a rapid innate response. The LD₅₀ of strain CO92 in this animal using a subcutaneous route of infection is > 10^7 CFU (compared to < 10 CFU in other murine models). Surprisingly, SEG mice exhibited a more rapid dissemination than the C57BL/6 strain. This systemic disease was of short duration but resulted in early colonization of liver, spleen, and lung. Colonization was associated with rapid cytokine release. The induced cytokines Granulocyte Colony Stimulating Factor (G-CSF), Keratinocyte-derived Chemokine (KC), Macrophage Cationic Peptide-1 (MCP-1), Interleukin 1α (IL-1α), and Interleukin 6 (IL-6) are associated with somatic cells (endothelial cells, fibroblasts, and keratinocytes) as well as bone-marrow-derived cell lines. MCP-1, G-CSF, and IL-6 may be responsible for the rapid appearance of PMN and monocytes in the SEG mice. The F4/80+ CD11b- macrophages in the spleen rapidly expanded in the SEG mice early in infection; these phagocytes are part of the innate response to bacterial pathogens in the septicemic animal.

In contrast, systemic disease in the C57BL/6 mice was delayed but continued until the death of the animal [37].

Insight into the protein-protein interactions of Y. pestis and mammalian cells is another approach to studying the host-pathogen interaction. Human proteins targeted by Y. pestis are generally those involved in signaling pathways such as those controlling the cytoskeleton, focal adhesion, TLR signaling and MAPK signaling. These pathways influence the expression of innate immunity, including those involved in phagocytosis [155]. A Toll/Interleukin 1 Receptor (TIR)-domain protein of Y. pestis was identified via bioinformatics; the purified protein appears to interact with MyD88 and, thus, may inhibit intracellular signaling from TLR. However, the role of this protein in pathogenesis is currently unknown [110].

### 11.2.3 Identification of Virulence Factors

Genetic analyses, both traditional and molecular, have been essential in unraveling the unique qualities that make Y. pestis a successful pathogen in both the flea and the mammalian host. Common molecular approaches include site-directed mutagenesis, and transposon mutagenesis. Curing of the native plasmids also contributed essential information.

Methods designed to identify gene expression of the pathogen inside the host (or the phagocytic cell), such as Transposon Site Hybridization (TraSH) and In Vivo-Induced Antigen Technology (IVIAT) have been useful in identifying potential new targets for medical countermeasures in Y. pestis and other pathogens. Andrews et al. [10] identified 20 proteins expressed in vivo that reacted with immune sera; these included Type V autotransporters.
and components of Type III and Type VI secretion systems. The outer membrane protein OmpA was recently identified as an essential virulence factor using TraSH [14]. Similarly, the locus galU (associated with antimicrobial peptide resistance) was identified in co-culture with primary murine macrophages [71].

Protein microarrays were used to identify antibody responses in pooled plague sera, either absorbed using laboratory-grown organisms or unabsorbed. In this manner, proteins were identified that were growth temperature regulated but not expressed in typical laboratory growth conditions. This is another promising method for identifying virulence factors and potential protective antigens produced in the host that are not observed in vitro [80].

*In silico* whole-genome transcriptomic analyses comparing gene expression in *Y. pestis* and *Y. pseudotuberculosis* revealed that a number of virulence-associated genes, including genes encoded by the *Yersinia* virulence plasmid, were expressed at a higher level in *Y. pestis* than in the progenitor organism *Y. pseudotuberculosis*. In addition, the *ail* locus and genes within the High Pathogenicity Island also were transcribed more efficiently in *Y. pestis*. Furthermore, the induction of pYV-associated genes at 37°C was significantly higher in *Y. pestis* [27]. This study emphasizes the importance of regulatory network changes in the evolution of *Y. pestis* from *Y. pseudotuberculosis*.

### 11.2.4 Virulence Mechanisms

Most strains of *Y. pestis* carry three plasmids two of which are unique to this species: pMT (or pFra), which encodes the F1 capsule and pPCP, which carries the gene for the virulence factor plasminogen activator. The third plasmid is common to the human pathogenic *Yersinia* species and is known as pCD (calcium dependence), pYV (*Yersinia* virulence), or pLcr (low calcium response). This plasmid, which is responsible for the synthesis of a number of anti-host factors, is an absolute requirement for virulence [99]. The contribution of these plasmids to virulence will be described below.

**Type III Secretion System** – Like a number of other Gram-negative pathogens, the human pathogenic *Yersinia* species possess a Type III secretion system that enables an organism in close contact to host cells to deliver toxic proteins directly into the eukaryotic cell cytosol [31], [32]. In the case of the *Yersinia* species, this system is encoded on the pYV plasmid, which encodes the components of the Low Calcium Response (LCR). Toxic activities of the LCR effector proteins, designated Yops (*Yersinia* outer protein), include disruption of the cytoskeleton, interference with phagocytic activity, prevention of pro-inflammatory cytokine synthesis, inhibition of the oxidative burst, and induction of programmed cell death (apoptosis). Yop delivery is necessary for growth of *Y. pestis* in the liver and spleen [101]. Specifically, YopM appears to induce a global depletion of Natural Killer (NK) cells, while YopH, a protein tyrosine phosphatase, inhibits host cell phagocytosis by dephosphorylating several focal adhesive proteins and inhibiting calcium signaling in neutrophils. YopE, YpkA and YopT are also antiphagocytic; these toxins act to inhibit cytoskeletal mobilization. YopJ plays an anti-inflammatory role by inhibiting inflammatory cytokine production and inducing apoptosis in macrophages [123], [31], [69]. Overall, the effect is that of paralyzing professional phagocytes. It is clear why the pathogen-host interaction mediated by the Type III secretion system has been designated the “*Yersinia* Deadly Kiss”.

Worthy of note is another virulence factor associated with the Type III secretion system. LcrV (historically known as V “virulence” antigen) is an important protective immunogen in new-generation plague vaccines. This protein serves a number of roles for the pathogen: as regulator of Yop transcription, for translocation of Yops into the host cell, and as a virulence factor in its own right [99], [123]. LcrV appears to stimulate production of the immunosuppressive cytokine interleukin 10 (IL-10) through interactions with Toll-like receptors 2 and 6 as well as CD14 signaling. These effects appear to be mediated by the N-terminal portion of...
YERSINIA PESTIS

LcrV [117], [97]. Repression of pro-inflammatory cytokines is presumed to be a result of the IL-10 induction. In addition, LcrV released from the cell appears to interact directly with IFN-γ and may contribute to immunosuppression through this binding [55].

The secretion mechanism includes an “injectisome” that can be visualized as a needle-like structure using electron microscopy. The Type III secretion injectisome consists of a cylindrical basal structure spanning the two bacterial membranes and the peptidoglycan, connected to a hollow “needle” [40], [32]. The needle is tipped by a structure that allows formation of pores in the host cell membrane and the length of the needle is governed by a protein deemed the “molecular ruler”. At body temperature, the secretion apparatus is synthesized on the outer surface of the bacterial cell. Contact with the host cell induces transcription of the Yops and opens this secretion channel that allows the Yops to be translocated through the membrane and into the host cell [40], [32]. YopK (YopQ) controls the rate of Yop injection from within the host cell [39]. Under certain environmental conditions, proteins with adhesin activity (Ail, Pla, Psa) appear to facilitate Yop delivery [49].

F1 Capsular Antigen – The F1 capsule, encoded by the largest plasmid of Y. pestis (pMT), is produced in large quantities by Y. pestis in vivo and when cultured in the laboratory at 37°C. This gelatinous envelope is generally thought to protect the organism from host phagocytic cells by interfering at the level of receptor interaction in the phagocytosis process [43]. It likely acts in concert with the Type III secretion system to provide Y. pestis with protection from phagocytes. Although the vast majority of natural isolates produce the capsular antigen, F1-negative strains have been isolated from rodent hosts and reported from one human case [148], [137], [149], [9]. In the laboratory, spontaneous mutants defective in F1 production have been obtained from immunized animals, from cultures treated with anti-serum containing F1 antibody, and from chronically infected rodents [137], [149], [9]. Examination of isogenic F1 positive/negative strain pairs revealed that F1 is not an absolute requirement for virulence in the mouse and the African green monkey models, including aerosol models, although mutations leading to loss of the capsular antigen do lead to an increase in time to death in the mouse [149], [35]. Older studies suggesting a role of F1 in the infection of guinea pigs and rats utilized F1-negative strains that were not genetically defined and, thus, are more difficult to interpret. However, they suggest that the importance of F1 in pathogenesis may vary with the species of the host. The fact that F1-negative strains are relatively rare among natural isolates suggests that the capsular antigen, or other gene products encoded by this plasmid, may play an important role in the maintenance of the disease in animal reservoirs. Historically, F1 has been of immense importance as a diagnostic reagent, as it is specific to Y. pestis. It is the major antigen recognized in convalescent sera of humans and rodents [15], [23]. It is also a highly effective protective immunogen.

Plasminogen Activator – The virulence factor Plasminogen activator (Pla) is encoded by a 9.5 kb plasmid, pPCP1, unique to Y. pestis. Inactivation of the pla gene leads to a significant attenuation of virulence from a subcutaneous but not an intraperitoneal or intravenous route of infection in mice, suggesting that Pla promotes dissemination of the organism from peripheral sites of infection and plasminogen-deficient mice are 100-fold more resistant to Y. pestis than normal mice [99], [57]. Although Pla is necessary for full virulence in some Y. pestis strains, a few strains that are Pla- and appear to be fully virulent have been identified among natural isolates or generated in the laboratory [138], [139], [151]. Presumably, these isolates synthesize other proteins that substitute for Pla function.

Fimbrae – The so-called pH 6 antigen is a fimbrial structure on the surface of Y. pestis that is necessary for full virulence in the mouse model. It has been proposed that pH 6 antigen mediates attachment of the organism to host cells via binding to glycosphingolipids. The temperature and pH of the environment tightly control the biosynthesis of these fimbrae; the expression of pH 6 antigen is most efficient in vitro with a growth temperature between 35 – 41°C and a pH range of 5.0 – 6.7. This suggests that, in vivo, the adhesin activity is likely to be
expressed only in specific microenvironments such as the phagolysosome, necrotic tissue, or in an abscess. Indeed, intracellular association with macrophages in the laboratory induces synthesis of the fimbrae [156]. More recent data, however, suggests that the pH 6 antigen does not enhance adhesion to mouse macrophages, but rather promotes resistance to phagocytosis [64]. Recent data suggest that this protein is not an essential virulence factor in wild-type Y. pestis; it is thought that the use of laboratory-passaged strains may have influenced the results of previous studies [12]. Alternatively, there may be redundancy of some functions in Y. pestis as implied by the work of Felek et al. (2010) [49].

Iron Sequestration – Acquisition of nutrients in the host is an essential part of pathogenesis. In the mammalian host, iron is sequestered from invading pathogens; therefore, the level of free iron in the extracellular milieu is less than that necessary for bacterial growth. Like many bacteria, Y. pestis possesses a high-affinity iron uptake system that is capable of procuring this essential nutrient from the host. Strains that do not produce the low molecular weight iron chelator, known as yersiniabactin, or those unable to transport yersiniabactin are highly attenuated by subcutaneous route of infection and somewhat affected in pneumonic models as well. Such strains are capable, however, of infecting via the intravenous route (septicemic model). The genes encoding this iron transport system are situated on a chromosomal pathogenicity island known as the Pigmentation locus (Pgm) [100], [139].

Phage Shock Protein Response (PSP) – The PSP is almost ubiquitous among microbes; homologues are found in numerous Gram-positive and Gram-negative bacteria, as well as archaeabacteria and even chloroplasts. This regulon appears to respond to environmental stressors, including disturbances in the cell envelope and changes in the proton motive force that are induced by impaired inner membrane integrity (reviewed by Darwin [34], [158]. For pathogens, environmental stressors triggering the PSP regulon likely include environments within the host and, indeed, the PSP response is associated with virulence in Salmonella enterica, Y. enterocolitica, Y. pseudotuberculosis, and Y. pestis [34], [93], [68]. For Y. pestis, it is required for virulence by both the aerosol and subcutaneous routes of infection [93].

Twin Arginine Transport (Tat) – Gram-negative bacteria have numerous ways to transport molecules across their membranes. One of these mechanisms is the Tat pathway. The Tat pathway secretes folded proteins that are identified by an N-terminal signal peptide containing a twin arginine motif across the inner membrane. The TatA (Twin Arginine Transport) gene product is an important virulence factor of Y. pestis in both parenteral and aerosol models of infection. TatA mutants exhibit defective secretion/assembly of the F1 capsule on the cell surface. This demonstrates that the twin-arginine transport system is associated with secretion and assembly of a known virulence factor associated with the cell surface. However, the attenuation of Y. pestis tatA mutants cannot be explained by the defect in F1 synthesis; the tatA mutant is much more attenuated than mutants affected in the capsular synthetic genes per se [21].

Surface Structures – Bacterial surface structures such as porins and phage receptors have been implicated in virulence. Omp A, a major outer membrane porin was identified as an in vivo-expressed protein and subsequently proven to be essential for virulence [14]. Receptors for Y. pestis-specific bacteriophage also play an important role in virulence; these tend to be associated with various portions of the Lipopolysaccharide (LPS) inner and outer core [52].

Small RNAs – Post-transcriptional control of virulence determinant expression by small RNAs was recently documented in Y. pseudotuberculosis. Most of these are shared with Y. pestis, although expression varied between the two pathogens. Identification of regulons governed by small RNAs may lead to identification of virulence factors previously unknown [72].
11.3 VACCINES

The first plague vaccine, consisting of killed whole cells, was developed in 1897. In the 1940s, the U.S. Army began developing an immunogenic and less-reactogenic vaccine from an agar-grown, formalin-killed, suspension of virulent plague bacilli. With minor modifications, this is the same procedure that was used to prepare the licensed vaccine, Plague Vaccine U.S.P. Plague Vaccine – U.S.P. (also known as the Cutter vaccine) was routinely given to military personnel stationed in Vietnam, and other individuals such as field personnel working in plague endemic areas with exposure to rats and fleas and laboratory personnel working with Y. pestis. However, this vaccine was discontinued by its manufacturers in 1999 and is no longer available. Although Plague Vaccine U.S.P. was effective in preventing or ameliorating bubonic disease, as seen by the remarkably low incidence of plague in U.S. military personnel serving in Vietnam [26], data from animal studies suggest that this vaccine did not protect against pneumonic plague [102]. A similar vaccine is still manufactured in Australia.

The former Soviet Union and many other Nations have traditionally focused on live attenuated vaccines, with tens of millions of humans receiving the live plague vaccine. Many investigators continue to believe that live attenuated vaccines are preferable to sub-unit vaccines. Live plague vaccines have been used alone and also with considerable success in a prime-boost strategy with sub-unit vaccines. Even the most recent reviews on plague vaccination continue to revisit the appeal of live attenuated vaccines [51]. Generally, live attenuated vaccines bestow a longer immunity with fewer doses than sub-unit vaccines. Presumably, live vaccines provide additional, currently undefined, antigens that enhance immunity. Furthermore, the living organism interacts directly with immune cells in what is essentially a low-grade infection; this better reflects the natural host-pathogen interaction than a sub-unit vaccine combined with an adjuvant. Because of this, a live vaccine may stimulate T-cell-based immunity. Although the importance of antibody in plague immunity is well documented, a number of studies also support the role of cellular immunity in protection against plague. Animals immunized with live vaccine preparations have survived Y. pestis challenge with very little measurable antibody. A recent extensive review of both recombinant and live plague vaccines is available [51].

Recombinant Vaccines – Recombinant plague sub-unit vaccine development has largely centered on two antigens: F1 and V. F1, the capsular antigen of Y. pestis, appears to prevent phagocytosis of plague bacilli, while V antigen has a key role in the translocation of the cytotoxic Yops into host cells, as well as stimulating the production of immunosuppressive cytokines [94]. These antigens have been studied as both a co-mixture (F1+V) and a fusion protein (F1-V). The F1-V vaccine, currently in clinical trials, consists of a recombinant fusion protein purified from E. coli and was developed by Army scientists at the U.S. Army Medical Research Institute of Infectious Diseases [60], [8]. A similar candidate, developed at Porton Down, the biodefense laboratory in the United Kingdom, is a recombinant protein-based vaccine, consisting of two separate proteins F1 and V [143], [144], [145]. The separate proteins are then combined (2 parts F1 to 1 part V) to form a sub-unit vaccine. This vaccine candidate was demonstrated to be safe and immunogenic in clinical trials [146]. In some cases, an altered form of V lacking the immunomodulating function has been used in vaccine studies and appears to retain antigenicity [108].

The ability of the F1 and V antigens, specifically F1-V, to stimulate a robust humoral response is demonstrated by studies where it was used as a part of a heterologous display platform to improve immunogenicity of other antigens. F1-V itself was fused to an HIV-1 envelope residue and, when part of a prime-boost protocol with a cholera toxin B sub-unit fused with that same HIV residue, significantly enhanced the humoral immune response to the HIV envelope protein [85].

In addition to the protein sub-unit vaccines described above, many studies have used the F1 and V antigens in other formulations that are designed to decrease the requirement for boosters and/or stimulate mucosal
immunity. An F1-V nanoparticle-based vaccine platform administered intranasally to mice is reported to have provided protection with a single immunization. This vaccine contained both soluble F1-V as well as F1-V within nanoparticles; both were required for maximal protection. The small number of animals per group in this study is of concern, however [130]. F1 and V have been displayed on the capsid of adenovirus vectors defective in replication; these vectors infect antigen-presenting cells such as dendritic cells and, thus, may produce a more robust immune response [20]. V antigen epitopes coupled to palmitate and encapsulated in polylactide-co-glycolide microspheres were used to immunize mice intranasally; this produced a significant IgG response in the lung [159]. A DNA vaccine was adjuvanted with the chemokine lymphotactin and administered parenterally; in this case, better protection was observed using an intramuscular rather than an intranasal route [154]. CpG ODN 2006 augmented the response to F1-V in mice [6].

Alternative expression systems have also been explored: F1 and V have been expressed in plants using all three known possible strategies: nuclear transformation, chloroplast transformation and plant-virus-based expression vectors and some success has been reported with oral administration of plant-based vaccines [36], [5]. Oral vaccines are attractive options due to the ease of administration.

**Live Vaccines** – The vast majority of live vaccines are derived from the attenuated *Y. pestis* strain EV76. Our own experience has confirmed that there are a number of EV76 strain variants in use across the world with significant variation in plasmid size and composition. The variation between strains is likely responsible for some of the conflicting data regarding vaccine efficacy and reactogenicity. EV76 is a pigmentation deficient strain of *Y. pestis*. The Pigmentation locus (Pgm) is an unstable pathogenicity island of approximately 100 kb encoding the yersiniabactin iron sequestration system, which is known to be required for virulence. Another gene product encoded within the Pgm locus is ripA, which appears to repress production of nitrous oxide by macrophages. While the deletion of the Pgm locus does lead to significant attenuation in EV76 strains, there are some potential drawbacks in using a Pgm strain as a live vaccine. First, the outer membrane receptor for yersiniabactin, Psn, has shown some promise as a protective antigen (outer membrane proteins associated with iron transport are among the most highly induced proteins *in vivo*). There are additional surface proteins encoded by Pgm that might also function as protective antigens. Secondly, there has been some concern that the predominant antigen seen by the host in an EV76 infection is the F1 capsular antigen. Antibody to F1 does not provide protection against strains lacking this antigen. An excellent review that discusses live vaccine development quite extensively is that of Fedodorova and Motin (2011) [51].

A recent study in rhesus macaques compared recombinant F1 + V with the EV76 vaccine. Both vaccination protocols protected these primates; however, V titers were poor in the EV76-vaccinated animals. IL-12 levels were higher with the live vaccine. It is unclear how this data reflects the human response [107]. Rhesus macaques are generally considered to be a poor model for plague [3].

A live oral vaccine consisting of a *Y. pseudotuberculosis* strain producing the *Y. pestis* capsular antigen was recently demonstrated to protect mice against both F1+ and F1- strains of *Y. pestis*. Both the F1 and other *Yersinia* antigens were recognized. Such a vaccine would be particularly useful in endemic areas [38].

It is important when developing new vaccines, particularly those designed to protect against biological threat agents, that we address the issue of vaccine resistance. One of the problems with the previously licensed plague vaccine is that the protective antigen in the preparation was primarily the F1 capsular antigen. Although the vaccine was protective against “typical” *Y. pestis* in many animal models, it did not protect against genetically engineered F1-negative strains. These strains maintained virulence despite the loss of capsule [149]. This example illustrates the importance of choosing essential virulence factors to be part of any new sub-unit vaccine. Genetic engineering of an organism to deliberately overcome a vaccine by removing a protective antigen will not produce an efficient pathogen if that protective antigen is an essential virulence factor.
With an F1-V-based vaccine, protection against F1 negatives strains relies on the V antigen component of the fusion protein. It is of some concern that there is evidence of V antigen heterogeneity within Yersinia species (Y. pestis versus Y. pseudotuberculosis versus Y. enterocolitica) and it appears that cross-protection is not complete [112]. Thus, a natural variant in lcrV or a genetically engineered strain expressing a V antigen distinct from the V antigen in the vaccine (particularly if F1-negative) could conceivably be “vaccine-resistant”. This makes a live vaccine expressing a number of potential protective antigens appealing. A Pgm+/F1-negative attenuated strain might present important antigens that the Pgm-strain does not (Psn and other Pgm-associated surface proteins) as well as presenting additional antigens typically masked by the F1 capsular antigen. Such a vaccine might address some of the concerns about natural or genetically engineered strains lacking F1 and/or expressing an aberrant V antigen. Alternatively, additional protective antigens necessary for virulence could be included with an F1-V-based vaccine.

11.4 THERAPEUTICS

Antibiotics – Since 1948, streptomycin has remained the treatment of choice for bubonic, septicemic, and pneumonic plague and is FDA-approved for the treatment of plague. However, streptomycin is rarely used in the United States and supplies of this antibiotic are limited [7]. Although not approved by the FDA for the treatment of plague, the Working Group on Civilian Biodefense recommends gentamicin as an alternative to streptomycin. Although there are no controlled comparative trials for therapy for plague infections, a recent review of 75 cases of human plague in New Mexico demonstrated that gentamicin alone or in combination with a tetracycline, was as efficacious as streptomycin for treating humans infected with plague [19]. Alternate regimens recommended by the Working Group on Civilian Biodefense include doxycycline, ciprofloxacin or chloramphenicol [65]. Chloramphenicol is indicated for conditions in which high tissue penetration is important, such as plague meningitis, pleuritis or myocarditis. It can be used separately or in combination with an aminoglycoside. In April 2012, the U.S. Food and Drug Administration is convening an Anti-infective Drug Sub-committee meeting to review data in support of the safety and efficacy of ciprofloxacin and levofloxacin in the treatment of pneumonic plague. This pathway may lead to new indications for these quinolones. (http://www.fda.gov/AdvisoryCommittees/Calendar/ucm295163.htm).

The Working Group on Civilian Biodefense has also proposed recommendations for antibiotic therapy in a mass casualty setting and for post-exposure prophylaxis. Since intravenous or intramuscular therapy may not be possible in these situations, oral therapy preferably with doxycycline or ciprofloxacin is recommended [65]. If treated with antibiotics, buboes typically recede in 10 to 14 days and do not require drainage. Patients are unlikely to survive primary pneumonic plague if antibiotic therapy is not initiated within 18 hours of the onset of symptoms. Without treatment, mortality is 60% for bubonic plague and 100% for the pneumonic and septicemic forms [79].

Asymptomatic individuals such as family members, health care providers, or other close contacts with persons with untreated pneumonic plague, should receive antibiotic prophylaxis for 7 days. Close contact is defined as contact with a patient at less than 2 meters [65]. Prophylaxis is also recommended for laboratory workers exposed to an accident, which may have created an infectious aerosol. Doxycycline is the preferred antibiotic, while ciprofloxacin or chloramphenicol are alternatives. The Working Group for Civilian Biodefense recommends that contacts that develop fever or chough while receiving prophylaxis seek prompt medical attention and begin parenteral antibiotic treatment [65]. Hospital personnel who are observing recommended isolation procedures do not require prophylactic therapy, nor do contacts of patients with bubonic plague.

However, people who were in the same environment and who were potentially exposed to the same source of infection as the contact cases should be given prophylactic antibiotics. The CDC also recommends that prophylactic antibiotics be given to persons potentially exposed to the bites of infected fleas (during a plague
outbreak for example) or who have handled animals known to be infected with the plague bacterium. In addition, previously vaccinated individuals should receive prophylactic antibiotics if they have been exposed to plague aerosols.

Natural antibiotic resistance is rare in *Y. pestis*; however, a chilling report appeared in 1997 of a human isolate in Madagascar resistant to streptomycin, tetracycline, chloramphenicol, ampicillin, kanamycin and sulfonamide. A transmissible plasmid (pIP1202) was responsible for the Multi-Drug-Resistant (MDR) phenotype of this isolate, suggesting a potential for transfer to other *Y. pestis* strains in nature [53]. This plasmid is very closely related to transmissible MDR plasmids of *Y. ruckeri* and *Salmonella enterica* serotype Newport SL254 identified in the United States [136]. More recently, a multi-drug-resistant strain of *Y. pestis* was isolated from a rodent in Mongolia [70]. Russian scientists have published descriptions of multiply drug-resistant plague vaccine strains produced in the laboratory; these techniques could conceivably be used on virulent strains as well [115]. Ciprofloxacin-resistant isolates have been obtained in the laboratory from attenuated strains [82]. If *Y. pestis* is used as a biological weapon, antibiotic resistance is a possibility; the stability and transmissibility of the MDR pIP1202 plasmid in *Y. pestis* suggests that such a strain could be engineered in the laboratory via conjugation without modern molecular technologies.

The U.S. Army Medical Research Institute of Infectious Diseases program in Bacterial Therapeutics screens next-generation antibiotics and novel anti-microbials for activity against a diverse panel of *Y. pestis* strains on an ongoing basis. Compounds are evaluated *in vitro and in vivo* using the mouse model of pneumonic plague.

**Passive Protection** – Passive protection of mice using sera from animals or humans receiving plague vaccine has been effective [9], [50]. This approach to treating pneumonic disease has not, however, been extended to humans. Xiao *et al.* (2010) [153] identified one anti-F1-specific human monoclonal antibody and two anti-V-specific human monoclonal antibodies by panning a naïve phage-displayed Fab library against the F1- and V-antigens. The Fabs were converted to IgG1s and their binding and protective activities were evaluated. A synergistic effect was observed when the three fully humanized antibodies were combined and used to treat mice infected with *Y. pestis*.

**Adjunctive Therapies** – Pathogen-specific bacteriophage therapy has been pursued for decades and has been approved in some countries for the treatment of infectious disease. A number of *Y. pestis*-specific bacteriophages have been identified and it has been proposed that a cocktail of these phages would address the concern of selecting phage-resistant mutants *in vivo*. The inclusion of phage specific to receptors that lie in surface structures required for virulence would insure that if such mutants were selected, the resulting strain would be attenuated in the host [52].

Adenosine Receptor (A(1)AR) antagonists may have proven to be useful adjunctive therapies; these compounds block the lung injury caused by bacterial lipopolysaccharide. Rats were better protected with ciprofloxacin + A(1) AR than with ciprofloxacin alone and showed less lung damage with the combined therapy [147].

Lovastatin, a cholesterol-lowering drug, is reported to moderate the effects of sepsis and was shown to enhance survival of mice in a plague model. However, the efficacy of this class of drugs in the treatment of human sepsis has not yet been proven [74], [13].

### 11.5 ANIMAL MODELS

A recent review of animal models for biodefense describes plague animal models in detail [3].
Mice – The most widely used animal model for plague in recent years is the mouse (*Mus musculus*). This model offers a number of advantages for investigators interested in pathogenesis, vaccine development, or evaluation of therapeutics. Practical considerations include the low cost of the animal, as well as the obvious advantages in terms of space requirements, and ease of handling. FDA-approved vaccines have previously been evaluated in the mouse model [141] and the immune response to *Y. pestis* in infected mice is similar to that of humans [15]. The mouse responds vigorously to antigens known to be important in human immunity such as F1, while some other models, such as the guinea pig, may require use of adjuvants not approved for human use to obtain a significant response [133]. Mice can also be used in models of passive protection [9]. The existence of numerous inbred strains and “knockout” mice enable investigators to dissect the immune response to vaccines and infection [44], [45], [58].

Both outbred strains of mice, such as the Swiss-Webster, and the BALB/c inbred strain have been employed in vaccine efficacy testing and characterization of the immune response [9], [116], [48], [54], [84], [81], [127]. For testing therapeutics, outbred Swiss-Webster, OF1, and Porton strains of mice have been used along with the inbred BALB/c strain [113], [25], [109], [122], [153], [13]. Some subtle variation between inbred strains in their response to a plague vaccine has been reported [66] and it has been suggested that some inbred mice may exhibit exaggerated responses to DNA-based vaccines [22]. In addition, live vaccines appear to be more virulent in mice of certain haplotypes; murine MHC classes H-2k and H-2b tolerate the live vaccine EV76 better than mice of haplotypes H-2 and H-2b [96]. Differences in subcutaneous (s.c.), intraperitoneal (i.p.), and intranasal LD50 values among BALB/c, NIH/s, (inbred), and Porton outbred mice have been reported, but the statistical significance of this is unclear, as confidence limits for the LD50 were not given [114]. Recently, inbred and knockout mouse strains have been useful in dissecting the immune response to plague in mice [44], [45], [58], [128], [129]. Females are generally used because males can be more aggressive and infighting among male mice has complicated interpretation of some experiments [66].

The mouse is highly susceptible to infection by *Y. pestis* by parenteral, intravenous, and aerosol routes. Thus, bubonic, septicemic, and pneumonic plague can be modeled. Bacterial strains selection is not complex in this model; susceptibility of outbred Swiss-Webster mice to a panel of genetically and geographically diverse strains of *Y. pestis* has been reported [152]. The mouse, like the non-human primate, is sensitive to infection by strains expressing the F1 capsular antigen and to F1-negative strains [152], [137], [149], [35]. The LD50 of wild-type *Y. pestis* in mice when administered by the subcutaneous route (mimicking the flea bite) is generally between 1 and 10 Colony-Forming Units (CFU) [99].

Small-particle aerosols of *Y. pestis* produce primary pneumonic plague in the mouse [9], [113]. As reviewed by Meyer, the lesions observed in mice after inhaling *Y. pestis* are quite similar to those of human primary pneumonic plague [88]. The investigators found little evidence of cross-infection between cage mates. It is thought that the lack of cross-infection stems from the physical structure of the mouse respiratory system, which prevents particles exhaled by the infected animal from reaching the lung of the contact animal. In this respect, the mouse is not a good model for examining the spread of pneumonic plague between animals. Aerosols with variable particle sizes may yield disease characterized by cervical buboes and septicemia rather than pneumonic disease; this could mirror reports of pharyngeal plague in humans [88], [87]. The whole-body aerosol LD50 of *Y. pestis* strain CO92 (one of the best characterized and most commonly used strains in current plague research) in Swiss Webster mice has been reported to be 2.1 X 10³ and 6 X 10⁴. Differences in aerosol generation systems and in the calculation of inhaled dose may be responsible for the observed variation in LD50 [3].

Intranasal models of respiratory infection, rather than aerosol exposures have been employed extensively in recent years. It is clear that the mouse is susceptible to infection by this route and that pneumonia results from installation of the pathogen in the nares. Estimated LD50 values for *Y. pestis* strain CO92 by intranasal infection...
are routinely one to two logs lower than the values reported when an aerosol challenge is used. It is not yet clear if this reflects damage to the pathogen during the aerosolization process or if it is indicative of different disease processes. Meyer reported that 10% of the inoculum reaches deeper respiratory passages when anesthetized mice are exposed intranasally [160]. He also noted that, after intranasal installation, the early lesions were observed in the bronchi; peribronchial masses of bacteria were observed before cellular infiltration in the alveoli. This is in contrast to the pathology of disease produced by small-particle aerosols. Time to death was similar to that observed with aerosol-induced disease. As the disease progressed, however, it came to more closely resemble human pneumatic plague. It is of concern that mutants showing significant attenuation by subcutaneous or aerosol routes are not necessarily attenuated in the intranasal model [21]. For this reason, virulence testing should not rely entirely on intranasal models.

It seems logical that intranasal installation might, in some respects, mimic a polydisperse aerosol rather than the small-particle aerosols generated by a Collison nebulizer (see the discussion under guinea pig below). It was recently shown that there are clear pathological differences in mice exposed to large-particle and small-particle aerosols in mice [125]. This pathological difference was seen with NALT damage and secondary pneumonia in large-particle exposure while primary pneumonia was the result of small-particle aerosols. This indicates that the method of aerosol exposure is a critical parameter that should be properly selected to support experimental aims. For instance large-particle exposure may be appropriate for prediction of person to person spread while small-particle aerosols would be recommended for testing protection against a deliberate aerosol release. A side-by-side comparison of disease kinetics in mice exposed to both types of aerosols and intranasally at various doses would be very useful in comparing the intranasal, small-particle aerosol, and polydisperse aerosol models. Ideally, the studies would be performed simultaneously using the same inoculum preparation and source of mice. This would minimize the large number of variables that currently plague the available data.

Septicemic plague can be induced in the mouse by intravenous (i.v.) challenge by fully virulent organisms and some therapeutics have been assessed in this model. More importantly, the i.v. virulence of Pigmentation-deficient (Pgm-) strains of *Y. pestis*, which are highly attenuated by the s.c. route, has enabled numerous investigators to safely assess the importance of several *Y. pestis* virulence factors under biosafety Level-2 conditions [99]. Because the organism does not normally have direct access to the bloodstream, i.v. models do not reflect naturally acquired disease. However, they do encompass an important part of the disease progression (septicemia followed by seeding of the spleen and liver) and are invaluable to scientists lacking higher containment facilities.

**Guinea Pig** – Like the mouse, the guinea pig is an attractive model in terms of expense, space, and ease of handling. Historically, however, there have been some problems with the use of this model. Numerous investigators have reported difficulties in successfully vaccinating guinea pigs with killed, whole-cell plague vaccines or with antigenic extracts, including F1 capsular antigen, although these preparations were highly effective in the mouse, the rat, and the non-human primate [90], [121], [77], [133], [28], [67], [106]. In some cases, incorporation of oil-based adjuvants was necessary to achieve protection from *Y. pestis* challenge in the guinea pig. Passive protection models, in which immune sera were administered to guinea pigs before challenge, have generally not been useful. Live attenuated vaccines have generally been more successful in protecting guinea pigs [121]. However, serious concern was raised regarding the use of this animal as a model for live plague vaccines when some vaccine strains that were essentially avirulent in the guinea pig proved fatal when tested in non-human primates [89].

The small animal model of choice for plague in the Former Soviet Union (FSU) is the guinea pig rather than the mouse. Anisomov has stated that guinea pig virulence is the best predictor of likely virulence for humans [11]. The emphasis on live bacterial vaccines in the FSU might also have made the guinea pig model appealing, as it is known to respond well to this type of vaccination.
Like the mouse, the guinea pig is considered to be highly susceptible to infection by *Y. pestis*, with a s.c. LD$_{50}$ < 10. However, there are striking differences in the sensitivity of these rodents to certain strains of *Y. pestis*. For example, unlike the mouse and non-human primate, the guinea pig is relatively resistant to infection by non-encapsulated strains (F1); the capsule is an essential virulence factor in this animal [137]. When the guinea pig is infected parenterally, the course of the disease is protracted when compared to many other models and is not always dose-related [135], [137].

The aerosol LD$_{50}$ for the Hartley guinea pig with strain *Y. pestis* strain CO92, the most commonly used isolate in the United States at this time, is ~ 40,000 CFU, similar to that of the mouse [137]. In aerosol studies of mixed particle size, infection of the guinea pig initiated a disease characterized by cervical and laryngeal edema, lymphadenopathy, hemorrhagic nodes, septicemia, and hemorrhage of the intestinal wall. Miliary abscesses of the spleen were present. Approximately one-quarter of animals had evidence of pneumonia; however, this appeared to be a secondary lung infection rather than primary [88], [87], [124], [42]. Monkeys exposed under the same conditions developed a primary pneumonic plague. Based on these results, Strong concluded that the infection in guinea pigs originates in the mucous membranes of the mouth and throat. Invasion of local lymph nodes is then followed by septicemia and, in some cases, secondary pneumonia.

**Rat** – Plague models of a number of species (*Rattus norvegicus, R. rattus, R. alexandrinus*, as well as Sprague-Dawley and Wistar laboratory rats [90], [28], [161] have been described. This genus is more resistant to infection by *Y. pestis* than either mice or guinea pigs, with the s.c. lethal dose approximately 1000-fold higher, depending on the strain of *Y. pestis* and the type of rat. Resistance to plague was noted in laboratory rats and in rats captured from both endemic and non-endemic areas [28]. Various challenge routes have been used with this rat, including s.c., intradermal, aerosol, and intranasal. Williams and Cavanaugh [140] found that the intranasal route was not as reliable as an aerosol in establishing pneumonic plague, as the intranasal infection often involved the tonsils and larynx rather than primary pneumonic disease. They felt, however, that the intranasal challenge route was a more stringent test of vaccines than an s.c. challenge and was, therefore, suitable for efficacy testing. A rat oral infection model was developed by feeding rats infected tissues; approximately 22% of the rats that succumbed to plague became bacteremic [161].

Otten [162] demonstrated that both wild-caught *R. rattus* and laboratory rats could be protected with live attenuated strains of *Y. pestis*. Rats were protected by the same cell fractions (predominantly F1) as mice and monkeys. They responded to live attenuated, whole-cell (killed), and F1-based vaccines with significant F1 titers; these titers appeared to correlate with protection from *Y. pestis* challenge [90], [140]. It has been demonstrated that protective antibody is passed to new-born rats by their immune dams in utero [163].

A recent study reported that 500 CFU of *Y. pestis* strain C092 was lethal to 100% of Brown Norway, Sprague Dawley, and Wistar rats when administered intradermally in the lower back. Some animals survived, however, when the pathogen was injected intradermally into the ear [164]. Currently, the Brown Norway inbred rat is the most utilized for models of both bubonic and pneumonic plague; the genome of this strain has been completely sequenced, which makes it particularly attractive as a model. Kinetics of disease progression, identification of virulence factors, and transcriptomic analyses have all been reported in the rat [165], [166], [167], [30]. The course of disease appears to be similar to that of the human. The intranasal LD$_{50}$ for *Y. pestis* CO92 was reported to be approximately 200 CFU while the aerosol LD$_{50}$ was calculated to be 1.6 x 10$^3$ CFU [166], [167].

**Non-Human Primates** – Non-Human Primates (NHPs) have been used for over a century as a model for plague infection, pathogenesis, and vaccine efficacy. Primates, like humans, are an incidental host for plague. However, there are some differences in plague susceptibility between NHPs and humans and between different species of NHPs. The extent of and basis for these differences are not completely understood. Comparing results from the...
literature is difficult as often the source of the monkeys used was not reported, and other variables such as the strain of plague, route, and dose of \( Y. \) \textit{pestis} confound interpretation. Several species of NHPs have been described in the literature as models to study plague pathogenesis and vaccine efficacy.

**Rhesus Macaque** – Rhesus macaques (\textit{Macaca mulatta}) were used extensively in older plague vaccine studies but are not the NHP model of choice. They have been described as more resistant to s.c. plague infection than other primates, including humans. The s.c. LD\(_{50}\) for the rhesus is apparently several million organisms, far above the predicted infective dose of several hundred to several thousand organisms in humans [46], [62]. The rhesus monkey is also somewhat resistant to aerosolized \( Y. \) \textit{pestis}. The calculated aerosol LD\(_{50}\) for rhesus is 20,000 inhaled organisms, while the LD\(_{50}\) for humans is estimated to be about 3,000 organisms [118], [120], [47]. However, a lowered resistance to plague has been observed in rhesus after intratracheal challenge with a calculated LD\(_{50}\) of 100 CFU [46]. Although infection by the intratracheal route does lead to the development of pneumonic plague, there are some differences in the pathology when compared to aerosol-exposed animals. Most notably, the nature of the pneumonia after intratracheal instillation is often more confined than that seen with aerosol delivery, and there is evidence in some primates that the pneumonia may be coincident with a primary septicemia [47]. While the rhesus can develop acute plague pneumonia, a large number of monkeys exposed to aerosolized plague will develop a protracted disease with unique lesions referred to as chronic pneumonic plague [111]. Chronic plague is a very infrequent finding in humans. However, an important observation regarding the two forms of pneumonic plague in the rhesus monkey is the notation that the numbers of viable bacilli were “controlled” in the chronic form of disease. Thus, the study of chronic plague in rhesus may lead to a better understanding of important host factors required for an enhanced protective response to aerosolized \( Y. \) \textit{pestis}.

Conversely, the observed lesions in acute pneumonic plague in rhesus monkeys closely match those in humans (for a review, see Ref. [3]). Fever has been reported to manifest early in the disease and 2 to 4 days after the animal becomes febrile, the blood pressure and hematocrit begin to fall, with death occurring within 24 hours [47]. The levels of circulating eosinophils decline during the course of disease. Animals develop tachypnea and are rapidly prostrate. In the acute form of the disease, rhesus monkeys develop lobar pneumonia similar to human disease Pneumonic plague, but not bubonic plague, in rhesus has been reported to cause an early disruption of liver function with rapid colonization of the liver. This observation appears to be the opposite of what happens in humans; however, interpretation is complicated by the use of the intratracheal challenge route in this rhesus study, as intratracheal infection may result in direct plague septicemia.

Finegold examined plague-infected rhesus monkeys for evidence of disseminated intravascular coagulation. Monkeys exposed to aerosols demonstrated a time-dependent increase in clotting times, partial thromboplastin times, mean prothrombin times, and circulating fibrinogen with a concomitant decrease in platelet counts [168]. This phenomenon has been described to variably occur in humans and is likely tempered by the use of antimicrobials in the human cases. Collectively, the data suggest that rhesus macaques may not be the best NHP model for plague vaccine studies although their use in this context has historical precedent. Rhesus may be more useful for pathogenesis and innate immunity studies.

**Cynomolgus Macaque** – The cynomolgus monkey (\textit{Macaca fascicularis}) has been used in plague vaccine trials since the beginning of the 20\textsuperscript{th} century. Importantly, in the last 5 years the cynomolgus macaque has become the most utilized NHP model for plague pathogenesis and vaccine studies [145], [169], [33], [91], [29]. The susceptibility to plague has been described to be similar to the rhesus by the s.c. and intratracheal routes. However, a review of the literature suggests a large variance in susceptibility [89]. Whether this difference is real or an artifact of experimental variables remains to be determined. Work at USAMRIID suggests that the cynomolgus is highly susceptible to aerosolized plague with an LD\(_{50}\) of approximately 300 inhaled organisms.
YERSINIA PESTIS

(Adamovicz and Pitt, unpublished data). This value was determined via a staircase method. More recent studies suggest that the LD_{50} for small-particle aerosols is even lower with values of 24 [134] or 66 [132] CFU as calculated by probit analysis or linear regression respectively.

The clinical and pathological responses of the cynomolagus macaque are similar to human disease, although subtle differences can be discerned. As with human pneumonic plague, cynomolagus macaques infected by the aerosol route manifest a fever generally 2 days after infection. Tachycardia and tachypnea are observed and the animals become lethargic. The animals develop detectable rales and lobular and lobar consolidation [89]. Bacteremia can only be detected in the peripheral circulation within 24 hours of death and death usually occurs from day 3 to 5 after exposure. The gross pathology of the lungs appears to be similar to human disease with the exception that the development of fibrinous pleuritis is notably reduced. This may be a temporal phenomenon as closer examination of lung lesions reveals damage consistent with fibrinous pleuritis (for a review, see Ref. [3]). The dissemination of bacteria outside of the lung appears to be reduced in cynomolagus macaques with lower levels in the spleen and peripheral circulation although this may reflect a dose phenomenon. The necrosis and hemorrhage of the lung, as well as that of the mediastinal lymph nodes, is reduced compared to noted human lesions, again this may be a dose effect or temporal phenomenon. The most recent published studies on the pathology of primary pneumonic plague confirm earlier observations and extend the findings such that previous patho-pneumonic findings such as fever and or difficulty in breathing may not always manifest in infected animals [132], [134], and there is variability in histopathology [75]. However, these studies all conclude that clinical symptoms, gross lesions, and histopathology were all consistent with end-stage human primary pneumonic plague.

Cynomolagus macaques make a robust, though variable, response to plague antigens. F1-V vaccinated macaques make an antibody response to Fraction one (F1), V antigen, LPS, Yop B, YopD, and YopM after aerosol plague challenge (Adamovicz et al., unpublished). This model has also been used to test killed whole cell, live attenuated, and F1+V recombinant plague vaccines [89], [144], [145] or to the recombinant fusion protein vaccine rF1-V [91], [50], [83]. The killed vaccines induced protection against parenteral but not aerosol challenge, while the live attenuated and recombinant vaccines protected the majority of animals against significant pneumonic plague morbidity and mortality. Measures of antibodies against F1 and V antigens seem to be the most promising correlate of immunity [170], [83]. While absolute titer seems to correlate with protection, the production of antibody to specific epitopes appears critical. The clinical and pathological results collected to date indicate that the cynomolagus macaque is an excellent model for plague pathogenesis and vaccine studies. Additional genetic data for comparative genomic studies are required to determine the ability of this macaque to reflect the human response to infection and treatment.

Vervet – The African Green Monkey (AGM, Cercopithecus aethiops) species consists of several sub-species with varied innate resistance to plague. Those described as originating from Kenya were susceptible to infection and mortality by an EV76 vaccine strain while Ethiopian green monkeys were not killed by the vaccine strain [89], [172], [171]. The response of a third sub-species currently located on the Caribbean island of St. Kitts, originally derived from South Africa, has not been determined. The ability to infect and kill certain sub-species of monkey with a human vaccine strain calls into question the use of African green monkeys for plague studies. While susceptibility to wild-type organisms is desirable, the ability to resist attenuated plague organisms is equally important. Note, however, that there are significant differences between EV76 strains and it is not clear if the aforementioned studies employed the same challenge organism.

Vaccine studies on non-Kenyan species of African green monkeys have been extremely productive. African green monkeys, like cynomolagus macaques, have an inhaled aerosol LD_{50} of about 300 organisms of wild-type Y. pestis. The clinical manifestation of plague in St. Kitts-derived monkeys is similar to humans and other NHPs that develop acute pneumonic plague (for a review, see Ref. [3]). The pathology has been reported to be similar
to humans; however, there did not appear to be a correlation with pathology and challenge dose. In contrast, animals challenged s.c. exhibited longer survival and protracted pathology with lower challenge doses [172]. An LD$_{50}$ for the s.c. route was not calculated; however, it was estimated to fall between several hundred and several thousand organisms- similar to the predicted LD$_{50}$ for humans. Conversely, the LD$_{50}$ by the intradermal route has been reported between 5 – 50 CFU, which may be significantly lower than in humans [173].

African green monkeys originating in South Africa or Ethiopia have been used in vaccine studies with mixed results. Although the animals exhibited a robust anti-F1 antibody response, an oral live attenuated plague vaccine protected three of six vaccinated monkeys from pneumonic plague, while a recombinant plague vaccine protected only seven of 28 animals from aerosolized plague (Adamovicz, unpublished data, [173]). Similar mixed partial protection results were also observed with various live vaccine trials in African green monkeys challenged by aerosol or s.c. [89]. The variable response to all plague vaccines tested to date, as well as an obvious “susceptible” plague phenotype, make interpretation of these vaccine studies difficult. Collectively, these data raise concerns about the utility of using African green monkeys for plague vaccine trials. However, the African green monkey has been found suitable for evaluation of plague therapeutics [78].

**Other Models** – A number of other animals have been utilized in the study of plague. These include rodents such as the multimammate mouse, the cotton rat, ground and rock squirrels, and voles. Larger animals include the rabbit, Langur monkeys (*Semnopithecus or Presbytis entellus*), Sacred baboons (*Papio hamadryas*), and the marmoset (*Callithrix* sp.) For more information on these animal models, see the review by Adamovicz and Worsham [3].

### 11.6 CONCLUSIONS

Plague is a zoonotic infection caused by the Gram-negative bacillus *Yersinia pestis*. The disease is maintained in nature, predominately in urban and sylvatic rodents and flea vectors. Humans are not necessary for the persistence of the organism, and acquire the disease from animal fleas, contact with infected animals, or, rarely, from other humans, via aerosol or direct contact with infected secretions. To be able to differentiate endemic disease from plague used in biological warfare, medical officers must understand the typical way in which humans contract plague in nature. First, a die off of the mammalian reservoir that harbors bacteria-infected fleas will occur. Second, troops who have been in close proximity to such infected mammals will become infected. By contrast, in the most likely biological warfare scenario, plague would be spread via aerosol. Person-to-person spread of fulminant pneumonia, characterized by blood-tinged sputum, would then ensue. If, on the other hand, an enemy force were to release fleas infected with *Y. pestis*, then soldiers would present with classic bubonic plague before a die-off in the local mammalian reservoir occurred.

The intentional release of *Y. pestis* would have significant public health ramifications. If introduced into rodent hosts in areas currently not endemic for plague, the disease could be introduced into new ecosystems. Furthermore, primary pneumonic plague is difficult to diagnose early in the disease and the treatment window is very short. Individuals with the pneumonic form of the disease are capable of spreading the disease person-to-person.

Since *Y. pestis* is a member of the *Enterobacteriaceae*, genetic methods learned in studies with *E. coli* or other enteric bacteria could provide the foundation for unscrupulous scientists endeavoring to genetically manipulate the organism. The existence of stable MDR in a human isolate of *Y. pestis* demonstrates that the MDR phenotype is not necessarily incompatible with virulence. A multitude of enteric MDR plasmids transmitted via conjugation have been described and could easily be transferred to *Y. pestis* without the need for recombinant
DNA technology. It is imperative that new classes of antibiotics and adjunctive therapies are evaluated for suitability in the treatment of plague. The mouse and the NHP are the most appropriate models for such investigations.

There is currently no licensed vaccine for plague in the United States and licensure of the candidate currently in clinical trials (F1-V) is likely some years away. This product is clearly superior to the whole cell killed vaccine previously in use in the U.S. However, virulent strains lacking one of the two protective antigens (F1) have been isolated from nature or created in the laboratory. For persons infected with an F1-negative strain, immunity must rely solely on their response to the V antigen component of the vaccine. The possibility of heterogeneity in V antigen is also of concern. It is desirable, therefore, to identify additional antigens to bolster recombinant vaccines or to identify acceptable live attenuated vaccines. Delivery systems that enhance mucosal immunity or provide protection with a single dose of vaccine are also avenues to be vigorously pursued.

11.7 REFERENCES


YERSINIA PESTIS


Disclaimer: Animal research at The United States Army of Medical Research Institute of Infectious Diseases was conducted and approved under an Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.
Chapter 12 – *FRANCISELLA TULARENSIS*

Patricia L. Worsham¹ and Jiri Stulik²

¹: Division of Bacteriology  
U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD  
UNITED STATES  

²: Department of Molecular Pathology and Biology  
Faculty of Military Health Sciences, University of Defence, Kralove  
CZECH REPUBLIC  

Corresponding author (Worsham): patricia.l.worsham.civ@mail.mil

12.1 INTRODUCTION

The bacterial species *Francisella tularensis* is the causative agent of the zoonotic disease known as tularemia. This small, pleomorphic Gram-negative bacillus is highly infectious in humans via a number of routes, including the skin, mucous membranes, gastrointestinal tract and respiratory tract. The genus and species names are derived from the scientist who recognized the common etiologic agent between the varied forms of the disease (Francis) and Tulare County California, where the organism was first isolated from a ground squirrel die-off. Tularemia is a zoonotic disease: small mammals including mice, squirrels, rats and rabbits are natural reservoirs. It is widely dispersed throughout the Northern Hemisphere with foci in North America, Europe, and northern Asia. Human infection usually occurs through environmental exposure such as contact or ingestion of contaminated food, water or soil, bites by infected arthropods, or by direct contact with infected animals or their tissues [48]. In North America, it has often been associated with the skinning and consumption of rabbits, thus the name “Rabbit Fever” [41]. Infectious aerosols may be generated from dusty areas contaminated by infected animals, or produced by the action of lawn mowers or brush cutters [63]. A human outbreak associated with wild-caught prairie dogs was localized to a pet shop selling the animals in 2002 [4]. It is suspected that the prairie dogs became infected through consumption of infected carcasses in the wild. Tularemia often presents with non-specific flu-like symptoms such as headache, fever, chills, nausea, diarrhea and pneumonia. Most of the symptoms are non-specific and can be easily misidentified as other forms of febrile diseases. A useful table for differential diagnosis has been prepared by the World Health Organization [113]. Ulceroglandular tularemia, characterized by a primary lesion at the site of infection, generally occurs when the organism comes in contact with the skin; this may be the result of handling infected animals/animal tissues or arise from arthropod-borne transmission (ticks, mosquitoes, or biting flies). Less often, disease results from exposure via the conjunctiva (oculoglandular tularemia) or ingestion of contaminated water or meat (oropharyngeal tularemia). The local lymph nodes become involved with all of these forms of the disease. Oropharyngeal tularemia may be mistaken for streptococcal disease; if so, this would often result in ineffective treatment (β-lactams). Respiratory tularemia, the most severe form of the disease, arises from an aerosol exposure such as the “lawn mower” tularemia reported among landscapers in Martha’s Vineyard in 2000. It is this route of exposure that is of greatest concern from a biodefense perspective. Patients may present with symptoms of pneumonia or non-specific symptoms such as nausea and vomiting. Hilar adenopathy, pneumonic infiltration, and pleural effusion may be noted. The high morbidity and mortality associated with respiratory tularemia, the extremely low infectious dose (< 10 organisms in humans), and the hardiness of *F. tularensis* in the environment make this a particularly dangerous pathogen [48]. The high infectivity of the organism increases
the potential for laboratory-acquired infection; one study estimated that tularemia is the third most common laboratory acquired infection [77]. Historical and recent reports highlight the risk of infection in laboratory scientists, including immunized individuals [71], [95], [56]. The respiratory route appears to be the most common route of infection in the laboratory [71]. When the route of infection is unknown, the disease is sometimes referred to as “typhoidal” tularemia. Human to human spread of tularemia has not been reported [113].

*F. tularensis* is typically divided into sub-species, the most virulent being *F. tularensis* subsp. *tularensis*, also known as “Type A”. This is the sub-species found in North America. The most commonly used laboratory strain of the A-type is the SCHU S4 strain. *F. tularensis* subsp. *holarctica* (Type B) causes a milder form of the disease in humans and is the primary cause of tularemia in Europe. Sub-species *tularensis* can, in turn, be further subdivided into additional distinct clades, based on variable number of tandem repeats, geographical location and severity of human disease [74]. Other sub-species, such as *mediasiatica* and *novicida* (possibly a unique species) are virulent in animals but are rarely associated with human disease in immunocompetent individuals. *F. novicida* and the Live Vaccine Strain (LVS), an attenuated derivative of *F. tularensis* subsp *holarctica*, are highly pathogenic in mice, hence are used as model organisms. *F. tularensis* is not closely related to other pathogenic bacteria.

*F. tularensis* is a facultative intracellular pathogen; it targets macrophages, but also appears to survive in lung epithelial cells, dendritic cells, neutrophils, and hepatocytes. Within monocytes, the bacteria interfere with phagolysosome development. The organisms subsequently escape to the cytoplasm, replicate, and trigger phagocyte death and lysis. Mutants that fail to prevent phagosome/lysosome fusion or are unable to escape from the phagosome are highly attenuated in animal models. Unfortunately, most of the studies examining the pathogenesis of *F. tularensis* and the host response to this organism have been carried out with the attenuated Type B Live Vaccine Strain (LVS) or other sub-species rather than the more virulent Type A. Results from one sub-species cannot always be extrapolated to another; thus, it is imperative to assess the role of potential virulence factors in the pathogenesis of fully virulent Type A and B strains rather than in surrogates such as LVS or *F. tularensis* subsp. *novicida*.

### 12.2 IDENTIFICATION AND LABORATORY GROWTH

Although a robust survivor in the environment, *F. tularensis* is a slow-growing, fastidious organism in the laboratory. The species is a strict aerobe and utilizes a limited number of carbohydrates. Sulphydryl supplementation (cysteine, cystine, thiosulfate or IsoVitaleX) enhances culture. CO₂ also enhances growth; however, even under the most favorable conditions, colonies only reach the 1 – 2 mm size after more than 48 hours. Traditional media for isolation of Gram negatives, such as MacConkey agar, do not promote growth. Heart agar, Thayer Martin agar, or chocolate agar supplemented with sulphydryl compounds are required. Plates inoculated with clinical samples should be incubated for more than 7 days [59]. A defined growth medium for research laboratories is also available [17].

In virulence studies involving aerosolized bacteria, the choice of growth medium impacts the survival of the organism in aerosols, with Brain Heart Infusion (BHI) broth being the medium that best stabilizes the bacteria [38]. There is also evidence that certain commonly used growth media affect bacterial structure in such a way that the cells aberrantly elicit pro-inflammatory mediators in *in vitro* models [94]. This is likely due to fragility of the bacterial cells, with associated lysis and release of molecules, such as DNA, that stimulate innate immunity. In these studies, BHI-grown cells appear to more closely resemble host-adapted cells from tissue culture or infected tissues, than the Mueller Hinton Broth (MHB)-grown cells. The latter elicited a much stronger pro-inflammatory response. Such data suggest that the choice of growth conditions may have a profound impact
on the interpretation of historical data and demonstrate the need to revisit some earlier experiments. Growth conditions should be carefully considered in designing future experiments.

A growth medium supplemented with antibiotics (colistin, amphotericin, lincomycin, trimethoprim, and ampicillin) is useful for culturing animal carcasses in the field that are suspected to carry \textit{F. tularensis} \cite{75}. Due to the growth inhibition of contaminating flora on standard growth media, recovery of the organism is greatly improved when this medium is used. Mice have also been used to recover \textit{F. tularensis} from contaminated samples.

\textit{F. tularensis} can be cultured from skin lesions, lymph node aspirates, pharyngeal washings, or sputum specimens. Blood cultures are often negative, despite the fact that the organism is known to cause septicemia. The Gram counterstain, safranin, is poorly taken up; this is an important indicator of the possibility of \textit{F. tularensis} when positive and negative controls behave as expected. The organism can be identified by a direct fluorescent antibody test (FITC-labeled rabbit anti-\textit{F. tularensis} conjugate), a slide agglutination test using commercially available hyper-immune serum prepared in rabbits, PCR (based on 16 s ribosomal RNA sequences and the species-specific \textit{lpnA} gene), or immunohistochemical staining \cite{113}. A four-fold increase in titer between acute and convalescent titers is considered to be confirmation of tularemia \cite{48}, \cite{15}.

\subsection*{12.2.1 Genomics and Molecular Fingerprinting}
Genomic studies on \textit{F. tularensis} have been facilitated by the completion of sequencing of the genomes of many strains of \textit{F. tularensis}, including subsp. \textit{tularensis}, \textit{holarctica}, \textit{mediasiatica} and \textit{novicida} \cite{103}. Despite marked differences in their virulence and geographical origin, there is more than 95 – 98 % overall identity among the four sub-species with an average of ~ 1700 Open Reading Frames (ORFs) \cite{57}. Approximately 30% of the annotated genes in \textit{F. tularensis} are hypothetical proteins of unknown function. It seems quite likely that some of these novel ORFs are involved in virulence.

\textit{F. tularensis} has a small bacterial genome considering the variety of ecological niches and the wide host range this organism is adapted to. The relative genetic homogeneity of \textit{F. tularensis} ssps. may be related to the intracellular lifestyle of the bacterium, since stable bacterial lifestyle correlates with genomic stability. The evolutionary relationships of \textit{F. tularensis} sub-species are complex and apparently involve both genomic acquisition and genomic decay in the more virulent strains, suggesting a role in pathoadaptation to infect mammals \cite{83}, \cite{18}. Earlier analyses of unidirectional genomic deletion and single nucleotide variations have indicated that the four sub-species of \textit{F. tularensis} have evolved by vertical descent \cite{99}. The genomes of the \textit{novicida} ssps. strains have > 97% identity to \textit{tularensis} ssps. and contain the highest percentage of intact ORFs and the least number of insertion elements compared with the virulent strains. Genome-wide SNP phylogenetic studies have shown that differentiation of sub-species \textit{novicida} predated differentiation of sub-species \textit{tularensis} and \textit{holarctica} from a common ancestor. Genomes of strains of \textit{F. tularensis} ssps. \textit{holarctica} have a greater degree of heterogeneity than strains belonging to other \textit{F. tularensis} sub-species, which may be partly due to the highest number of disrupted ORFs as well as insertion and transposable elements incorporated in the genome. The observed increase in disrupted genes in \textit{F. tularensis} ssps. \textit{holarctica} lineages suggests that this sub-species has been undergoing a reductive evolution to become more niche-adapted. A putative pathogenicity island encoding functions essential for survival in macrophages has been identified in \textit{F. tularensis} sub-species \textit{tularensis} and \textit{holarctica} and appears to include a homologue to Type VI secretion systems \cite{5}.

Bioinformatic analysis has revealed that this species has low levels of homology to other Gram-negative bacteria, lacking homologues to many virulence factors important to other bacterial pathogens. This has made exploration of bacterial pathogenesis particularly difficult for this organism. Furthermore, tools for genetic manipulation of \textit{F. tularensis} have only relatively recently become available \cite{116}.
12.2.2 Use as a Bioweapon

There is concern that *F. tularensis* could be used as a biological weapon; both the United States and the Former Soviet Union (FSU) weaponized this organism during the Cold War. Although there is speculation that it was used by the Soviets against the German Army in World War II, this was not proven and could have been due to endemic disease.

The most serious scenario is one in which a large-scale aerosol release takes place over a highly populated area. The low infectious dose would lead to a large number of incapacitating casualties and, in some cases, death. The resulting disease would be difficult to distinguish from other febrile illnesses such as influenza. Early in the disease course, symptoms would also be similar to the disease resulting from exposure to other agents such as *Yersinia pestis* or *Bacillus anthracis* [48].

12.2.3 Host-Parasite Relationship

The initial stages of infection by *F. tularensis* are remarkable for the lack of pro-inflammatory response [25], [109]. It appears that this organism possesses multiple anti-host mechanisms that contribute to this phenomenon. *F. tularensis* may initially evade the host innate immune system through resistance to the complement-mediated Membrane Active Complex (MAC). Although the mechanism of this early avoidance of innate immunity is uncertain, it has been demonstrated that the bacteria bind serum factor H, a protein that cleaves complement C3b to the inactive form iC3b. Not only does this affect generation of the MAC, but opsonization with iC3b also enhances the efficiency of bacterial uptake by the very phagocytes that promote growth of the organism (reviewed by Bosio [12]). The O-antigen of *F. tularensis* also fails to activate TLR 4, an important component of the pro-inflammatory response [32]. In addition, *F. tularensis* directly affects production of lymphocytes. Following aerosol exposure to a Type A strain, but not a less virulent Type B strain, mice exhibited destruction of the thymus and depletion of immature CD4 and CD8 thymocytes by day 4 of infection [19].

It has been demonstrated that in murine aerosol models using strain SCHU S4 that the organism replicates rapidly within host cells without eliciting TNF-α, IL-1β, IL-6, IL-12, and IFNγ [2], [11], [109]. This is in contrast to other respiratory pathogens, including *Y. pestis*. A number of transcriptomic changes in the lungs of mice infected with SCHU S4, including induction of some genes associated with anti-inflammatory pathways, have been noted. However, the resulting downstream effects on these pathways have not yet been well characterized. In addition, there is evidence that the organisms induce production of the anti-inflammatory cytokine TGF-β [11], although the contribution of this cytokine to the repression of innate immunity is not clear since introduction of antibodies to TGF-β failed to significantly decrease bacterial load in this study. The SCHU S4 strain also fails to activate dendritic cells in the lungs of mice.

The intracellular cycle of *F. tularensis* consists of several major steps: entry into the cell, a rapid phagosomal escape, an active cytosolic multiplication and subsequent dissemination by host cell lysis. The bacteria are able to enter the cell via a number of mechanisms: if opsonized by complement, the cells enter through complement receptor 3 or the scavenger receptor, while antibody-opsonized bacteria enter through the FCγ receptor. Unopsonized bacteria may enter via the mannose receptor and surface nucleolin (reviewed by Asare and Kwaik [3]). Lipid rafts also appear to play a role in the entry process, at least in the LVS strain [100].

Complement factor 3-opsonized *F. tularensis* enters host macrophages by inducing the formation of unique spacious pseudopod loops [20]. When a Type A strain is opsonized via complement C3, there is a remarkably muted inflammatory response by the phagocyte. In contrast, when a Type A strain is incubated in C3-depleted sera, there is less uptake by the macrophage but a significant inflammatory response. The end result of C3 opsonization of a Type A strain is enhanced phagocytosis with an impaired host response [26]. *F. novicida*
uptake, even in the presence of C3, led to a robust inflammatory response in these same studies. It appears that the Type A strain Schu-S4, but not F. novicida, is able to inhibit the pro-inflammatory response of human cells via inhibition of MAPK activation and down-regulating TLR2-dependent responses. It should be noted that the C3 opsonin-mediated inhibition of MAPK is specific to human-derived macrophages and is not observed when murine macrophages were used. Differences between mice and primates in their innate immune systems and signaling pathways are becoming increasingly recognized [115], [26].

Once inside the cell, the bacterium arrests maturation of the phagosome at a late endosomal-like stage. Following partial acidification, but prior to phagosome-lysosome fusion, the bacteria escape from the phagosome and begin replicating in the cytosol. Depending on the bacterial strain and phagocyte cell type, organisms can escape as early as 15 – 30 min after phagocytosis [87]. Hundreds of gene products appear to be associated with the ability to escape and replicate in macrophages (reviewed by Asare and Kwaik [3]). Some of these are required for both escape and replication, while others contribute only to replication. Activation of the inflammasome, initiated by bacterial DNA, is associated with the escape of F. tularensis to the cytosol and leads to the activation of caspase 1, which promotes pyroptosis.

F. tularensis also modulates apoptosis in phagocytes. Between 6 and 12 hours post-infection, the organism induces caspase 1 and caspase-3, which initiate pyroptosis and apoptosis respectively. However, cell death is not observed until several hours later. As it escapes the phagosome, the pathogen is also promoting anti-apoptotic pathways. This delay in host cell death appears to be associated with activation of the anti-apoptotic factor NFκB that promotes viability of the host cell. Escape from the phagosome appears to be necessary for the nuclear translocation of NFκB [87]. Since NFκB is also involved in the induction of pro-inflammatory cytokines, it is likely that this process is tightly regulated by the bacterium to allow the organism to fine-tune its environment. It should be noted that this particular study utilized F. tularensis sub-species novicida, however. It is not yet known if the human-virulent sub-species behave in the same way.

Although the majority of in vitro studies have focused on macrophages and other monocytic cells, it appears that F. tularensis also has profound effects on neutrophils. Unlike monocytes, neutrophils are normally turned over rapidly. Schwarz et al. [91] demonstrated that LVS and SCHU S4 replicated in neutrophils (PMN) while inhibiting both the intrinsic and extrinsic apoptotic pathways. The increase in longevity of PMN was observed at a low multiplicity of infection – even in PMN that were not infected. This suggests a factor present in the tissue culture medium, produced by either the pathogen or the infected host, which inhibits the process of apoptosis. Since PMN apoptosis and turnover is an essential part of regulating the inflammatory response, this phenomenon may contribute to the tissue necrosis associated with the disease.

12.2.4 Virulence Factors

Due to the highly infectious nature of F. tularensis, many laboratories have chosen to study the LVS strain or one of the less virulent sub-species of F. tularensis. This choice enhances laboratory safety as well as enabling the work to be conducted at a lower level of biocontainment and without the restriction of select agent regulations. However, the resulting data may not necessarily be relevant to the fully virulent organism. This should be kept in mind while reviewing the literature.

A series of genome-wide screens of banks of transposon insertion mutants have been developed to find novel F. tularensis virulence genes [42], [96]. The identified genes can generally be grouped into four distinct functional categories: bacterial cell envelope, the Francisella pathogenicity island, chaperone proteins, and genes involved in bacterial metabolism.
Mutants in capA, capB, and capC genes have been repeatedly isolated from in vitro and in vivo screening approaches, establishing that these genes contribute to pathogenesis. Mutagenesis has demonstrated that the capBCA locus is essential for full virulence of *F. tularensis*. CapBCA appears to play a role in intracellular growth of the bacteria, although its precise role is not yet clear [97]. It is interesting to note that this locus was designated “cap” due to a small amount of homology to the capsular operon of *Bacillus anthracis*. In *B. anthracis*, the CapB protein homologue is involved in synthesis of the Poly-D-Glutamic Acid (PGA) anti-phagocytic capsule. Attempts to identify PGA associated with *F. tularensis*, however, have been unsuccessful.

The LPS O-antigen moiety has been shown to be important for intracellular survival of *F. tularensis*. Mutants devoid of O antigen (i.e. mutants in the wbt gene cluster) generally show impaired intracellular multiplication. Their increased susceptibility to killing by serum is likely to account, at least in part, for their reduced mouse virulence [81].

A Type IV pilus-like structure (Tfp) is required for virulence of *F. tularensis*; such structures are typically used by pathogens to mediate adhesion, promote motility, and aid in protein secretion. They are more often associated with non-intracellular pathogens. Nevertheless, the Tfp appears to serve an important function for *F. tularensis*. The less virulent Type B strains carry more pseudogenes (inactive copies) encoding Tfp than the Type A organisms. It has been proposed that these differences may reflect the different environmental niches of the two strain types [86]. The attenuation of LVS, when compared to clinical Type B strains, may be due to the complete loss of the Type IV pilin and a hypothetical outer membrane protein FTT10918 associated with virulence. In both cases, the deletions appear to be due to homologous recombination between direct repeats [85].

A 33 kb pathogenicity island, designated FPI (Francisella Pathogenicity Island), was identified by computer-assisted analysis of the newly sequenced genomes of LVS and *F. novicida* strain U112 [67]. The FPI is duplicated in all the subsps of *F. tularensis*, but is present in a single copy in *F. novicida* and *F. philomiragia*. Systematic mutational analyses of the FPI-encoded genes have shown that most of them participate in bacterial virulence [28], [13], [58], [60]. In addition, six FPI genes, including all the genes of the igl locus, were identified repeatedly in screens, confirming the key role of FPI-encoded proteins in *F. tularensis* virulence. These gene products are required for replication within macrophages and for escape from the phagolysosome. Part of the FPI encodes a Type VI-type secretion system. In other pathogens, Type VI systems are known to provide a mechanism for the “injection” of effector proteins into the host cytosol. It is likely that this system assists the bacterial cell in controlling its intracellular environment [58].

Six genes encoding proteins with functions in protein quality control were also repetitively identified as necessary for virulence: the peptidases Lon and CphB [96], [112]; the DsbA and DsbB proteins [78], involved in disulfide bond formation; the heat shock protein HspG [111]; and the heat-shock associated chaperone ClpB [24], involved in protein folding. Owing to their pleiotropic mode of action, these chaperones are likely to play an important role in the adaptation of *F. tularensis* to the stressful environment encountered during infection.

A number of essential metabolic genes have also been identified. These pathways may function in utilization of nutrients obtained from the host cell or in biosynthesis of essential building blocks of bacterial cell growth. Induction of the stringent response strongly influences the expression of virulence factors by *F. tularensis*; this stress response to carbon or nitrogen starvation is shared by many other pathogens (reviewed by Meiborn and Charbit [66]).

### 12.3 Vaccines

Efforts to develop a safe, effective vaccine against *F. tularensis* have primarily focused on three strategies:
• Live attenuated bacteria (such as the LVS vaccine);
• Inactivated whole cells; and
• Sub-unit vaccines.

The most successful approach to date is the attenuated Live Vaccing Strain (LVS) that (in humans) is administered through a scarification process. A recent review of experimental vaccines against tularemia is available [7].

**Live Vaccines-LVS** – A live attenuated vaccine strain was derived from a Type B strain of *F. tularensis* in the Former Soviet Union (FSU) during the 1930s. It was transferred to the United States in 1956; studies of LVS in humans demonstrated that this vaccine is partially protective in human challenge models. However, it fails to reliably protect individuals exposed to a high dose aerosol of the more virulent Type A strain [88]. A single dose of LVS induces measurable anti-LVS antibody and lymphocyte proliferation for a majority of humans within 14 days of vaccination [106], [107], [108].

LVS was derived by repeated passage of a Russian vaccine strain on peptone cysteine agar, giving rise to two phenotypic variants distinguishable by their colony color, grey or blue, when viewed under oblique light. The blue variant was able to induce a protective immune response in mice, whereas the grey variant was overly attenuated and did not induce immunity. The blue variant underwent further manipulations including lyophilization and serial passage through mice before being designated as *F. tularensis* Live Vaccine Strain (LVS).

Scarification followed by aerosol immunization with LVS was evaluated, and although aerosol vaccination induced more solid immunity in volunteers, most humans have received LVS through scarification. Despite being used successfully for decades to immunize large numbers of people (reportedly millions in the FSU), LVS has as yet failed to achieve licensing by regulatory authorities. The reasons for this are complex, but include a lack of understanding of the basis of attenuation, residual virulence, and the issue of the mixed blue/grey phenotype in vaccine lots. The original parental strain is not available for genomic comparison with LVS; thus, the mechanism of attenuation cannot be definitively pinpointed. However, virulence in mice has been “restored” to the strain (LD$_{50}$ similar to other Type B strains) by introducing the Type IV pilus and an outer membrane protein [85].

This vaccine is under Investigational New Drug (IND) status in the United States and is administered to laboratory workers at risk of exposure. The incidence of typhoidal tularemia decreased in laboratory workers following introduction of the vaccine to individuals at risk of exposure. Although the incidence of ulceroglandular tularemia did not decrease significantly, milder symptoms were observed in LVS-vaccinated personnel [14].

Based on the intracellular nature of *F. tularensis*, cell-mediated immunity is thought to be critical for protection, particularly against Type A strains. CMI persists in LVS-vaccinated humans for three decades. Vaccinates showed higher proliferative responses and, out of 17 cytokines assayed, higher levels of MIP-1β, IFN-γ, IL-10, and IL-5 in response to recall stimulation than controls. During recall stimulation, expression of IFN-γ by CD4+CCR7+, CD4+CD62L+, CD8+CCR7+, and CD8+CD62L+ cells significantly increased in samples from vaccinated donors [35].

**Live Vaccines – Next Generation** – The availability of genome sequence data for a range of *Francisella* strains has facilitated the process aimed at targeting important virulence and metabolic genes. As a result numerous
attenuated strains capable of eliciting protective immunity, yet limited in their ability to survive, replicate, and cause disease were created [62], [84]. Nonetheless, even the attenuated vaccines that meet these criteria may represent considerable risk to immunocompromised individuals.

A prime-boost approach strategy using a live LVS variant unable to produce capsule and a boost with a recombinant *Listeria monocytogenes* strain expressing the virulence factor IgIC conferred slightly better survival against a 10 LD<sub>50</sub> challenge of a Type A strain than the LVS alone [51].

**Inactivated Whole Cells** – Bacteria killed by heat or chemicals have generally failed to elicit protective immunity in either humans or animal models. This is not surprising since *F. tularensis* is an intracellular pathogen. Nevertheless, the combination of paraformaldehyde-fixed *F. tularensis* LVS administered in conjunction with IL-12 [6], combined with pre-formed immune complexes and CpG oligonucleotides [37] or targeted to Fc receptors via anti-*F. tularensis* LPS mAb [79] has shown some promise in recent murine studies. However, significant protection against a Type A strain challenge has not been demonstrated.

**Sub-Unit Vaccines** – Sub-unit vaccines comprised of *F. tularensis* antigens constitute a third approach to immunizing against *F. tularensis*. To date, only the O-antigenic component of the LPS molecule derived from *F. tularensis*, was found to elicit protective immunity in mice to systemic infections by Type B (but not Type A) strains of *F. tularensis* [23]. Therefore, additional immunoprotective bacterial antigens must be identified in order to prepare a sub-unit vaccine composed of more than a single antigen in order to elicit protective immunity. Immunoproteomic approaches may identify immunogenic proteins synthesized within the mammalian host. New methods to identify membrane protein complexes will allow identification of immunogen combinations more likely to be seen in vivo by the host immune system than the individual sub-unit proteins [31].

**Other Approaches** – Gregory *et al.* [45] reported inducing immunity to tularemia utilizing a vaccine construct consisting of detoxified O-polysaccharide side chain-deficient LPS complexed with an outer membrane protein of *Neisseria meningitides*. This vaccine induces Ab specific to the glycolipid core found within Gram-negative bacteria and is not specific to *F. tularensis*. Some protection was observed against a low (10 CFU) challenge with a virulent Type A strain. Native outer membrane proteins provided some protection to mice against a low dose SCHU S4 challenge and diminished bacterial burden in the spleen, liver, and lung [49]. In another approach, epitopes recognized by T-cells obtained from humans previously infected with *F. tularensis* were used to develop an epitope-based vaccine [65]. HLA Class II “humanized” mice were vaccinated and challenged with a low dose of the LVS strain (5 LD<sub>50</sub>). All control mice died and 60% of the immunized animals survived. Vaccinated animals also had a lower bacterial burden than control animals and a rapid cytokine response [44].

### 12.3.1 Immunity in Humans and Mice

In humans, infection with *F. tularensis* followed by subsequent recovery results in long-term immunity; there are few documented cases of reinfection [14]. Based on the intracellular nature of *F. tularensis* growth in vivo, cell-mediated immunity is thought to be critical for protection, particularly against Type A strains [102]. The precise mechanism(s) of immunity are not well understood; however, long-lasting humoral and cell-mediated responses have been documented in individuals who have recovered from tularemia [55], [36]. There is evidence for continued synthesis of IgM, IgA, and IgG for years after infection, which is unusual for infectious diseases [55]. It is unclear what stimulates this continued response; perhaps some bacteria or debris remain intracellularly. Peripheral Blood Monocytic Cells (PBMC) from donors who had been immunized with LVS or had recovered from tularemia produced significantly more cytokines when stimulated with *F. tularensis* antigens than naïve donors. Expression of IFN-γ, MIP-1β, and CD107a by CD4+CD45RO<sup>+</sup> or CD8+CD45RO<sup>+</sup> T cells most strongly discriminated (presumed) immune and naïve persons [36]. In addition to Th1 responses,
cytokines associated with Th17 are also expressed when PBMC from LVS-immunized humans are stimulated with *F. tularensis* antigens [72].

Cell-mediated immunity is thought to be the dominant factor in murine resistance to tularemia; both CD4+ and CD8+ T-cells are key elements in the efficient resolution of primary and secondary infections. Both IFNγ and TNFα are also critical factors in primary host defences to systemic infections in mice. It is speculated that IFN-γ and TNF-α synergize to promote nitric oxide production, and to regulate iron homeostasis and pH thus, limiting *F. tularensis* survival within macrophages, the primary site of intracellular replication *in vivo* [21]. However, mice inoculated intraperitoneally (i.p.) with immune sera exhibit Fcγ receptor-dependent protection from lethal intranasal (i.n.) challenge, suggesting that B cells and antibody production might play an additional role in immunity, at least in this animal model [53].

**12.3.2 In Vitro Correlates of Immunity**

Although cell-mediated immunity is clearly important in protection against tularemia, there are no established *in vitro* correlates of immunity. In humans, LVS induces *F. tularensis*-specific antibodies. In addition, circulating memory T-cells found after immunization produce IFNγ, IL-17A, and IL-22 following stimulation with *F. tularensis* antigens [52]. However, the importance of these cytokines in protection of humans is unknown. In early studies, stimulation of human lymphocytes obtained from LVS-immunized humans did not correlate with serum agglutinating antibody activity [101].

In *vitro* correlates of immunity in the mouse have also been pursued. Ryden *et al.* [84] described the comparison of five SCHU S4 strains with defined mutations affecting virulence. Although the mutants varied in their ability to protect BALB/c mice from challenge, antibody responses to the live vaccine strains were not significantly different when assessed using immunoproteomics. However, protection was associated with serum levels of TNFα, IFNγ, and MCP-1. Other potential correlates of protection were identified by De Pascalis *et al.* [29], who compared the response to LVS isolates with a range of protective efficacy. The ability of immune lymphocytes to control growth of *F. tularensis in vitro* was correlated with protection, as was the up-regulation of IFN-γ, IL-6, IL-12Rβ2, T-bet, SOCS-1, and IL-18bp. Due to the different bacterial and mouse strains, it is difficult to directly compare these two studies.

**12.3.3 Therapeutics**

Although it can be difficult to diagnose, tularemia does generally respond to antibiotic therapy. Streptomycin has been used extensively for the treatment of tularemia. Although there are adverse effects associated with this antibiotic, it is still considered to be the drug of choice by the U.S. Center for Disease Control based on efficacy and the Food and Drug Administration (FDA) licensure [15]. Treatment with gentamicin is an alternative possibility. Aminoglycoside treatment should continue for a 10-day period. Ciprofloxacin is not yet FDA-approved but has been efficacious in the treatment of tularemia; there is increasing amount of information documenting its safety and efficacy and also its suitability for the treatment of pregnant women and children [113], [48], [110]. Chloramphenicol is not recommended to be the first choice for therapy as it has been linked with serious side effects. Both chloramphenicol and tetracycline/doxycycline have been associated with a higher rate of relapse than the aminoglycosides. Ceftriaxone and other B-lactams are ineffective [33]. Erythromycin resistance is found in many European strains [113].

As prophylaxis for potential laboratory exposures, doxycycline (100 mg BID X 14 days) is generally recommended [16]. Ciprofloxacin is not FDA-approved for prophylaxis but has shown promise in many studies. Thus, it is listed as an alternative to doxycycline by the CDC [113], [16], [56].
Despite the availability of antibiotics for the treatment of tularemia, the potential exists for antibiotic-resistant 
*F. tularensis* to be developed by an adversary in the laboratory. Ciprofloxacin-resistant strains have been created 
via serial passage on media containing the antibiotic and the methodology has been published in the open 
literature [61]. During the Cold War, both the U.S. and the FSU constructed strains resistant to chloramphenicol 
and tetracycline [30]. Streptomycin resistance has been introduced in some laboratory studies [113].

Immune sera protects against lethal intranasal challenge with LVS in a murine model when administered 
24 – 48 hours post-exposure; however, it is unclear if this approach would be effective against a Type A strain 
challenge or in another animal model [53]. Protection required expression of Fcγ on phagocytes and production 
of IFNγ, but was independent of complement.

12.3.4 Animal Models

In nature, *F. tularensis* is capable of infecting hundreds of species. These include mice, voles, rats, squirrels, 
lemmings, rabbits, cats, and prairie dogs [1]. Humans are often infected through direct exposure to infected 
animals or through arthropod vectors. In the laboratory, development of animal models for tularemia has focused 
primarily on the mouse. Although this is the most economic model in terms of cost and space, the murine model 
has some drawbacks, particularly for vaccine efficacy testing. Other rodents, such as voles, guinea pigs and rats 
have also been employed. Larger animals (non-human primates, rabbits) have been evaluated as well, although 
much of this work is several decades old.

Mouse – Mice are commonly used as models for many infectious diseases. However, the attenuated human live 
LVS vaccine kills mice. Furthermore, mice that do survive LVS challenge are unprotected when challenged with 
a virulent Type A strain by the aerosol route [92]. This is of particular concern in the development of vaccine 
candidates, since the mouse model may very well yield data that does not reflect the way a particular product 
would perform in humans. However, despite their extreme sensitivity to lethal disease caused by *F. tularensis*, 
including strains highly attenuated in humans, mice continue to be the most commonly used animal model for 
tularemia. Advantages to the mouse as a model, in addition to the cost, include the diversity of murine strains, 
including those carrying knockout mutations in genes contributing to the immune response.

Due to the very high susceptibility of mice to wild-type strains, many investigators interested in immunity to 
tularemia and host-pathogen interactions have focused on the LVS strain, which is lethal for mice at higher doses 
than fully virulent strains, is safer for laboratory workers, and does not require BLS-3 containment. 
Unfortunately, the results cannot necessarily be extrapolated to fully virulent strains of *F. tularensis* or other 
animal models. An extensive review of LVS pathogenesis in the mouse was presented by Adamovicz and Waag 
[1]. Lethality of LVS in mice varied significantly by the route of infection with mice generally being the most 
sensitive to intraperitoneal (i.p.) challenge, followed by the intravenous, intranasal, aerosol, and intradermal (i.d.) 
routes [43]. For BALB/c or C57BL/6 mice, the i.p LD₅₀ is less than 5 CFU, while the i.d. LD₅₀ is > 10⁶ CFU [40]. The aerosol LD₅₀ is > 10³, logs higher than that observed with SCHU S4 (< 10 CFU).

A comparison of the LD₅₀ values for LVS using various routes of infection in several mouse strains suggests that 
the mouse could contribute to a better understanding of the role of host genetics in innate immunity to infection 
by this strain. Susceptibility to LVS varies between strains; C3H/HeJ, CBA/J, C57BL/6J, and A/J strains are 
more sensitive than SWR/J, SJL/J, BALB/cJ, AKR/J or the outbred CD-1 [92], [1].

Recently, Type A strains have been more commonly used in the laboratory than in previous decades; although 
challenging when using a mouse model, this is the appropriate route for development of medical countermeasures. 
Type A and B strains of *F. tularensis* have LD₅₀ values of 10 – 20 in BALB/c and C57BL/6 mice infected by
aerosol exposure [22]. The Type A strains have a shorter mean time to death than the Type B. Intradermal LD₉₀ for A- or B-type clinical strains are ~ 20 CFU. Type A *F. tularensis* are highly virulent for all mouse strains and by all routes of infection.

Mice are commonly used to evaluate the effect of specific mutations on the virulence of *F. tularensis* and to explore innate and acquired immunity [2], [21], [24], [60], [84]. Mice have been useful as a screening tool for therapeutic compounds with activity against *F. tularensis* [54], [76], [98]. Despite the disadvantages of the mouse model, it has been invaluable in understanding *F. tularensis* pathogenesis.

Mouse primary cells and cell lines have been extensively used for examining the intracellular existence of *F. tularensis*. More recently, some human cell lines have also been used. Differences between mice and humans in the host cell pathways and their interactions with *F. tularensis* have been noted [115], [26].

**Rat** – Rats are more resistant to infection by *F. tularensis* than mice. Significant differences between rat strains have been observed; for example, Fischer 344 rats were much more susceptible to an aerosol challenge with a Type A strain than the Sprague-Dawley strain [50]. Unlike mice, however, the Fischer rats were relatively resistant to LVS respiratory challenge [80]. Furthermore, this rat strain was partially protected from aerosol challenge by a Type A strain after LVS vaccination [114]. Additional studies in the rat suggest that disease progression differs between animals infected intranasally versus by aerosol [50]. Intranasal models of respiratory disease have become popular as substitutes for aerosol challenge; these results suggest that intranasal infections should be used with caution as models of pneumonic disease. A live vaccine based on *F. novicida* was recently shown to be somewhat protective against a low dose of SCHU S4 [93] in the rat. Although the rat has not been used often as a model for glandular tularemia, the pathology appears to be similar to that of humans and it may be an appropriate model for this form of tularemia.

**Rabbit** – Wild rabbits and hares are natural hosts for *F. tularensis*. As a model of pathogenesis, domestic rabbits resemble humans in terms of their relative sensitivity to Type A and Type B strains of *F. tularensis*. Intradermal infection of rabbits results in a disease similar to human ulceroglandular tularemia [89]. They also develop lethal disease from aerosol exposure, developing systemic disease with a pattern of disease progression similar to that of humans [8], [82]. *In vitro*, rabbit macrophages respond differently to Type A strains than to LVS; the phagocytes were more efficient at controlling LVS than the highly virulent strain [70]. Recently, rabbits were used to evaluate an improved LVS vaccine [73]. Direct comparisons between mice, guinea pigs and rabbits suggest that when comparing virulence between bacterial strains, the subcutaneous (s.c.) rabbit model is particularly useful for identifying strains that are of intermediate virulence [10].

**Non-Human Primates (NHP)** – Naturally occurring tularemia in NHP has been reported in zoos and primate centers. In a German zoo holding 35 Cynomolgus macaques (*Macaca fascicularis*), 12 NHP seroconverted and 6 animals succumbed to disease in a two-year period. The pathological findings were similar to those of humans with ulceroglandular disease, with local lymphadenopathy and systemic spread to liver, spleen, and lung. In this case, a murine tularemia outbreak is thought to have been the source of infection, either by ingestion of rodents by the NHP, tick-borne organisms, or contamination of water/food [64]. Similar pathology was observed in an African Green monkey (*Chlorocebus aethiops*) in a Hungarian zoo. The primates in this particular facility were described as “regularly hunting and consuming” rodents and birds [46]. In both zoos, Type B *F. tularensis* was isolated from the NHP. In the United States, an outbreak of tularemia in rhesus macaques (*Macaca mulatta*) occurred in 2010 in a primate center. Four animals were euthanized due to severe disease, while two more died within a day of birth. This was a Type B strain rather than the more virulent Type A strain type that is also found in North America. This primate center is also focusing now on rodent control efforts [39].
In the laboratory, Cynomolgus macaques (Cynos) are susceptible to infectious aerosols of both Type A (SCHU S4) and Type B strains, although the Type A strain is more lethal [104], [47]. Cynos vaccinated with LVS intracutaneously or by aerosol and challenged with $10^5$ CFU of SCHU S4 by the aerosol route develop a self-limited disease with a delay in dissemination and decreased bacterial burden when compared to unvaccinated controls, which die within 7 days. Immunization by aerosol appears to be more effective than other methods of administering LVS. Unvaccinated Cynos are less sensitive to a Type B strain than the Type A, with a mortality rate of 18% at an inhaled dose of $10^6$ CFU. In early studies, vervet monkeys (Chlorocebus sp.) appeared to be more susceptible to *F. tularensis* than Cynos, although the vervets were challenged intranasally rather than by aerosol [9]. More recently, it was found that African Green Monkeys infected with *F. tularensis* by aerosol develop lethal multi-systemic disease that particularly targets the lungs and lymphoid tissues; based on a limited number of animals, it appears that this model is suitable for further development [105]. The rhesus macaque has also been used as a model, with an estimated i.d. LD$_{50}$ of ~10 CFU and aerosol LD$_{50}$ of 14 CFU for a small particle aerosol of a Type A strain [34], [27], [90]. Marmosets (*Callithrix jaccus*) are also susceptible to *F. tularensis* and were used for a recent study evaluating the efficacy of levofloxacin in the treatment of tularemia [68], [69]. In all of these NHP models, infection with a Type A strain is fatal if the animals are not vaccinated with *F. tularensis* by aerosol or treated with antibiotics. Although there is not a great deal of recent work with NHP models of tularemia, these NHP appear to be promising models for both vaccine and therapeutic efficacy trials [34].

12.4 CONCLUSIONS

Both the United States and the Former Soviet Union (FSU) weaponized this organism during the Cold War. The high morbidity and mortality associated with respiratory tularemia, the extremely low infectious dose (< 10 organisms in humans), and the hardiness of *F. tularensis* in the environment make this a particularly dangerous pathogen. The most serious scenario is one in which a large-scale aerosol release takes place over a highly populated area. The low infectious dose would lead to a large number of incapacitating casualties and, in some cases, death. The resulting disease would be difficult to distinguish from other febrile illnesses such as influenza. Early in the disease course, symptoms would also be similar to those resulting from exposure to other agents such as *Yersinia pestis* or *Bacillus anthracis*. Unlike *Y. pestis*, however, person-to-person transmission has not been documented for this disease.

The lack of a licensed vaccine is a significant gap for countermeasure development. Live attenuated vaccines, such as LVS, have been used extensively in humans. However, LVS has only an IND status in the U.S. Although this vaccine appears to protect humans against some forms of the disease, it is not efficacious against higher doses of a Type A strain when individuals are exposed by the aerosol route. Furthermore, it has not been licensed, primarily due to a lack of understanding of the basis of attenuation, the residual virulence, and the issue of multiple phenotypes in vaccine lots. In terms of protective efficacy against an intracellular pathogen, a more genetically defined live vaccine strain would likely be preferable over a killed or sub-unit vaccine for future development. However, the U.S. FDA is less likely to license a live vaccine than a killed or sub-unit product and this is driving research toward the latter areas. Current efforts to better understand the immune response, particularly cell-mediated immunity to tularemia, will assist in identifying new approaches. These may consist of *F. tularensis* protective antigens expressed by other organisms capable of intracellular growth. Advances in bacterial genetics and proteomics will provide important information about immunoprotective antigens. This is reflected by rapid progress in sequencing of genomes of *F. tularensis* strains and identification of a plethora of genes encoding possible candidates for virulence factors. Unfortunately, most of these genes exhibit no homology to known virulence factors of other microbial species; thus their biological function is unclear. Novel adjuvants should also be explored. Correlates of immunity are poorly understood and must be pursued in appropriate animal models.
Despite the availability of antibiotics for the treatment of tularemia, the potential exists for antibiotic-resistant *F. tularensis* to be developed by an adversary in the laboratory. Recent advances in genetic modification of *F. tularensis* have increased the possibility of constructing a multi-drug-resistant strain. Development of antibiotics with novel modes of action that would be useful for a number of biothreat agents may be facilitated as we gain a better understanding of bacterial pathogens, common virulence mechanisms, and their host interactions.

The extreme susceptibility of the mouse to *F. tularensis* is a challenge when attempting to identify promising vaccine strategies. Although the majority of vaccine studies have utilized mice, these animals respond very differently to LVS than humans. Mice are susceptible to lethal disease caused by this live vaccine strain. Furthermore, it is extremely difficult to protect mice against a Type A strain, particularly when using a respiratory challenge; thus only very low challenge doses may be used. Direct comparisons of live vaccine studies have been hampered by the diverse bacterial strain backgrounds and the lack of consistency in the mouse strains used. Animal model development for tularemia, including animals other than the mouse, should be emphasized in future efforts.

12.5 REFERENCES


[24] Conlan JW, Shen H, Golovliov I, Zingmark C, Oyston PC, Chen W, House RV and Sjostedt A. Differential ability of novel attenuated targeted deletion mutants of Francisella tularensis subspecies tularensis strain...


[92] Shen H, Chen W and Conlan JW. Susceptibility of various mouse strains to systemically- or aerosol-initiated tularemia by virulent type A Francisella tularensis before and after immunization with the attenuated live vaccine strain of the pathogen. Vaccine. 2004 Jun 2;22(17-18):2116-21.


Disclaimer: Animal research at The United States Army of Medical Research Institute of Infectious Diseases was conducted and approved under an Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.
Chapter 13 – BURKHOLDERIA PSEUDOMALLEI AND BURKHOLDERIA MALLEI

Joseph C. Larsen
U.S. Department of Health and Human Services
Office of the Assistant Secretary for Preparedness and Response
Biomedical Advanced Research and Development Authority, Washington, DC
UNITED STATES

Corresponding author (Larsen) email: joseph.larsen@hhs.gov

13.1 INTRODUCTION

Burkholderia pseudomallei is a Gram-negative, facultatively anaerobic, motile Bacillus that is the causative agent of melioidosis. The bacterium is a soil saprophyte, often present in wet soils and rice paddies in Southeast Asia. Melioidosis occurs after the organism contaminates superficial breaks in the skin or via inhalation. The clinical presentation of melioidosis ranges from an acute febrile illness to disseminated septicemia [74]. The septicemic form of infection is rapidly fatal, with a mortality rate of approximately 40%, and death occurs 24 – 48 hours following the onset of symptoms [75]. Abscess formation in the lungs, liver, spleen, and skeletal muscles are the hallmarks of infection. Sub-clinical infections are common and may reactivate after numerous years of dormancy. Impairment of the immune system, diabetes, alcoholism, renal disease, malignancies, steroid therapy, and tuberculosis are all risk factors for melioidosis [12].

B. mallei is a Gram-negative, aerobic, non-motile Bacillus that causes glanders. It is a host-adapted pathogen that is not capable of environmental persistence. Because of quarantine and other measures, glanders has been eradicated from most countries. Small pockets of zoonotic endemicity still exist in the Middle East, Asia, Africa, and South America [11]. Glanders is highly transmissible among solipeds (horses, donkeys, and mules). In horses, acute glanders presents as fever accompanied by necrotic ulcers and nodules in the nasal passages. Lymph nodes of the neck and mediastinal regions are enlarged and pneumonia with dissemination to internal organs can occur. In humans, the disease can be acute or chronic. With respiratory exposure, acute febrile illness with ulcerative necrosis of the upper respiratory tract can occur. Patients present with mucopurulent discharge from the nose, lips, and eyes. Neck and mediastinal lymphadenopathy, pustular skin lesions, and septicemia can follow. The mortality rate in the pulmonary form of glanders is 90 – 95 % if untreated and 40% if treated with antibiotic therapy [11].

B. mallei was introduced as a biological weapon early in the twentieth century [11]. During the First World War, German sympathizers in various countries infected equines destined for conflict areas with B. mallei [22], [34], [50]. Combat operations were affected by infection of humans and horses [73]. In 1925, the Geneva Protocol prohibited the use of bacteriological warfare [50]. Prior to the Second World War the Soviet Union and Japan studied the use of B. mallei as a biological weapon [34], [50]. During the Second World War, the Japanese infected Chinese prisoners with B. mallei [50]. Several countries, including the United States and the Soviet Union, studied B. pseudomallei for potential offensive intent [72], [80]. In 1972, the “Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction,” was introduced [34] which prohibited the offensive research on biological agents. Since then, the Soviet Union was accused of using B. mallei in Afghanistan during its occupation in the early 1980s [50]. There have been no reports of the malicious use B. mallei or B. pseudomallei in recent years.
13.2 GENOMICS

The genome sequences of *B. pseudomallei* and *B. mallei* have been reported [28], [43]. The *B. pseudomallei* genome consists of two chromosomes of 4.07 and 3.17 Mb. There is evidence of significant horizontal gene transfer, indicated by the presence of 16 different genetic islands distributed across the genome. These regions possess homology to genes associated with mobile genetic elements, such as insertion sequences, plasmids, and bacteriophage. The *B. pseudomallei* genome contains multiple genes predicted to be involved in environmental survival and virulence. Drug resistance genes, including predicted drug efflux pumps, β-lactamases, and aminoglycoside acetyltransferases were identified, reflecting the intrinsic high levels of drug resistance exhibited by *B. pseudomallei*.

Numerous protein secretion systems typically involved in pathogenesis of other bacterial agents were identified in the genome of *B. pseudomallei*. Several protein secretion systems (Types I, II, and V) and three Type III Secretion Systems (T3SS) are predicted to be encoded in the genome. The *B. mallei* genome possesses four gene clusters encoding Type VI Secretion Systems (T6SS). Genes predicted to encode cell surface adhesion proteins and fimbriae were also detected [28]. The *B. mallei* genome is significantly smaller than *B. pseudomallei*, consisting of 5.7 Mb residing in two chromosomes (3.5 and 2.3 Mb). The genome harbors insertion elements that account for approximately 3.1% of the sequence. These elements likely mediate the extensive genome-wide insertion, deletion, and rearrangements that have resulted in the genome reduction observed relative to *B. pseudomallei*. Approximately 1,400 genes present in *B. pseudomallei* are either missing or are variant in *B. mallei*. Flagellar and chemotaxis genes, for example, have undergone frameshift or insertional mutation, resulting in defective proteins critical for function. This suggests that portions of the *B. mallei* genome are undergoing active genomic decay, possibly to evolve further to a lifestyle within a mammalian host [43].

Comparative analysis of *B. pseudomallei* and *B. mallei* with other non-pathogenic *Burkholderia* species provides insight in the mechanism of divergence and evolution of these organisms. It is believed that *B. mallei* evolved from a single strain of *B. pseudomallei* [19]. Much of the gene loss observed by *B. mallei* is due to the presence of Insertion Sequence (IS) elements. Homologous recombination across these numerous IS elements led to the extensive genomic reduction observed with this organism [33]. All isolates of the non-pathogenic *B. thailandensis* can utilize L-arabinose as a sole carbon source, although *B. pseudomallei* cannot. Analysis of the *B. pseudomallei* and *B. mallei* chromosome revealed the presence of a deletion of the arabinose operon in all strains examined [38]. The entire *B. thailandensis* arabinose operon was cloned into *B. pseudomallei*. The resulting strain exhibited a decrease in virulence (25- to 50-fold) in the Syrian hamster model compared to the parent, wild-type strain [38]. Microarray analysis revealed that a number of genes in the Type III Secretion System (T3SS) were down-regulated when cultured in the presence of arabinose. This suggests that the loss of the genes required for the metabolism of arabinose was a pathoadaptive mutation that may provide *B. pseudomallei* with a selective advantage for survival in animal hosts [38]. Similar pathoadaptive mutations are present in a number of bacterial pathogens, suggesting that the loss of genes incompatible with a pathogenic lifestyle is a common mechanism of niche adaptation [35].

A number of studies were conducted to identify genes required for virulence or host survival. Transposon mutagenesis was utilized to screen for *B. pseudomallei* mutants attenuated in their ability to invade cells in vitro and survive in vivo [44], [10]. Mutant genes involved in amino acid biosynthesis, capsular polysaccharide synthesis, DNA replication and repair, a putative lipoprotein, ABC transporters, and a putative oxidoreductase were found to be significantly attenuated in vivo, suggesting that these pathways play a critical role in allowing for survival and replication within the mammalian host.

A set of 650 putative *B. pseudomallei* and *B. mallei* virulence-related genes were compiled utilizing a computer-based analysis of the genomes of *B. pseudomallei* and *B. mallei*, and 5 non-pathogenic *Burkholderia* species.
To rapidly evaluate these mutants in an animal model, a wax moth (Galleria mellonella) larvae system was developed [53]. Pathogenic strains of B. pseudomallei and B. mallei, but not non-pathogenic-related bacteria, were found to be highly pathogenic for this insect. Three new mutants in B. mallei were identified that were reduced in their lethality in the wax moth larval model. These genes encode proteins with homology to a polyketide synthase, a putative exported protein in Bordetella bronchiseptica, and a protein involved in amino acid biosynthesis [53].

Expression analysis of B. pseudomallei in the Syrian hamster was conducted to determine the bacterial genes that are up- or down-regulated in the mammalian host [65]. Syrian hamsters were administered intraperitoneal or intranasal challenges of B. pseudomallei, liver, lungs, and spleens were collected, and expression analysis performed using whole genome microarray. Expression levels were compared to bacteria grown in culture broth. In concurrence with the aforementioned study, genes for specific energy production pathways and amino acid biosynthesis were up-regulated. A phospholipase C, which is a virulence factor in several other bacterial species and a two-component regulatory system were detected. Aside from genes for the biosynthesis of amino acids, there is no common set of genes detected in these studies that would suggest a core genetic armamentarium that is required for host colonization or pathogenesis. The inability to identify a common set of virulence genes in these organisms has impeded our understanding of the molecular mechanism of pathogenesis of these agents. It may be that the model systems employed to date are not entirely reflective of the host environment and thus have failed to identify bacterial factors that are essential for productive in vivo infection.

13.3 VIRULENCE DETERMINANTS

13.3.1 Capsule

Bacterial surfaces are commonly decorated with various polysaccharide structures, including lipopolysaccharide and capsules. These structures contribute to the pathogenesis of a number of bacterial pathogens. Subtractive hybridization was utilized to identify genetic loci within the B. mallei and B. pseudomallei genomes that are not present in the related, non-pathogenic B. thailandensis to identify genes that contribute to virulence [47], [14]. Sequencing of these loci revealed the presence of genes involved in the biosynthesis, export, and translocation of capsular polysaccharide. Mutation of these genes in B. mallei results in strains that fail to react with polyclonal antisera raised against the B. pseudomallei capsule, suggesting that the capsules are structurally similar between the two species. B. mallei capsule mutants display significant attenuation in the Syrian hamster challenge model and the BALB/c aerosol challenge model. There was a greater than 10^5 -fold difference in lethal dose (LD50) between the wild-type and capsule mutants in the Syrian hamster model. There was a 10^3-fold difference in LD50 in the mouse model between the wild-type and mutant strains. Capsule mutants in B. pseudomallei display similar reductions in virulence with LD50,8 of 10^5 Colony Forming Units (CFU) in the mouse aerosol challenge model. Further, the addition of purified capsule decreased the LD50 (from 3.5 × 10^5 to 34 CFU) of B. pseudomallei capsule mutants in the Syrian hamster [48]. These data suggest that the capsule is an essential virulence determinant in B. mallei and B. pseudomallei. Studies to determine the mechanistic contribution of the capsule to the virulence of B. pseudomallei were performed [48]. The B. pseudomallei capsule was required for persistence in the blood as capsule-deficient mutants were recovered from the several organs of the Syrian hamster at lower levels than wild type. Purified capsule restores the ability of acapsulated strains to survive in human serum. Western blot and immunofluorescence analyses demonstrated that the deposition of complement factor C3b is enhanced in acapsular mutants, suggesting that the persistence of B. pseudomallei in blood is because of the prevention of opsonization by complement.
13.3.2 Protein Secretion

T3SSs are Gram-negative protein secretion systems, ancestrally related to flagella, capable of injecting protein substrates into the cytosol of a eukaryotic cell. Present in a number of plant and animal pathogens, T3SSs are critical for pathogenesis. Both *B. mallei* and *B. pseudomallei* encode a T3SS, which shares genetic similarity to T3SSs found in *Salmonella* and *Shigella flexneri* [68]. Mutation analysis demonstrated that the T3SS is required for intracellular growth and phagosomal escape, suggesting that protein secretion through this system is critical to the pathogenesis of *B. pseudomallei* and *B. mallei* [49], [68], [60]. Mutants in the *B. mallei* T3SS are attenuated in the BALB/c mouse aerosol challenge model and the Syrian hamster intraperitoneal challenge model [68]. The role of specific secretion substrates of the *Burkholderia* T3SS has been investigated. BopE, a protein encoded within the *Burkholderia* T3SS loci, is secreted in a T3SS-dependent manner. Mutants in BopE have reduced ability to invade non-phagocytic cells. BopE shares homology to SopE, a secreted T3SS effector protein involved in the uptake of *S. typhimurium* by non-phagocytic cells [82]. When expressed in eukaryotic cells, BopE-induced cytoskeletal rearrangements. *In vitro*, BopE possesses guanine nucleotide exchange activity for Cdc42 and Rac1 proteins involved in actin cytoskeleton regulation [59]. These data suggest that BopE is injected in T3SS-dependent mechanisms into cytosol of non-phagocytic eukaryotic cells where it induces the necessary cytoskeletal rearrangements to facilitate cellular invasion. Although this process is well studied in organisms such as *Salmonella* and *Shigella*, the characterization of a secreted virulence factor in *Burkholderia* allows for mechanistic commonalities to be identified across these diverse bacterial pathogens. Mutation of a predicted structural component of the T3SS, BipD, results in significant attenuation following intraperitoneal or intranasal challenge in mice [58]. BipD shares homology with SipD, a T3SS protein in *Salmonella* that is required for the secretion of protein substrates [9]. The course of bacterial replication in the liver and spleen of mice after challenge was markedly reduced in *BipB* mutants. These data implicate T3SS as a major contributor to the molecular pathogenesis of *B. mallei* and *B. pseudomallei*.

*B. pseudomallei* and *B. mallei* are capable of intracellular survival and spread. To facilitate this process, *B. pseudomallei* and *B. mallei* utilize actin-based motility, a process that employs elements of the cellular cytoskeleton to propel the bacterium through the eukaryotic cell cytosol. *Shigella flexneri*, *Rickettsia rickettsii*, and *Listeria monocytogenes* all possess cell surface proteins that facilitate this process and that nucleate and polymerize actin [18]. These proteins, known as autotransporters, are a class of bacterial proteins that mediate their own secretion and/or membrane localization [26]. A computational search of the *B. pseudomallei* genome revealed the presence of 11 predicted autosecreted proteins [57]. One of these proteins, designated BimA, possesses proline-rich region commonly found in proteins that stimulate actin polymerization [27]. Mutation of BimA abolished the ability the *B. pseudomallei* to form membrane protrusions in *vitro*, suggesting a defect in actin-based motility. Inactivation of BimA did not affect the function of the T3SS, as the bacteria retained the ability to escape from the phagosome. *In vitro* studies determined that BimA directly interacts with actin [57]. A series of cellular localization studies demonstrated that the actin-based motility involves only a sub-set of cellular proteins involved in intracellular spread of other bacterial pathogens, suggesting the molecular mechanism of intracellular spread employed by *Burkholderia* may differ from other bacterial species [5]. The *in vivo* contribution of BimA to the pathogenesis of *B. mallei* was assessed [54]. Mutation of BimA did not affect virulence in the Syrian hamster, suggesting that cell-to-cell spread and actin-based motility may not be required for virulence. In 2007, Schell et al. reported the presence of a Type VI secretion system (T6SS) in *B. mallei* [54]. T6SS is a newly identified family of Gram-negative protein secretion system that has been implicated in the pathogenesis of *Vibrio cholerae* and *Pseudomonas aeruginosa* [40], [46]. Both *B. mallei* and *B. pseudomallei* contain additional genes that possess homology to T6SSs, suggesting there may be multiple T6SSs in pathogenic *Burkholderia* species. SDS-PAGE and mass spectroscopy were used to identify proteins secreted into culture supernatants upon induction of protein secretion. A number of proteins were identified. One protein, Hep1, was found to be secreted in a T6SS-dependent manner. Hep1 and other T6SS genes are
essential for full virulence in the hamster model of infection. In these experiments, groups of five hamsters were challenged at increased doses (10^3 – 10^4 CFU) with wild-type B. mallei or T6SS mutants. All hamsters challenged with 10^2 – 10^4 CFU of wild-type B. mallei succumbed to infection. All of the hamsters challenged with the mutant strains survived. Analysis of a mutant T6SS mutant, TssE, demonstrated that the system is not required for phagosomal escape, but was essential for multi-nuclear giant cell formation and intracellular spread, suggesting the T6SS plays a critical role in the pathogenesis of B. mallei [54], [6].

Proteins secreted by B. mallei are currently undergoing characterization. TssM, a protein under control of the virAG regulon and is genetically linked to one of the B. mallei T6SS loci [55]. TssM was found to be secreted into culture supernatant in vitro and to possess deubiquinase activity. The protein is not secreted in a manner dependent on the T6SS, but was expressed within 1 hour of infection of macrophages, suggesting a role in vivo. Additional studies have been conducted to elucidate the role of TssM in the pathogenesis of B. pseudomallei. TssM was shown to interfere with several key signaling proteins such as TNFR-associated factor 3, TNFR-associated factor 6, and IκBα. A TssM mutant strain demonstrated a more pronounced inflammatory response and infected mice succumbed more rapidly to infection, suggesting that TssM play a significant role in the modulation of the host response [61]. The identification of additional T6SS substrates and their respective role in host – pathogen interactions will be essential to understanding the molecular mechanism of pathogenesis of these agents.

### 13.3.3 Quorum Sensing

A substantial number of bacterial species utilize a cell density-dependent system of genetic regulation known as quorum sensing. These systems are based upon the production of N-acetyl homoserine lactones known as autoinducers. The LuxI protein is responsible for synthesis of the autoinducer. The LuxR family of proteins are transcriptional regulators that respond to sufficient concentrations of autoinducer. The amount of autoinducer present in the environment is dependent on the cell density of the bacteria. Quorum sensing systems induce or suppress the expression of multiple target genes and are involved in pathogenesis. A computational search of the B. mallei genome revealed the presence of two LuxI and four LuxR homologs. A search of the B. pseudomallei genome identified three LuxI and five LuxR homologs. Quorum sensing mutants of both B. mallei and pseudomallei were attenuated in the BALB/c mouse aerosol challenge model and the Syrian hamster intraperitoneal challenge model [69], [70]. Significant increases in LD_50s and increased survival rates and decreased organ colonization were exhibited by the quorum sensing mutants. The identity of genes regulated by the B. mallei and B. pseudomallei quorum sensing systems is unknown. An understanding of the contribution of these genes to pathogenesis will provide valuable information regarding the disease process.

### 13.3.4 Host Response

Interferon gamma (IFN-gamma) is a major contributor to host survival in an acute and chronic B. pseudomallei infection models. Taylor Outbred (TO) mice infected with 10^6 CFU of B. pseudomallei present with acute septic shock and death within 2 days following exposure [51]. At lower doses, TO mice are able to clear the initial infection from the liver and spleen, but the organism persists at other sites resulting in a chronic infection that lasts 2 to 16 months and eventually results in death. Administration of a monoclonal antibody against IFN-gamma lowered the LD_50 from 5 X 10^5 to approximately 2 CFU. Further, bacterial burdens were 8,500-fold and 4,400-fold higher in the liver and spleen. Studies in IFN-gamma knock out mice have confirmed these results, as IL-12 or IFN-gamma knockout mice succumb to infection much more rapidly than wild-type mice (less than 4 days versus 30 days) [23]. These results suggest that IFN-gamma plays an essential role in host protection from acute B. pseudomallei infection.
Analysis of cytokine levels between the innately susceptible mouse strain BALB/c and the resistant strain C57BL/6 provides additional insight to the immunopathology of melioidosis [66]. A comparison of these responses showed that production of cytokines associated with the development of TH1 or TH2 immune responses were minimal or non-existent. The relative induction of cytokines, particularly IFN-gamma was greater in BALB/c mice, suggesting a role of this cytokine in the immunopathology of melioidosis.

In C57BL/6 mice, neutrophils are rapidly recruited to the lungs following intranasal exposure to B. pseudomallei [48]. Prevention of neutrophil infiltration by antibody depletion results in a 1000-fold increase of bacterial loads in the lungs four days following infection. Levels of TNF-alpha, IFN-gamma, and IL-6 were significantly decreased in neutrophil depleted mice [16]. C57BL/6 mice incapable of producing IFN-gamma were sensitive to infection, with mice succumbing to infection 3 days following challenge. These data suggest that neutrophils play an important role in the early immune response against B. pseudomallei.

The role of cell mediated responses to B. pseudomallei infection in humans was examined [49]. Peripheral Blood Mononuclear Cells (PBMC) were collected from 13 individuals who were exposed to B. pseudomallei in Papua New Guinea. Of these individuals 5 had a clinical history of melioidosis and 8 were seropositive but had not become clinically ill [2]. Lymphocyte proliferation and IFN-gamma levels were determined following in vitro antigen stimulation using a B. pseudomallei lysate. There was a statistically significant difference in both lymphocyte proliferation and IFN-gamma production in individuals with sub-clinical melioidosis. Reasonably, cell mediated immunity may be an important factor in the outcome of infection with B. pseudomallei.

Interleukin-18 was shown to play a role in the outcome of B. pseudomallei infection, as well. IL-18 KO mice have increased bacterial loads, elevated liver inflammation, and show an accelerated mortality following experimental infection [77]. Additional clinical studies demonstrated the correlation between reduced IL-18 and IL-18BP levels and positive clinical outcomes of severe melioidosis patients [77].

Toll-Like Receptors (TLR) are essential components of the innate immune response that are capable of detecting conserved molecular patterns across microbial species. The contribution of TLRs to the host defence against melioidosis was assessed [78]. The expression pattern of TLRs in monocytes and granulocytes was determined. Increased expression of several TLRs including TLR1, TLR2, TLR4, and CD14 were associated with B. pseudomallei infection. Using cell lines transfected with either TLR2 or TLR4, it was demonstrated that the LPS from B. pseudomallei signals through TLR2 and not TLR4. Intranasal infection of TLR2 or TLR4 knockout mice showed increased survival rates in TLR2 knock out mice, with 40% of mice surviving until the end of the 6 week observation period. In contrast, all wild-type and TLR4 knockout mice died 5 days after infection. At 72 hours post-infection there was a statistically significant difference in the bacterial loads in the lung and spleen in the TLR2 knockout mice. These results suggest that B. pseudomallei LPS signals through the TLR2 pathway and this signaling contributes negatively to the pathology of melioidosis. Further supporting these data, CD14, a pattern recognition protein involved in the detection of several ligands from Gram negative pathogens contributed to the deleterious response to B. pseudomallei infection [79]. CD14 knock out mice intranasally inoculated with B. pseudomallei demonstrated reduced lethality and bacterial burdens compared to wild-type. This suggests that CD14, possibly through its interaction with TLR2, is crucially involved in the innate recognition of B. pseudomallei but that interaction is ultimately detrimental to mounting an effective the host response.
13.4 MEDICAL COUNTERMEASURE DEVELOPMENT

13.4.1 Current Therapies

At present, there are no FDA-approved anti-microbials for either Post-Exposure Prophylaxis (PEP) or treatment for *B. pseudomallei* or *B. mallei* infection. All antibiotics used for PEP or for treatment would be used off-label or would be granted Emergency Use Authorization status by the FDA.

Conventional treatment of melioidosis is divided into two phases; the intravenous intensive phase and the oral eradication phase. Ceftazadime, meropenem, imipenem, or amoxicillin-clavulanate are given intravenously until the patient’s temperature has returned to normal for more than 48 hours. Following the resolution of the acute phase, doxycycline and trimethoprim-sulfamethoxazole are given in combination for 12 – 20 weeks to ensure eradication [81]. Even with prolonged antibiotic therapy, patient relapses are common [17], [13]. Treatment of *B. pseudomallei* is difficult because of its natural resistance to a number of antibiotics. The presence of several multi-drug efflux pumps implicated in resistance to aminoglycoside and macrolide antibiotics in *B. pseudomallei* have been reported [37], [8], [39]. Typically, *B. pseudomallei* are sensitive to trimethoprim-sulfamethoxazole, chloramphenicol, tetracyclines, third generation cephalosporins, carbapenems, and amoxicillin-clavulanate [74].

There is much less information describing the treatment or management of patients infected with *B. mallei*. Laboratory exposures have been reported and provide some insight into the antibiotic treatment regimens required to resolve the disease [7]. The antibiotic sensitivity profile of *B. mallei* is similar to *B. pseudomallei*, except that *B. mallei* is sensitive to gentamicin and certain macrolides (clarithromycin, azithromycin) while *B. pseudomallei* is resistant [11].

13.4.2 Animal Models

The development of animal models which recapitulate or closely mimic human pathology of infection is critical to obtaining regulatory approval of medical countermeasures. This is particularly evident with many biodefense pathogens, as medical countermeasure efficacy cannot be evaluated directly in human clinical studies. At present, there are no animal models for *B. pseudomallei* or *B. mallei* which fulfill the requirements necessary to support the regulatory approval of a candidate medical countermeasure. There are three routinely used small animal models to study the pathogenesis of and immunological response to *B. pseudomallei* [62], [29], [31]. Most studies have utilized mice to identify virulence factors, and due to the availability of laboratory reagents, characterize aspects of the immune response to infection. There is significant variance in the susceptibility, pathology, and outcome of infection with several different mice strains [62]. Common differences observed include variation in susceptibility dependent on: route of challenge, variance in the location and severity of abscesses, and the presence of latent infection. The hamster and infant diabetic rat models are utilized experimentally, albeit with much less frequency than the murine model. Hamsters are extremely susceptible to infection, with the LD$_{50}$ calculated as approximately 10 CFUs [15], [30]. Infant diabetic rats are less susceptible, with LD$_{50}$s calculated at approximately $10^3$ – $10^6$ CFUs, depending on the challenge strain used. Further, the rats are only susceptible up to 4 weeks old. Both the hamster and infant diabetic rat models are acute models of infection [62].

Large animal model development and characterization of *B. pseudomallei* infection has lagged significantly behind the small animal models. There are preliminary reports of the experimental evaluation of melioidosis in goats [41]. Abscesses resulted from infection, but the anatomical locations were dependent on the route of inoculation. Further, the clinical presentation of horses inoculated intratracheally with *B. mallei* was reported [32]. The horses presented with the clinical signs of glanders and *B. mallei* were collected from additional
anatomical sites, suggesting bacterial dissemination. The use of horses, while costly, could represent a useful model for the evaluation of candidate medical countermeasures.

There are a limited number of reports describing naturally occurring infections of Non-Human Primates (NHP) with *B. pseudomallei* [56], [63]. In macaques, clinical signs of infection included wasting, cough, nasal discharge, and mild respiratory disease, which can lead to acute fulminate bronchopneumonia with abscess formation. There are no published reports of aerosol exposure of NHPs with *B. pseudomallei* or the evaluation of candidate medical countermeasures using NHPs as a model system.

### 13.4.3 Candidate Anti-Microbials

Passive immunotherapies have been evaluated for their ability to provide protection from *B. pseudomallei* and *B. mallei* exposure. Monoclonal antibodies targeting capsular polysaccharide, LPS or surface proteins developed, pooled or tested individually for their ability to protect mice from a lethal challenge of *B. pseudomallei* [29]. The antibodies were administered immediately prior to challenge to mimic high titers of antibodies that would be induced following vaccination and not to mimic a post-exposure prophylaxis of treatment of infection. At high challenge doses (greater than $10^6$ CFU), none of these antibodies were protective. At challenge doses of $10^4$, the antibodies were effective at decreasing the mortality from the initial phase of infection, but provided little protection upon repeated administration at later time points. These data would suggest that antibody therapy does not allow for the elimination of the bacteria and thus allows for the establishment of a chronic infection. Antibodies targeting *B. mallei* LPS were evaluated for their ability to protect mice from lethal challenge [64]. In this study, the antibodies were administered 18 hours prior to challenge with $2 \times 10^4$ CFU. All antibodies were provided significant protection from the acute phase of illness. Since these studies concluded on day 14 following challenge, it is impossible to determine if these antibodies provide any significant protection from possible recrudescent infection.

Immunomodulatory therapies, which stimulate the innate immune response, have been evaluated as candidate medical countermeasures. Cationic Liposome DNA Complexes (CLDC) were administered to mice before or shortly following lethal challenge of *B. mallei* and *B. pseudomallei*. Complete or nearly complete protection was observed and protection appeared dependent on the presence of IFN-$\gamma$ [20]. When CLDC treatment was combined with anti-microbial therapy (ceftazadime) 100% mice challenged with lethal doses of *B. pseudomallei* survived the acute phase of infection [45]. Further, approximately 55% of mice survived until day 60 post-challenge and spleens, lung, and livers of these animal all had decreased bacterial burdens, suggesting that the combination of CLDC and anti-microbial therapy may aid in the clearance of the organism and prevention of recrudescent infections. It is important to note, however, that the administration of therapy began 6 hours following challenge, and would be considered a more general use prophylaxis versus post-exposure prophylaxis or treatment. Studies providing data on the therapeutic window of opportunity for CLDC therapy for *B. pseudomallei* have not been reported.

As aforementioned, exposure to *B. pseudomallei* often results in a chronic and/or latent pulmonary infection. The identification and characterization of bacterial or host factors involved in the establishment or maintenance of persistence will be critical to the development of therapies that lead to eradication. Other bacterial pathogens, such as *Mycobacterium tuberculosis*, require enzymes involved in fatty acid metabolism to establish bacterial persistence [36]. One of these enzymes, isocitrate lyase, was identified as a factor involved in the establishment of persistent infection in *B. pseudomallei* [71]. Inhibiting the activity of the *B. pseudomallei* isocitrate lyase resulted in a reversion of an experimental pulmonary chronic lung infection to an acute state. Inhibition of the enzyme did not attenuate the virulence of *B. pseudomallei*, as the resulting acute infection was lethal in the absence of anti-microbial treatment. The development of isocitrate lyase inhibitors, however, may facilitate the prevention of chronic or recrudescent infections with *B. pseudomallei*. 
Antisense therapy has been a candidate anti-microbial area which has shown promise in recent years. Phosphorodiamidate Morpholino Oligomers (PMOs) are modified oligomers, which are resistant to degradation by RNases. When conjugated to anti-microbial peptides, these PMOs possess increased cell permeability. A peptide modified PMO targeting the acyl carrier protein, which is an essential bacterial gene involved in lipid biosynthesis, was evaluated for its ability to treat *Burkholderia cepacia* infection [21]. The peptide modified PMOs targeting acyl carrier protein were bactericidal *in vitro* and treated mice demonstrated a significant increase in survival compared to controls. While *B. cepacia* is not a direct comparator to the disease caused by *B. pseudomallei* or *B. mallei*, these results provide essential proof of concept for the potential utility of this treatment in a related bacterial species.

### 13.4.4 Candidate Vaccines

A number of different approaches have been utilized in developing a candidate *B. pseudomallei* or *B. mallei* vaccine, which were reviewed recently [3]. Irrespective of the vaccination platform or strategy employed, none of the vaccines reported to date provide sterilizing immunity following challenge, as evidenced by the substantial bacterial loads in the spleens and livers of animals that survive lethal challenge. Heat inactivation of whole cells results in protection of approximately 50% of vaccinated animals and fails to result in sterilizing immunity [52], [76]. Depletion studies suggest that B-cells, TNF-α, and IFN-γ contributed to the partial protection observed.

Live, attenuated vaccines of *B. mallei* and *B. pseudomallei* were developed and tested. A capsule mutant and a branched chain amino acid auxotroph were evaluated for their ability to protect against lethal aerosol challenge of *B. mallei* [67], [68]. The capsule mutant was found to induce a Th2-like response but was unable to provide any protection, with all animals succumbing to infection at day 5 post-challenge. In contrast, vaccination with the amino acid auxotroph induced a Th1-like response and resulted in 25% of the animals surviving 1 month post-challenge. An identical mutation in a gene involved in branched chain amino acid biosynthesis was introduced into *B. pseudomallei* and evaluated as a vaccine candidate [1]. The authors of this study reported that this construct possessed protective efficacy mice but did not state the proportion of animals that were protected. Mutants in the purine biosynthetic pathway were also examined [4]. A purN mutant demonstrated the greatest level of protection in mice, with 100% of animals surviving until day 30 post-challenge. Unfortunately, long-term survival was not evaluated.

Cell surface polysaccharides have served as candidate antigens for a *B. pseudomallei* vaccine [42]. Vaccination with LPS protected 60% of mice from a lethal *B. pseudomallei* challenge whereas vaccination with capsular polysaccharide resulted in no statistically significant increase in survival. Passive transfer studies using polyclonal sera derived from LPS vaccinated mice protected mice until 10 days post-challenge. When antibody administration ceased, however, mice succumbed to infection as soon as three days later. This suggests that cell surface polysaccharides alone are insufficient at providing robust immunity to *B. pseudomallei* infection.

A number of protein sub-unit vaccines have been examined. Results from an *in silico* search selected for the evaluation of 12 putative outer membrane proteins of *B. pseudomallei* [24]. Two of these proteins were successfully expressed and purified and were evaluated for their ability to protect mice against *B. pseudomallei* infection. One protein was capable of protecting 50% of the mice, although the mice were only evaluated until day 21 following challenge.

ATP binding cassette proteins, such as LolC homologs, were evaluated for their ability to protect mice from *B. pseudomallei* challenge [25]. In the presence of adjuvant, *B. pseudomallei* LolC was able to protect 80% of mice against challenge. Further, LolC was able to partially protect from challenge with a heterologous strain of *B. pseudomallei*, suggesting that these proteins could be exploited in the development of a broadly protective vaccine.
13.5 CONCLUSION

The intentional release of *B. pseudomallei* or *B. mallei* would have significant public health ramifications. The high degree of mortality, the logistically challenging long duration and combination anti-microbial therapy, and the significant possibility of patient relapse, would all confound a productive public health response. Therefore, there is a need for the development of novel medical countermeasures for these agents. A number of technical and scientific challenges to accomplishing this goal exist. The development of animal models which mimic the pathology of melioidosis and glanders in humans are required for the licensure or approval of any medical product under the United States Food and Drug Administration (FDA) Animal Rule. It will be critical to evaluate aerosol exposure to these agents in a number of animal species to best ascertain which models can be utilized most effectively to evaluate medical countermeasures. Further, the determination of factors involved in bacterial persistence and recrudescent infection will be critical in the design of therapies that facilitate the production of sterile immunity following infection. The development of model systems to study persistence could facilitate the identification of bacterial and host factors involved in the suppression and maintenance of the sub-clinical infection.

Significant progress has been made in elucidating the molecular mechanism of pathogenesis of *B. pseudomallei* and *B. mallei*. It will be critical to further identify and characterize secreted effectors from the T3SS and T6SS implicated in the virulence of these pathogens. Available data suggest that there is a strong interplay in the regulation of the T3SS and T6SS, and it will be necessary to characterize this regulation in further detail.

For vaccines, the identification of a vaccine platform, antigen, adjuvant, or combination thereof, which could induce sterilizing immunity, is a critical research gap. Several host factors have been identified as critical mediators in both productive and non-productive immunological responses. IFN-γ appears to be a critical mediator of clearance, and the identification of vaccine components which could stimulate IFN-gamma production may lead to vaccines with enhanced protection and clearance properties. A more detailed understanding of both the innate and adaptive immune responses to these agents would likely allow for better targeted vaccine development research.

The state of medical countermeasures for *B. pseudomallei* and *B. mallei* has lagged significantly behind many of the other bacterial threat agents. There are several reasons for this, including the classification and/or perception that these agents are not as great a threat as other bacterial threat agents, like *Bacillus anthracis* or *Yersinia pestis*. Regardless, there is an urgent need to increase research in this area to facilitate the development of effective medical countermeasures.

13.6 REFERENCES


Mima T and Schweizer HP. The BpeAB-OprB efflux pump of *Burkholderia pseudomallei* 1026b does not play a role in quorum sensing, virulence factor production or extrusion of aminoglycosides but is a broad-spectrum drug efflux system. Antimicrobial Agents and Chemotherapy 2010; 54:3113-20.


Pukatzi S, Ma AT and Sturtevant D. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system, 2006; 103:1528-33.


Chapter 14 – COXIELLA BURNETII

Hugo-Jan Jansen
Expert Centre Force Health Protection, Ministry of Defence
NETHERLANDS
Corresponding author (Jansen): hj.jansen.01@mindef.nl

14.1 INTRODUCTION

Q fever was first described in 1937 in Australia by Derrick, and as the etiologic agent of the disease was initially unknown it was called “Query fever”. The causative agent was discovered in 1937 and ultimately designated Coxiella burnetii to honour the contributions of both Cox and Burnet to the isolation and characterization of this new pathogen [69].

Coxiella burnetii is a small, Gram-negative, obligate intracellular bacterium. Although morphologically resembling Rickettsia, the organism is genetically and physiologically different and thought to be more similar to the genera Legionella and Francisella [60]. Hence, Coxiella and the family Coxiellaceae are classified within the order of Legionellales. C. burnetii replicates only within the phagolysosomes of eukaryotic host cells. Within its host cells, Coxiella grows and replicates in two morphologically distinct cell forms. The Large-Cell Variants (LCV) are pleomorphic and represent the intracellular metabolically active forms. In contrast, the Small-Cell Variants (SCV) are much smaller, rod-shaped, with a densely stained cell wall, and are metabolically inactive. The developmental cycle of C. burnetii has been described by McCaul and Williams [61]. The sporelike form is resistant to osmotic pressure, heat, drying and many common desinfectants [106], [29] and corresponds to the extracellular form of the organism. These features enable the bacteria to survive for long periods in the environment. Both large- and small-cell variants are produced during standard replication and can be found simultaneously [60], [65].

14.2 ZOONOSIS

C. burnetii is found world-wide and can infect many species of domestic animals and wildlife. Domesticated ruminants such as sheep, goats, and cattle seem to be the most common animal reservoirs and represent the most frequent source of human infection. Cats and dogs, particularly following parturition, are suspected sources in urban outbreaks of Q fever. Domesticated poultry such as chicken, geese, turkeys, and ducks may also be a source of Q fever. Wild rodents may be important reservoirs in some areas and ticks may play an important role in spreading coxiellosis among lagomorphs, wild rodents and wild birds. C. burnetii has also been isolated from a large variety of mammals and birds, such as horses, swine, camels, water buffalo, deer, pigeons, swallows, parrots, crows, geese. Antibodies have been found in coyotes, raccoons, opossums, badgers, jackrabbits, black bears, musk oxen, and other species. There are also reports of C. burnetii in tortoises and snakes [60], [7]. Recently marine mammals, like seals, porpoises and sea lions were found to be positive in Coxiella-specific PCR and antibody detection tests [52], [51].

In many animal species, infection with C. burnetii appears to be asymptomatic, although abortion in goats and sheep has been linked to infection. C. burnetii is excreted in milk, urine, and faeces of infected animals [76]. Most importantly, during birthing the organisms are shed in high numbers in the amniotic fluids and the placenta. The C. burnetii spore-like form is resistant to many adverse conditions and enables the bacteria to survive for long periods in the environment in dust and soil [60], [5].
14.2.1 Transmission

Humans usually acquire Q fever by inhalation of air containing airborne barnyard dust contaminated with *C. burnetii* in dried placental material, birth fluids, and excreta of infected domesticated animals. Other ways to acquire infection are direct contact with infected animals, or processing of infected animals or their products, such as wool. People at higher risk of acquiring Q fever are thus those working with infected domesticated animals, such as farmers, veterinarians, sheep and meat workers, and laboratory workers.

Humans are often very susceptible to the infection, and very few organisms may be required to cause disease. The ID$_{50}$ was determined to be as low as 1 – 10 organisms [106], [97], [55], [62]. An outbreak in an urban school implicated the air conditioning system [1]. Compared to inhalation, ingestion of contaminated unpasteurised dairy products, followed by regurgitation and inspiration of the contaminated food, is a less common mode of transmission. A literature survey concluded that the oral exposure route in animals is not very efficient in comparison with the inhalational, subcutaneous or intraperitoneal route [32]. Other modes of transmission to humans, including tick bites and human-to-human transmission, are rare [14]. Finally, the European Centre for Disease Control (ECDC) estimated the risk of acquiring the infection through blood, cells, tissues, organs from contaminated donors as small [25].

According to a recent literature search, 53 lab-confirmed outbreaks of Q fever in humans have been published during the years 1981 – 2007. The following 6 countries experienced the largest number of confirmed outbreaks: Australia (5), Canada (5), France (6), Germany (9), UK (5), USA (5). The remaining 18 outbreaks were reported from various parts of the globe. Most often, 26 of the 53 outbreaks, sheep were regarded as source of infection; goats in 6. Among the other animal sources, cats were suspected in 3 smaller outbreaks. Larger outbreaks were reported from East European countries, but laboratory confirmation was not available [70].

14.2.2 The Outbreak in the Netherlands

The largest outbreak to date is recorded in the Netherlands. During 2007 – 2010, an unprecedented 4028 people fell ill, and 18 died as recorded by the Dutch National Institute of Public Health and the Environment [74]. Those patients who died suffered underlying chronic health disorders in addition to their Q fever infection [100], [49]. The percentage of cases that required hospitalisation was around 20% [100]. This is unusually high compared to the 2 – 5 % that is recorded [73] and suggests that many mild cases remained unnoticed or unreported (personal communication). By studying the relation between seroconversion and the number of reported cases, it was estimated that the number of reported acute infections correspond to over 44000 people infected [102].

In 2009, preliminary typing results from Multiple-Locus Variable-number tandem repeat Analysis (MLVA) of *C. burnetii* from a variety of human and animal clinical samples linked to the outbreak obtained from different locations in the Netherlands were reported. These indicated that the Q fever outbreak in the Netherlands involved multiple genotypes of *C. burnetii* – and because most of the genotypes differed only by a single repeat difference, they might represent microvariants of a hyper-virulent strain that had been introduced in the Dutch animal population [53], [75]. However, to-date no proof has been documented of a strain with increased virulence. Single-nucleotide-polymerorphism-genotyping studies from clinical samples indicated that at least five different genotypes of *C. burnetii* contributed to the outbreak(s) in the Netherlands [42]. In contrast, in dairy goat herds in the Netherlands, a single genotype predominated, possibly indicating that this type spreads more easily [75]. Subsequent Multi-Spacer Typing (MST) confirmed that the outbreak was caused by a single MST genotype, MST33, from dairy goats [98].
Retrospective analysis of available epidemiological and surveillance data suggests Q fever as a likely cause of clusters of lower respiratory tract infections in the proximity of infected small-ruminant farms in 2005 – 2006, and indicated that the outbreak started well before 2007 [103]. The outbreak was linked to 74 dairy goat and 2 diary sheep farms on which C. burnetii was found in abortion and parturition fluids, or detected by PCR screening of tank milk. Likely, dust from barn yards contaminated with C. burnetii was picked up by the wind and spread to neighbouring communities in what is essentially a densely populated area. Epidemiological analysis and environmental sampling during the Dutch outbreak indicates that the effective range of airborne spread of C. burnetii via contaminated dust may be up to 5 km [100], [82], and that the organism survives in the environment for at least 1 year [22]. Using similar wind-borne transmission, an outbreak of Q fever in the town centre of Cheltenham, England in 2007, likely originated from local farms between 2 and 4 km away [107]. In an earlier outbreak of Q fever, infection in 51 persons in Scotland was tied to a meat processing plant and appeared to spread via aerosol for at least half a mile [108] – and in Jena, in a Q fever outbreak linked to sheep, people living up to 400 meters away were found to be at higher risk of infection [34]. Other recent outbreaks of Q fever in urban areas have been reported in among others Israel [1], Bulgaria [67], Italy [84], and Germany [71]. The differences in distance that the infectious dust particles seem to spread may be due to environmental and meteorological parameters. Environmental parameters that are strongly linked to airborne dispersion of contaminated dust are vegetation density and groundwater level as an indicator of soil humidity [43]. The spread of C. burnetii contaminated dust from farms into the environment in areas were soil is moist and fully covered, such as pastures and grasslands, was found to be low as indicated by an absence of Q fever cases in surrounding villages, whereas in areas with dry and bare soil in spring, e.g. crops like corn, many urban Q fever cases were found.

The outbreak in the Netherlands was finally brought under control by nation-wide vaccination of dairy goats in combination with improvements in the monitoring and notification systems, hygiene measures, restrictions in transport of animals and manure to and from infected farms, restrictions in reproduction and culling of pregnant animals on infected farms [100], [74]. Vaccination of goats and sheep appears to be an effective measure to reduce prevalence, bacterial load, and shedding of Coxiella bacteria into the environment from vaccinated animals as compared to unvaccinated animals [40].

The origins of the outbreak and its management were evaluated in 2010 [27]. It was concluded that despite the many uncertainties at the time and the learning curve for all involved, government could and should have acted with greater urgency. Most likely, upscaling of the dairy goat industry and urbanisation of rural areas contributed to a situation in which a large outbreak was enabled by a combination of large numbers of infected goats keep close to a large susceptible population in a densely populated area, and environmental and meteorological factors that favoured transmission. Due to the large numbers of infected people involved, future complications such as an increase in cases of chronic Q fever are to be expected. Chronic Q fever is a condition linked to considerable morbidity and mortality [48] and strategies are required to timely detect these patients [101].

14.2.3 Clinical Manifestations

In humans, most Q fever infections result in asymptomatic seroconversion and remain largely unnoticed. However, about 40% of people infected with C. burnetii show signs of clinical illness and infection leads to a febrile disease, pneumonia and/or hepatitis [60], [28], [5], [50], [56]. After an incubation period of 9 – 39 days, usually 2 – 3 weeks [14], most acute cases of Q fever begin with sudden onset of one or more of the following: high fevers (up to 40 – 41 °C), severe headache, general malaise, myalgia, confusion, sore throat, chills, sweats, non-productive cough, nausea, vomiting, diarrhea, abdominal pain, and chest pain. Fever usually lasts for 1 to 2 weeks. Weight loss can occur and persist for some time. 30 to 50 % of patients with a symptomatic infection will
develop pneumonia. Additionally, a majority of patients have abnormal results on liver function tests and some will develop hepatitis. In general, most patients will return to good health within several months without any treatment. Still, a considerable portion, namely 30% of patients who suffered an acute infection, report chronic fatigue and long-term impaired health that lingers for over a year afterwards [56], [104].

Chronic Q fever, which is characterised by infection that persists for more than 6 months, is a much more serious disease. Patients who have had acute Q fever may develop the chronic form between 1 to up to 20 years after initial infection. Chronic infections are observed in approximately 5 percent of cases and result most often in vascular infection (57%) or endocarditis (35%) [48]. Pregnant women and persons with heart valve disorders or impaired immunity, transplant recipients, patients with cancer, and those with chronic kidney disease are at increased risk of developing chronic infections [5], [28]. Q fever in pregnancy, whether symptomatic or asymptomatic, may also result in adverse pregnancy outcomes [17]. About 1 – 2 percent of people with acute Q fever die of the disease, usually those with endocarditis and underlying health disorders. The mortality in patients with chronic Q fever was found to be 13% [48].

14.2.4 Military Relevance of Q Fever

*Coxiella burnetii* has many attributes that would make this agent suitable for intentional use as a biological weapon or bioterrorism. It is a highly infectious agent that is resistant to heat and drying, it can be dispersed by air and inhaled by humans. A single *C. burnetii* organism may cause disease in a susceptible person. Further, it will survive in the environment for a long time. On the other hand, a relative long incubation period, low mortality, and many asymptomatic infections would make this agent mostly an incapacitating agent. In case of intentional use, clinical presentation would be similar to naturally occurring cases. This agent has been developed for use in biological warfare in several Nations and is considered a potential terrorist threat [16], [31], [26], [30]. The Q fever organism is classified by the U.S. Centers for Disease Control and Prevention as a Category B biological agent and, in fact, although not thought to be related to bioterrorism, was spread by mail in a post office in Oxfordshire, United Kingdom, in 1987 [109].

In addition to its potential to be intentionally used as an incapacitating agent, many outbreaks among military personnel have been documented since World War II demonstrating that Q fever is a disease of military relevance in particular in Southern Europe, the Balkans and Middle-East [106], [89], [90], [44]. Q fever has been frequently diagnosed in soldiers returning from recent operations in Iraq and Afghanistan [3], [72], [8], [37].

14.2.5 Genomics

All *Coxiella burnetii* isolates carry either an autonomously replicating plasmid, or have plasmid-like sequences integrated into their chromosome. Whether the plasmid sequences are integrated or autonomously replicating, the strict conservation of plasmid sequences suggests that these sequences play a crucial role in life of *Coxiella burnetii* [12]. The first fully sequenced genome of *C. burnetii* RSA493 (Nine Mile Phase I), including its QpH1 plasmid, was published in 2003 [85]. Since, the genomes of many more strains (e.g. 5J108-111 Dugway, RSA331 Henzerling, Cb109, Q154 K, Q177 Priscilla, Q321) including their plasmids (QpDG, QpH1, QpH1, QpRS, QpRS, respectively QpDV) have been fully sequenced and are accessible in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) [47], [57]. In addition, the genome of plasmid-less strain Q212 G genome and the QpDV plasmid of strain R1140 have been sequenced and are accessible too. Genome sizes of *C. burnetii* strains range between 1.9 – 2.1 Mbp and contain between 2060 to 2380 genes. Plasmids size between 32 – 54 kbp and may contain 34 – 59 genes. The genomes of obligate intracellular living bacteria were thought to contain no or few IS elements as there is little opportunity for gene transfer. Yet, IS elements have been described in at least four *Rickettsia* species [12]. Similarly, the genome of *C. burnetii* strain RSA493 was found to contain 29 IS elements.
In addition, there are 83 pseudogenes caused by frame shifts, point mutations or truncations, suggesting ongoing reduction of the genome. The presence of mobile elements and genome size reduction may indicate that the transition of *C. burnetii* to its intracellular life style is relatively recent [85], [10]. Comparative genomics within the genus *Coxiella* and its adaptations to a life style within a eukaryotic host has been described [12].

### 14.2.6 Molecular Typing

*C. burnetii* was considered to be homogeneous by analysis using serology or 16S rRNA [91], [10]. Typing methods differ in their discriminatory power, depending on the power required and method used. Basically, *C. burnetii* can be divided into six main genomic groups depending on different restriction endonuclease pattern determined with SDS-Page. These groups are also presenting distinct plasmid types - , QpH1 (3 groups), QpRS, QpDG, and a chromosomally integrated type [38], which can be subsequently subdivided into smaller branches. The relative limited number of genomic groups, 4 to 8, was found using among others genome sequencing and analysis of plasmid type [12], analysis of variable number of the IS1111 insertion sequence [23], by endonuclease digestion of genomic DNA [38], or microarray [10]. Additional genomic diversity was found by Restriction Fragment Length Polymorphism (RFLP) of 80 isolates revealing 20 different restriction patterns [45]. However, these techniques require isolation and culturing of the organisms, which is time-consuming and not easy. Multi-Spacer Typing (MST) yields 30 genotypes [35], and by Multiple-Locus Variable-Number of Tandem Repeats (VNTR) Analysis (MLVA) of 36 genotypes are revealed [4], [29]. The discriminatory power of both MST and MLVA make them very suitable techniques for epidemiological studies permitting typing without the need to isolate strains. Another tool with high discriminatory power is canonical Single Nucleotide Polymorphism (canSNP) typing [57], [41]. SNP genotyping, MST and MLVA have been used in field studies [42], [20], [53], [74], [98], [58] and databases (MST, http://ifr48.timone.univ-mrs.fr; MLVA, http://minisatellites.u-psud.fr/MLVAnet) have been established to allow inter-laboratory comparison among others. Further, efforts to harmonise and standardise MLVA by deciding on marker panels to be used and allele calling are ongoing [87]. An overview of typing methods that have been used to characterise *C. burnetii* was published recently [88], [59].

There is controversy whether differentiation into genomic groups is linked to pathogenicity. It was reported that isolates associated with chronic disease contained the QpRS or chromosomally integrated plasmid, whereas isolates associated with acute disease possessed the QpH1 plasmid [80]. This association was supported by other studies [38], [35], [94]. However, other studies found the QpH1, QpRS or QpDV plasmid in both acute and chronic disease-associated isolates and argued that there are no specific genes responsible for a particular type of disease [96], [45], [93], [99]. For the disease to arise, genetic predisposition, immune status and other unique patient factors likely play a role [73], [6]. For example, patients with valvulopathy infected with *C. burnetii* are at increased risk to develop endocarditis. These patients also have increased levels of circulating apoptotic leucocytes and it was found that these apoptotic leucocytes inactivate monocytes and macrophages and contribute to bacterial persistence and replication inside these immune cells [13]. Still, striking differences in infectivity and virulence between *C. burnetii* strains have been observed in animal models [64], [92], [78]. Typically, isolates associated with chronic disease are less virulent in animal models as compared to isolates associated with acute disease, suggesting that bacterial factors contribute to the disease outcome. Similar to many infectious diseases, the disease outcome in the host likely is affected by both host factors (immune status and genetic predisposition) on the one hand and bacterial factors (e.g. virulence, plasmid type) on the other hand.

Further, differentiation into genomic groups reflects geographical spread and subsequent diversification and pathoadaptation. It is speculated that plasmid QpH1 is the ancestor plasmid and in some strains got lost over time whilst essential sequences for survival were integrated into the chromosome. Whereas in other strains, QpH1 developed into the QpRS plasmid, which in turn developed into the QpDV type [35]. The QpDG type has
COXIELLA BURNETII

the largest genome in size, has the least number of pseudogenes, is not associated with human disease and is less
pathogenic in animal models of Q fever. All this suggests that the QpDG plasmid lineage is the least
pathoadapted [12] and perhaps a recent event. The capabilities to type C. burnetii strains have significantly
improved over the years, but much remains to be learned about the epidemiology of the disease.

14.2.7 Virulence Determinants

Studies on C. burnetii always have been hampered by its intracellular life style and fastidious nature. Growth
outside a eukaryotic host is difficult and slow, and genetic tools have not been readily available until recently.
Further, C. burnetii is highly infectious, which requires BSL-3 laboratory facilities to study this organism and
limits exchange of strains.

To date, the only well-defined virulence factor of C. burnetii is Lipopolysaccharide (LPS). Full-length LPS is
required for C. burnetii bacteria to induce fever in the host, and to survive in the spleen in a guinea pig model.
These virulent strains are serologically characterized as Phase I. Strains with a truncated form of LPS, Phase II,
are avirulent. Phase II LPS consists of Lipid A and core sugars, but lacks the O-antigen sugars [10]. A strain with
intermediate length of LPS did cause fever, was not able to survive in guinea pigs [64]. Antigenic variation
within Phase I LPS has been observed and may be linked to the plasmid type [36]. Serial in vitro passaging in
cell culture or embryonated eggs of virulent Phase I strains increasingly shortens their LPS, thus ultimately
leading to a shift from Phase I to Phase II. This shortening is due to a chromosomal deletion and possibly also
due to mutation(s) in genes responsible for O-antigen biosynthesis. Full-length LPS masks toll-like receptor
ligands on dendritic cells, and thus prevents the dendritic cells to recognize the presence of the bacteria and
mature. This mechanism allows C. burnetii to evade the host immune system and replicate inside the host
without inflammatory cytokine production [86].

Another way for the C. burnetii to escape the host immune system is by its ability to replicate only within the
phagolysosomes of eukaryotic host cells. Its metabolism is adapted to the low pH, and all C. burnetii isolates
possess a variety of enzymes required to detoxify the phagolysosome [12]. One of these escape mechanisms is
the production of acid phosphatase by C. burnetii to inhibit NADPH oxidase mediated killing by reactive oxygen
intermediates [39].

The recent advances in whole genome sequencing and manipulation of obligate intracellular bacteria have
allowed the identification of putative virulence factors. Following the sequencing of the first Coxiella genome
[85], other genomes have been completed. Beare [12] compared the genomes of four C. burnetii strains, and also
with other obligate intracellular living organisms such as Legionella pneumophila and Rickettsia sp. Putative
metabolic pathways, secretion systems [19], and possible virulence factors have been identified. These putative
mechanisms form a foundation for others to study [15] and to (dis)prove expression in the phenotype and a
physiological role. Yet, indisputable proof is difficult to obtain due to lack of methods for site-specific gene
inactivation and complementation in bacteria associated with an intracellular life style. The difficulties and
genetic tools available haven’t been reviewed [11].

It is speculated that ongoing genome reduction and rearrangement in C. burnetii results in slower growth as
metabolic pathways are rendered inactive, similarly as is seen in Mycobacterium leprae [21]. Slow growth is
associated with chronic disease [110]. Ongoing genome reduction also results in the differentiation into genomic
groups, and may explain in part an association between disease type and genomic group.
14.2.8 Animal Models

To facilitate study of pathogenic mechanisms of C. burnetii, various animal models have been developed. Mice and guinea pigs are used most often. Mice are relatively cheap and easy to handle, but their clinical response to infection with C. burnetii may differ from the response in humans. Namely, immunocompetent mice require a large infectious dose in order to develop clinical signs and most often develop splenomegaly rather than fever as a primary indicator of disease. The use of inbred mice lowers the infectious dose required to develop clinical signs [83]. In contrast, guinea pigs are susceptible to C. burnetii and require an infectious dose (intraperitoneally) of approximately 10 organisms and do develop fever following infection, which more resembles the human clinical response.

To study Q fever pneumonia, an aerosol exposure apparatus was tested [92] in Severe Combined Immunodeficient Disease (SCID) mouse and these mice exhibited pulmonary lesions, but immunocompetent mice proved resistant to infection. This SCID mouse model is also suitable to study differences between C. burnetii strains in pathogenic potential to cause acute pulmonary disease. Infection of guinea pigs with C. burnetii via inhalation is another model to study acute Q fever associated pneumonia [79]. Clinical signs following infection and histopathological changes are similar to those observed in humans. Guinea pigs infected intraperitoneally develop dose-dependent fever and pathological changes associated with the liver. This would be a good model to study Q fever-associated hepatitis. On the other hand, intranasally infected guinea pigs develop more lung-associated disease. Depending on the route of infection, guinea pigs are a good model to study various clinical complications of Q fever and are suitable to study the efficacy of vaccines and antimicrobial compounds and regimens [79]. Dose-response relations between size of infectious dose and clinical outcome in various animal models, e.g. the SCID mouse [2] or the guinea pig model [79], have been modeled to predict dose-response outcomes in humans and risk of infection following exposure [95].

In addition to acute disease models, animal models to study chronic Q fever have been developed too. Andoh and colleagues used a SCID mice model [2] to study endocarditis. SCID mice intraperitoneally infected with C. burnetii developed similar heart lesions to those in man and died, whereas immunocompetent mice became asymptomatic and survived. Besides the heart, lesions were found in other primary organs too. SCID mice are highly susceptible to infection with C. burnetii and this model may be suitable to study endocarditis and disease in immunocompetent hosts. Another model to study chronic disease by intracellular bacteria such as C. burnetii is provided by Meghari and co-workers [63] by transgenic mice overexpressing IL-10 in macrophages. IL-10 is associated with increased susceptibility to and persistence of intracellular bacteria. Transgenic mice developed chronic infection and a strong antibody response, whereas wild-type mice did not. It can be concluded that various animal models are available to study acute and chronic Q fever.

14.3 DIAGNOSIS

An array of widely available methods to diagnose Q fever has been reviewed by Fournier and co-workers [28]. These comprise of DNA amplification, a wide variety of serological methods, and culture.

Culture of C. burnetii requires BSL-3 laboratory facilities due to its infectivity. Cells are grown in cell culture (e.g. Vero cells), embryonated hen yolk sacs, or laboratory animals. Unfortunately, the eukaryotic host background may impede interpretation of subsequent experimental results, and the sensitivity of this method for the purpose of diagnosis can be low. Culturing of C. burnetii in a synthetic medium and axenic growth has been described recently [66].

Serology is the gold standard for diagnosis of a Q fever infection in humans. A positive result can be either by seroconversion or a four-fold increase in antibody titer. Phase I LPS is characterized by full-length carbohydrate
chains – but after infection of the host, LPS progressively shortens until basically only the lipid A and core carbohydrate backbone remains. Phase II antibodies are expressed during acute infection, and antibodies against Phase I LPS are expressed during established infection. Thus, the serological differentiation between Phase II and Phase I LPS is essential in differentiation between acute and chronic infection, and for both types of antigens the rise in IgM antibodies precedes that of a rise in IgG-titer. The reference test for serological diagnosis of Q fever is the immunofluorescence assay test (IFA). Other suitable platforms for serological testing are Enzyme-Linked Immunosorbent Assay (ELISA) and complement fixation. However, serological cross-reaction is possible with other microorganisms, e.g. Legionella and Bartonella species [28], [60]. Discrimination between Coxiella strains using serological methods is not feasible as yet.

However, as it requires several weeks after infection to develop a serological response, serology is less useful in the early stages of disease or to timely detect an outbreak and other methods with high specificity and sensitivity are required to detect circulating C. burnetii bacteria. DNA amplification, e.g. conventional PCR and in particular real-time PCR, proved essential for early diagnosis of Q fever [81], [68]. Though sensitive, conventional PCR is time-consuming and laborious due to post-PCR processing. In-tube assays and real-time PCR reduce not only the time required to diagnosis, but also potential cross-contamination problems. Further, real-time PCR is suitable for high-throughput processing of samples for screening purposes [68]. Diagnosis of Q fever in deployed military personnel by PCR using the Joint Biological Agent Identification and Diagnosis System (JBAIDS) platform has been carried out in a deployed Combat Support Hospital and demonstrate the possibility of having a fieldable method to reduce the time required to diagnose Q fever [37]. In Dutch Q fever patients, seventeen days after onset of disease C. burnetii, DNA became undetectable as the serological response develops [81], although it can become positive again in chronic Q fever infection. The large outbreak of Q fever in the Netherlands required large cohorts of patients to be screened and allowed for laboratory follow-up. Algorithms have been developed for diagnosis of acute and chronic Q fever using a combination of serology, PCR and clinical presentation [101], [46].

Full-length LPS sterically impedes access of host antibodies to bacterial outer membrane proteins, which become accessible after an LPS-shift to Phase II [28]. Protein accessibility may not only explain why patients develop life-long seropositivity after acute infection, but also it is possible to identify and target these surface proteins by micro-array and proteomics to develop recombinant-protein based diagnostic methods to ascertain chronic Q fever infection, or to develop sub-unit vaccines [105], [9]. Advances in the field of improving Q fever diagnosis using C. burnetii proteomics have been reviewed [54].

14.4 MEDICAL COUNTERMEASURES

14.4.1 Anti-Microbial Treatment

Antibiotic treatment of Q fever is most effective when initiated within the first 3 days of onset of disease and doxycycline is the treatment of choice for acute Q fever. The use of antibiotics shortens duration of disease and reduces potential severe complications. A dose of 200 mg dd of doxycycline for 15 – 21 days is a frequently prescribed therapy. Quinolone antibiotics, e.g. moxifloxacin, have demonstrated good in vitro activity against C. burnetii and may be considered. Analysis of antibiotic regimens prescribed in the Dutch Q fever outbreak in 2007 and 2008 indicated that 200 mg doxycycline for 14 – 21 days in acute infection was found to be most protective against hospitalisation. As an alternative, 400 mg moxifloxacin was found to be a suitable as well and these data support the current guidelines that doxycycline is the first choice to treat acute Q fever infection [24].
Chronic Q fever is much more difficult to treat effectively and often requires the use of multiple drugs for long periods of time. The recommended dosage is 200 mg dd doxycycline and 3x 200 mg dd hydroxychloroquine for 18 months.

There is indication that long-term antibiotic therapy with cotrimoxazole (1920 mg dd trimethoprim/sulfamethoxazole) has the potential to prevent severe pregnancy outcomes [17], but the evidence is based on a case series without randomisation and without controlling for potential biases [25]. Serological control and treatment during and after pregnancy should also be considered aimed at prevention of chronic infection of the mother.

14.4.2 Vaccines

A formalin-inactivated whole-cell Q fever vaccine is produced and licensed in Australia since 1989 (Q-Vax; Commonwealth Serum Laboratories, Australia). Q-Vax is not licensed in other countries. It is prepared from the Phase I Henzerling strain of \textit{C. burnetii} grown in the embryonated egg yolk sacs and still the only approved human vaccine in the world. The vaccine is highly immunogenic and effective, but pre-vaccination testing is necessary due to induction of adverse effects when administered to persons who have earlier been infected with \textit{C. burnetii}. This renders the vaccine more suitable for defined risk groups than for general vaccination [60], [25]. The immunity is supposed to last 10 years. However the use of the vaccine is limited by the fact that revaccination/boostering is not possible.

Other vaccines that were developed are a chemovaccine from trichloroacetic-acid extracted antigens from the Phase I \textit{C. burnetii} Nine Mile strain and tested in Slovakia appears to be less reactogenic, but the protective effect is not fully evaluated [60]. A live-attenuated vaccine from the attenuated M44 strain was developed in Russia, but has been abandoned for safety concerns [6].

Vaccination against Q fever is usually recommended only to those for whom exposure to \textit{C. burnetii} is an occupational hazard, and the Q-Vax vaccine is very effective in these cases [33]. With Q fever increasingly being reported in urban areas, vaccination may be considered for those at risk of developing chronic infection, although the safety and efficacy for the general population and in particular risk groups have not been studied. In 2011 in the Netherlands, vaccination was offered to risk groups, e.g. patients with cardiac valve defects, who have not been exposed to \textit{C. burnetii} as determined by pre-vaccination skin testing in order to prevent new cases of Q fever and possible chronic disease. The efficacy and safety of the vaccine is being studied in these groups.

Studies are being carried out to develop new-generation sub-unit vaccines with less adverse side effects. The focus of these studies is as yet on identification of key target surface antigens and understanding of the host response [105], [18].

Studies into the efficacy of the available vaccines for animals showed that the CEVA Phase I vaccine (Coxevac; CEVA Santé Animale, France) is efficient, as it not only reduces the number of abortions in a herd, but in particular the amount of shedding [77], [40]. In contrast, the Merial Phase II (Chlamyvax FQ; i.e. combination vaccination against \textit{Chlamyphila} and Q fever) did neither [6]. Vaccination is less effective in pregnant animals [5], [77], [40], and neither does it completely prevent abortions or shedding nor clear \textit{C. burnetii} from the animal if infected prior to vaccination [60].
14.5 CONCLUSIONS

Q fever is endemic around the world. Military personnel are at risk of developing Q fever because they often operate in dry and dusty environments which they share with herds of goat, sheep and other animals that may harbor and excrete *C. burnetii* bacteria. Thus it may be no surprise and indeed Q fever has been found in military personnel operating in for example Iraq and Afghanistan. Further, *C. burnetii* may be used as biowarfare agent, and potentially can infect large numbers of people due to its very low infectious dose. Although Q fever usually remains asymptomatic or presents as a mild and self-limiting disease, Q fever may evolve into a serious or chronic disease in susceptible individuals. In addition, a considerable portion of persons who suffered acute Q fever are still plagued with fatigue for over a year afterwards. Unfortunately, experiences in France, the Netherlands, and elsewhere indicate that Q fever outbreaks and Q fever patients are easily missed and under-reported. Awareness is needed among physicians, veterinarians, and preventive medicine personnel as well as sensitive and specific diagnostic tools to timely detect an outbreak. Good monitoring and surveillance systems are required to assess the real magnitude of Q fever. Finally, medical countermeasures could be improved to better protect military personnel and/or the civilian population from Q fever infection. Suggestions are vaccines that are suitable for use in the general population and do not require pre-vaccination skin testing. In addition, current therapy to treat chronic Q fever consists of 18 months of multiple antibiotics and is linked to poor success and adverse effects. A better therapy to treat chronic disease more quickly and efficiently, and/or that prevents lingering fatigue would be of value.

14.6 ACKNOWLEDGEMENTS

The author wants to thank LCol Dr Dimitrios Frangoulidis for his valuable comments and proofreading of the manuscript.

14.7 REFERENCES


COXIELLA BURNETII


[57] Macellaro A, Svensson K, Sjödin A, Frangoulidis D and Forsman M. An improved canSNP-typing scheme for rapid and robust real-time PCR detection of Coxiella burnetii obtained by adding four new sequenced genomes. 21st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), May 2011, Milan, Italy.


Chapter 15 – A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE BRUCELLA SPECIES

John W. Cherwonogrodzky
Defence R&D Canada – Suffield
CANADA

Corresponding Author (Cherwonogrodzky): jcherwono@shaw.ca

ABSTRACT

It has been over a century since the identification of a bacterium as the causative agent of Malta Fever, and a long frustrating path to develop medical countermeasures, either as a pre-infection preventative measure or as a post-infection therapy. Brucella continues to be a public health, agricultural and defence threat, afflicting about 500,000 people and 10% of the livestock in developing countries, an easily acquired and easily grown pathogen for the bioterrorist. Aside from aggressive antibiotic therapy to clear an infection (and recent PCR results on sera suggest that the infections are never cleared) there are very few medical countermeasures against the Brucella species. However, within this decade there is likely to be several successes in this field, spurred by recent advances for sub-unit vaccines, anti-Brucella antibody characterization, liposomal delivery of therapeutics, immunomodulators, and innate immune responses. Sub-unit vaccines have the advantage of not being infectious, avirulent and for some not requiring cold chain storage. Where none currently exist, there is evidence that an effective vaccine will soon arise from key proteins, polysaccharides or a conjugate of these, either naturally extracted or synthetically produced. Although the journey for such a vaccine against brucellosis has been long and difficult, perhaps its greatest contribution will be newly discovered concepts and approaches applicable to treating other pathogens that have been equally difficult.

15.0 EXECUTIVE SUMMARY

Introduction or Background – Military personnel have the worst of both situations. Not only are they put in harm’s way and vulnerable to the worst possible assaults, but they are also in an environment with the poorest medical care. In the 1850s, it came to the attention of British medical officers that soldiers were becoming ill (about 500 cases a year) and dying (about 2% for military, 10% for civilians) from Malta Fever. In 1884, Dr. David Bruce was stationed at the military base and a few years later discovered the causative agent, the bacterium that is named after him. After a century of research and discoveries, this infectious agent is still present throughout the world, especially in under-developed countries where our troops have been or might be stationed. It is an agricultural, public health, military and terrorist agent of concern, easily acquired and cultured. Medical countermeasures are limited to antibiotics which, although these appear to return the patient to health after several months of aggressive regimes, have been found never to eliminate this intra-cellular facultative parasite. A literature review and analysis is given on the current state of knowledge on the medical countermeasures available to address this threat.

Results – The presented review is divided into medical countermeasures for protection and treatment. For the former, no vaccines for human use are available and other measures such as antibiotic prophylaxis are impractical. For the latter, the only approved recourse is to give patients combinations of antibiotics (doxycycline,
rifampin and streptomycin in high amounts, daily, for several weeks to several months. PCR analysis of sera from recovered patients suggest that the infection is never cleared.

Significance – After a century of research and observations of several laboratories throughout the world, these are indeed exciting times. Where only live attenuated strains existed as vaccines for animals, recent findings have found that sub-unit vaccines have the potential for protecting against the *Brucella* species. After infection, antibiotics are the treatment of choice, but their effectiveness is likely to improve with liposomal and invasive liposomal encapsulation. Our arsenal to treat this intra-cellular pathogen is being expanded with the discovery of intra-cellular acting antibodies and newly discovered non-antibody serum components (mannose binding lectins, defensins, etc.).

Future Plans – Where there were few medical countermeasures available to protect against or treat the *Brucella* species, there now appear to be several advances in several fields. All cannot be pursued, and the most important appear to be invasive liposomal encapsulation of antibiotics to improve clearance of the pathogen from within the cell and sub-unit vaccines to offer lifelong protection against infection. As part of the latter countermeasure, surrogate markers must be identified for establishing the level of protection. There are reports of others, in which it is cited that infected people or animals do not develop brucellosis, and our own studies, where it was observed that unvaccinated animals had resistance identical to polysaccharide vaccinated animals. Based on these observations, perhaps 20% of the military do not need vaccinations because they have already been vaccinated by natural exposure to cross-reactive antigens from other bacteria. Surrogate markers, either sub-groups of antibodies, other serum components, or cellular responses to antigens, need to be identified in order to determine both the level of protection for vaccinates and those already protected by cross-reaction/cross-protection.

15.1 INTRODUCTION

The *Brucella* are Gram-negative non-motile, non-sporulating, non-capsulated bacteria that appear as cocci or short rods, either singly, in pairs or short chains depending on how these are cultured. These do not secrete toxins nor enzymes such as proteases [1]. These differ from other pathogenic bacteria by having two chromosomes [2], [3], the ability to utilize erythritol, a sugar found in animal reproductive tracts [4], [5], an extensive collection of virulence factors [6], [7], and their ability to be facultative intracellular parasites [8]. For many years there has been a debate as to whether there are different species of *Brucella* within different hosts (e.g. *B. abortus* for cattle, *B. melitensis* for goats, *B. suis* for swine) [9], or a single zoonotic species (*B. melitensis*) with several variants or biovars [10]. With the discovery of *Brucella* with more varied genetic sequences, different metabolic profiles and antigens (e.g. *B. ceti* and *B. pinnipedialis* of marine mammals [11], *B. microti* from voles [12], *B. inopinata* from humans [13]) more recently an international taxonomic committee has recommended that the *Brucella* family be divided into species rather than *B. melitensis* biovars [14].

The *Brucella* species are zoonotic infectious agents, people usually acquiring this from infected animals or their products [15]. Although we tend to think of it as a modern disease, skeletal evidence suggests that 1.5 – 2.8 million years ago *Australopithecus africanus* had brucellosis [16] from meat in their diet [17]. Although the bacteria are zoonotic, these vary in their virulence for animals and humans. As an example, *B. ovis* is highly pathogenic for sheep but seldom causes illness in people [18]. For those species that are highly pathogenic for humans (e.g. *B. abortus, B. melitensis* and *B. suis*), these have a low attack rate [19], seldom kill (the mortality rate being between 2 – 4 % [19], [20]) and half of those infected will not show symptoms of illness [21]. The incubation period in the host is variable, usually taking about 3 weeks before the onset of symptoms [22] but in rare instances it might take several decades [23]. The species are minimally contagious. Although there may be transmission from the mother to the fetus [24] or the breast-feeding baby [25], exposure to infected blood [26] or by sexual contact [27], these aren’t spread by patient respiration or touch [28]. Infections of *Brucella* can be
readily treated with common antibiotics [21] and incidences of brucellosis rarely occur in developed countries [29].

Although the noted characteristics may suggest that it is of little concern as a threat, it is far from innocuous. Within livestock, wildlife and marine mammals, these will cause malaise, weight loss and abortions [29]-[31]. Humans are exceptionally sensitive to several Brucella species, suffering daily high undulating fever (from normal to 40°C), night sweats, incapacitation, muscle/joint aches, arthritis, bone deformities, headaches, swollen spleen/liver, and inflammation of the reproductive tract [22], [32]. Neurobrucellosis is very rare for patients that have been infected by land Brucella species [22], [33] but more frequent for those exposed to marine Brucella species [34]. Unlike mice which are naturally resistant to Brucella with an ID₅₀ of about 10,000 colony forming units (cfu) [35], humans may become infected with as little as 10 cfu [36]. Humans are also susceptible to most routes of infection, be these by finger-prick, contaminated skin, oral ingestion of contaminated dairy products or inhalation of aerosols [19]. For the latter, there have been incidences where those in a laboratory became infected when one person inoculated a plate on a benchtop rather than using the biosafety cabinet [20]. In another example, 45 students in second and third floor classrooms became infected (with one death) because of researchers working with Brucella in the basement [37]. Brucella are also the most frequent cause of laboratory-acquired bacterial infections [19]. Although it does not form a spore or protective capsule, Brucella is exceptionally tough in the environment, lasting 8 months in manure, 18 months in frozen milk, 6 months in cheddar cheese and 3 months in drinking water [18], [38]. Part of this resilience may be due to a yellow wax on the cell surface, about 1% of the cell’s dry weight, which when removed with organic solvents make the cell more sensitive to enzyme digestion, suggesting that this wax may contribute to its spore-like or pseudo-spore properties [39]. The bacterium also protects itself from degradation within the mammalian cell by creating a protective brucellosome [40], and by having components, such as Lipopolysaccharide (LPS), that is 10,000-fold less toxic than the LPS of Escherichia coli [41], that do not activate cellular defences [42]. Rather than being harmless, the Brucella spp. are sophisticated facultative parasites that use several mechanisms to persist within the host.

The bacterium can be easily grown and bulk-produced and was one of the first infectious agents to be weaponized by the USA [43] and the former Soviet Union [44]. The risk increases still further when one notes that there is a deficiency of medical countermeasures against this bacterium. There are currently no vaccines or antibody to protect humans [45], even aggressive antibiotic treatment of infections may result in relapses once these are discontinued [46], and a patient thought to have cleared the bacterium may have it resurface years later [23].

Additional cause for concern stem from its ubiquitous nature. It has been cited above that these Brucella have been found in livestock, wildlife and marine mammals, but insects [47], fish [48] and birds [49] are also susceptible. Ochrobactrum spp. are found naturally in the environment [50], are opportunistic infective pathogens and on occasion Brucella is misdiagnosed as the former [51]. There is now much controversy and discussion that perhaps some of the Ochrobactrum strains from the environment may appear similar to Brucella because these are Ochrobactrum-Brucella intermediates [52]. Within patients, medulloblastoma (brain tumours) may be a form of neurobrucellosis [53] and on rare occasions sterility in men could be caused by a Brucella infection [54].

In light of the above notes on Brucella’s virulence and the inadequate medical countermeasures to protect or treat casualties, these species are very real biological threats to civilian populations and national security. The following review offers insights into what can be done for now, and the new developments that will soon defend us from Brucella.
15.2 PREVENTATIVE MEASURES

15.2.1 Antibiotics

As vaccines do not exist to protect people from Brucella, potentially one can take antibiotics as a measure to prevent infection, similar to the military using anti-malarial drugs to protect soldiers going into a malaria-endemic area [55] or using antibiotics to treat combat-related injuries [56]. However this approach is impractical to protect against an unlikely illness such as brucellosis. Antibiotic use is more practical as a medical countermeasure after infection, and this will be reviewed in the next section.

15.2.2 Anti-Brucella Antibodies

There is substantial evidence that mouse monoclonal antibodies, directed against the outer membrane proteins [57], [58], lipopolysaccharide [59], [60] and polysaccharides [61], [62] of Brucella, are protective in the mouse model against brucellosis. The latter provided the best protection against smooth species to which humans are sensitive [63], [64]. To date no approved humanized antibodies are available for human use. As one cannot predict who will be exposed to Brucella or when, if ever, the use of anti-Brucella antibodies to protect the military or civilians is an impractical countermeasure.

15.2.3 Vaccines Against Brucella Species

15.2.3.1 Killed Cells as Vaccine Candidates

In 1897, Sir Almoth Wright (UK) vaccinated himself with heat-killed B. melitensis cells, then a few weeks later infected himself with viable cells. The intent was to apply basic principles of vaccination to develop a vaccine against Brucella. It didn’t work. He became debilitated for several weeks with high fevers, emotional outbursts and hallucinations [65]. As discussed by Harris [66], studies in France also showed that killed Brucella cells did not protect animals nor people from brucellosis. The inconsistent results, erythema, swelling and excessive local pain made the approach of using killed cells as vaccines fall into disfavor.

15.2.3.2 Live Attenuated Cells as Vaccine

There have been several successful trials in which live attenuated Brucella strains have been used in animals or people to prevent brucellosis. As the sheer number of these is beyond the scope of this review, and as far more capable experts have addressed this topic [67]-[71], only a few examples will be discussed to outline the different approaches.

The attenuated Brucella abortus strain 19 vaccine has had a major impact on the control of brucellosis in animals and to a lesser extent in humans. In 1923, an isolate recovered from the milk of a Jersey cow was sub-cultured by J.M. Buck and left at room temperature for a year [72]. These cultures were tested in guinea pigs, and it was found that the culture in the 19th tube, hence its strain designation, had lost its virulence. From the 1930s, this strain has been used throughout the world to protect livestock, especially cattle from B. abortus. A derivative of this strain, B. abortus 19-BA [73] has been used in the former Soviet Union to vaccinate people in high risk occupations. Unfortunately, strain 19 retained some of its virulence and on occasion caused brucellosis in people [74]. Other live attenuated strains, such as B. abortus RB51, have been developed that are less severe but the same issues of residual virulence arose [75], [76].

There have been several other attempts to genetically manipulate Brucella strains to create defective mutants that will both enhance protection of laboratory or farm animals against brucellosis and avoid illness caused by the
vaccine itself. Researchers have investigated bacterial structural proteins, enzymes, the synthetic pathway for carbohydrates and LPS, the synthesis of key nucleotides, acquisition of essential metals, stress adaptation, metabolism of substrates, efflux of toxic compounds, and components that manipulate the host mammalian cell [77]-[139]. There have also been the transfers of Brucella structural or virulence components into less harmful bacteria to create protective recombinants [140]-[148]. With hundreds of mutants created by manipulating several diverse structural or physiological pathways of different species of Brucella, there is a need to compare these so as to determine the best strain for protection without ill effects.

15.2.3.3 Sub-Unit Components as Vaccine Candidates

It has been discussed that killed Brucella cells are not protective, while live attenuated strains do offer protection in livestock or laboratory animals from brucellosis. To date, the lesson learned from the development of such vaccines has been that only “rough” living strains (those lacking Polysaccharide (PS) on their cell surface, on agar plates their colonies have a rough surface) have been effective while “smooth” strains (those coated with sugars) were ineffective. As surface proteins would be exposed on the former and masked on the latter, these Outer membrane proteins (Omps) have been investigated since the 1980s as virulence factors and vaccine candidates [149]. Most of the Omps did not play a role for either [150], but a few Omps did show vaccine potential, notably Omp25 and 31 [151]-[153], the latter being a haemin-binding protein [154]. In the mouse model, a DNA vaccine, coding for lumazine synthase and an Omp31 insert, provided superior protection against Brucella melitensis when compared to the widely used Rev 1 attenuated vaccine strain [155].

An alternative to protein sub-unit vaccines are the LPS, and more specifically the polysaccharide, PS, component. There has been a long rich history of investigations dealing with these. Huddleson and Johnson in 1933 prepared the filtrate reagent Brucellin which they used as a therapeutic for patients to clear their illness [156]. In 1939, Miles and Pirie reported that the predominant antigen in these extracts was a formamino dihydroxyl sugar [157], [158]. Over several decades, the Brucella PSs have been characterized [159]-[169] and as noted before, antibodies against these were protective in the mouse model. If PSs have the potential for being sub-unit vaccine candidates, why haven’t these been used? In part, the reason against their use as vaccines has been experimental evidence. As noted previously, only rough, i.e. strains lacking PS, were found to be effective vaccines in animals while killed or live smooth Brucella cells are ineffective, the PS were very poor antigens that did not induce significant titres of antibodies against Brucella [170], and this cellular component was tested in the mouse model challenged intranasally with Brucella melitensis and found not protective [170].

Appearances can be deceiving. Live rough Brucella strains used as attenuated vaccines have been found to produce PS, but some were defective for linking this to the bacterial surface [171] or produced very level levels [172], hence the colony rough appearance. Smooth killed cells did have PS, but these also had Lipid A as part of the LPS [173] and lipoproteins that interfered with the host’s immune response [174]. PS may be poor inducers of antibodies, but these are excellent immunomodulators of cell-mediated responses [175]-[178] that are more relevant for guarding against Brucella as an intracellular parasites. Brucella melitensis was particularly difficult to protect against, especially when given as an intra-nasal challenge. Curiously, for intra-nasal challenge, LPS containing polysaccharide provided protection for mice only when given by a different route [179].

Studies have found that the polysaccharide is indeed an effective sub-unit vaccine for protection against brucellosis in 3 animal models. Mice given a single low dose of Brucella abortus OPS (1 µg in sterile saline), without adjuvant, had 10,000-fold less Brucella in their spleens than unvaccinated control mice [180]. In a study in Colombia, three of four pregnant guinea pigs given 10 or 100 µg of polysaccharide had no sign of the bacterium in their blood or spleens [181]. For a large swine trial done in Venezuela, Brucella abortus and Brucella suis OPS, given
either by intra-muscular injection or orally, gave sows very high levels of protection against a field strain of
B. suis transmitted sexually by infected boars [182]. In this latter study, the polysaccharide vaccine used had
been stored lyophilized and kept on the shelf at room temperature for a decade before use, showing that cold
chain was unnecessary. More recently we have found that OPS and exopolysaccharide extracted from B. suis
145, which expresses both the B. abortus A-antigen and B. melitensis M-antigen, protected mice from all
3 species. A single dose of 1 µg vaccine, in sterile saline and without adjuvant, protected mice for 15 months
from B. suis 145 infection [unpublished results].

15.3 TREATMENTS AFTER INFECTION

15.3.1 Antibiotics

There have been several publications on the sensitivity and Minimum Inhibitory Concentration (MIC) of
Brucella species to different antibiotics in vitro [183]-[187]. However, as noted in the review by Young [21],
the usefulness of these may differ in vivo. Influencing factors may be the extent of intracellular penetration,
the blood-brain barrier in neurobrucellosis, the tissues invaded such as the heart or bone marrow, the age and health
of the patient, and their refusal to comply if they experienced adverse side-effects [22], [46]. It has also been
found that some antibiotics act synergistically, being more effective at lower concentrations for shorter times
when used together than each individually [188]. Taking all of this together, studies involving large numbers of
patients have found doxycycline with rifampin and streptomycin to be the most effective for clearance
of infection [46], [189]. It should be noted that the United States Army Medical Research Institute of
Infectious Diseases (USAMRIID) and the Centers for Disease Control and Prevention (CDC) recommend that
the treatment of brucellosis patients should be a combination of antibiotics in high amounts (doxycycline,
100 – 200 mg, oral; rifampin, 600 – 900 mg, oral; for acute complicated cases add 1 gram of streptomycin by
intramuscular injection), daily for several weeks to several months [36], [45].

Aside from the side-effects of patients taking some antibiotics, such as hearing loss from aminoglycoside use
[190] and photoallergy from those taking quinolones [191], there are other complications. If the patient has a
large load of Brucella in their tissues and the therapy is too effective, the sudden death of bacteria and the release
of toxic compounds may kill [192]. However, this rarely occurs because the bacterium has evolved not to
compromise its host, its sLPS being 10,000-fold less toxic than that of other bacteria such as Escherichia coli
[41]. With regards to clearance, after an aggressive antibiotic schedule, the blood cultures and bone marrow
sampling will likely be negative for the bacterium, the patient will feel fine, and after antibiotic use has stopped
they will continue to appear healthy. This may be a false sense of security. The bacterium is likely persistent at
very low numbers within cells, controlled by a vigilant immunity. From investigations using sensitive PCR
methods, it appears that patients that have recovered from brucellosis will unknowingly have the infection for
the rest of their lives [193]. Perhaps a key question is not how the pathogen continues to exist within the host for
so long, but how it is that the host’s immune system manages to have the upper hand over several decades.

15.3.2 Anti-Brucella Antibodies

In theory, once an infectious agent or toxin has entered the mammalian cell, antibodies which are outside in the
serum will not be able to play any role on threat agents inside. If this was always the case, it would not explain
how anti-botulinum anti-serum therapy can have a positive effect several days after a patient has been poisoned
[194]. That some antibodies can enter the mammalian cell and bind to antigens is evident in auto-immune
diseases such lupus erythritosis/nephritis, migraines and diabetes [195]. For brucellosis, human trials in the
1930s showed that giving anti-serum from recovered patients could in turn alleviate the symptoms of those with
A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE *BRUCELLA* SPECIES

acute infections [156]. For water buffalo, resistance to *B. abortus* has been correlated to antibody titres and enhanced control of intracellular replication of the bacterium [196]. The action of antibodies on pathogens within the mammalian cell has only been sparsely reported, but the current findings suggest the potential for exciting novel applications [197]-[200].

### 15.3.3 Vaccines

Vaccines are viewed as preventative measures to be given before infection, not as treatments after infection. Even with this dogma, concepts are beginning to change. Vaccination with melanoma-specific antigens enhanced survival of cancer patients [201] and it is now recommended that those infected with *Bacillus anthracis* receive both aggressive antibiotic therapy and protective antigen vaccination [202]. The immune responses may be different for these two examples, the first dealing with immunomodulation and an activation of cytokines, the latter an active immunization to deal with spores that germinate after antibiotic therapy has been discontinued. With regards to brucellosis, in the 1930s it was found that Brucellin, filter-sterilized culture filtrates of *B. abortus* or *B. melitensis*, reduced the severity and length of illness for patients [156]. Perhaps a sub-cellular vaccine can be developed that can be given after infection to facilitate clearance of the bacterium from the patient.

### 15.4 FUTURE DIRECTIONS FOR RESEARCH AND DEVELOPMENT OF MEDICAL COUNTERMEASURES AGAINST BRUCELLOSIS

#### 15.4.1 Sub-Unit Vaccines

There is no need to inoculate people with live attenuated strains if the “magic bullet” can be identified. A description has been given that DNA encoding specific outer membrane proteins of *Brucella* are more effective and cross-protective to other species than Omps by themselves [155]. A very low amount of single-dose of polysaccharides without adjuvant can give long-term cross-protective immunity against *Brucella* spp. in animal models [180]-[182]. In the swine model, oral vaccination was as effective as intra-muscular injections [182]. Although not reported, it was found during our PS vaccine preparation, that an equivalent amount of glycosylated proteins, removed by trichloroacetic acid precipitation, was as effective for protecting mice. Perhaps the latter is the best of both candidates, combining protective proteins and polysaccharides of *Brucella*. Instead of synthetically conjugating the two to improve their effectiveness, perhaps all that is needed is to use that which was made naturally. On the topic of glycosylated proteins, it used to be dogma that prokaryotes did not produce these. It is now known that bacterial flagellin, pilin and enzymes can be glycosylated [203]-[205]. Those of *Brucella* should be investigated for vaccine potential. Although *Brucella* is non-motile, it may resemble other bacteria, such as *Burkholderia mallei* [206] that has the gene for flagellin but is not motile.

#### 15.4.2 Immunomodulators

Until recently, immunomodulators were viewed as either suppressants of inflammation (e.g. corticosteroids) or crude particulate material that would irritate the body to respond to a foreign object. In our laboratory we have found that *Brucella* polysaccharides, either that of unusual composition such as the 4,6-dideoxy-4-formamido-D-mannose composition of the OPS, or that of unusual linkage such as the cyclic 1,2-beta-glucan, could double mouse macrophage activity against yeast zymosan (data not reported). Cytokines also have been shown to play a major role in clearance of this bacterium [207]. Where *Brucella* has commandeered the mammalian cell, it may be possible to override the cell’s incapacitation with potent immune stimulating compounds.
15.4.3 Liposome Encapsulated Antibiotics and Vaccines

Once the bacterium has invaded the cell and tissues, it is difficult to clear. To deliver the antibiotic in high concentration directly into the cell, liposome encapsulation has been evaluated. It was found that positively charged plurilamellar vesicles were the most effective for delivery of aminoglycosides into mouse monocytes [208]. The usefulness of liposomes has been extended for enhanced delivery of antigens and DNA vaccines [209]-[211]. Advances for this delivery vehicle for cell delivery continue to develop. As monocytes, macrophages and dendritic cells of the body contain mannose receptors [212], [213], recently a Japanese laboratory has improved on liposomal delivery of anti-cancer drugs into macrophages by coating liposomes with a synthetic oligomannose that bind to these receptors [214].

15.4.4 Antibody Therapy

There have been different descriptions and categories of anti-\textit{Brucella} antibodies. One group appears to be antibodies that are Type 1, have affinity to the length of the PS, are produced in high titre in infected animals, are non-protective, agglutinate bacteria and may enhance entry into the cell. The other group appears to be antibodies that are Type 2, have affinity to the tip of the PS (which may include the 3 sugar tag from the core), are produced in vaccinated animals, are protective, non-agglutinating at neutral pH and may inhibit entry into the cell [39], [215]-[218]. The latter may have protective or therapeutic value as medical countermeasures. In providing a therapeutic, two locations of the infectious agent have to be considered. The first is to destroy the bacterium outside the cell, either before it enters or after it is released from infected cells. Curiously, less is more in that low levels of antibody, not high amounts, allow complement killing of \textit{Brucella} in the blood [219]. The second is to enhance clearing once the bacterium gets inside the cell. It was discussed previously that in some instances antibodies may act inside the cell. Possible mechanisms may be antibody targeting the pathogen to lysosomes within the cell [220] or interacting with the cytosolic IgG receptor, TRIM-21, which targets the pathogen to a proteasome for its degradation [221].

15.4.5 Serum Components Other Than Antibodies

When one thinks of an immune response, one is likely to think of the production of antibodies and the induction of complement lysis against the threat agent. However, the body’s defences are far more diverse and robust than this single mechanism. Although \textit{Brucella} appear to have evolved a resistance to serum cationic peptides called defensins [222], with its polysaccharide coating composed of mannose derivatives, the bacterium has been found to be sensitive to the mannose binding lectin in the blood which also activates complement lysis [223]. On a similar theme, although the mechanism has been resolved with a fungal model, when the macrophage has been exposed to mannose polymers, the receptor dectin-1 can induce the macrophage mannose receptor to be shed, bind to the pathogen and enhance its destruction [224]. These serum components should be investigated further to determine if these play a role in long-term immunity against \textit{Brucella}.

15.5 REFERENCES


A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE BRUCELLA SPECIES


A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE *BRUCELLA* SPECIES


A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE BRUCELLA SPECIES


A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE BRUCELLA SPECIES


A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE BRUCELLA SPECIES


[112] Martinez M, Ugalde RA and Almiron M. Irr regulates brucebactin and 2,3-dihydroxybenzoic acid biosynthesis, and is implicated in the oxidative stress resistance and intracellular survival of *Brucella abortus*. Microbiology 2006;152(Pt 9):2591-98.


induced a better degree of protection against \textit{B. ovis} and a similar degree of protection against \textit{B. melitensis} than Rev.1 vaccination. Vaccine 2007 10 Aug;25(32):5958-67.


A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE BRUCELLA SPECIES


A REVIEW OF MEDICAL COUNTERMEASURES, PROTECTION AND TREATMENT, AGAINST THE BRUCELLA SPECIES


Chapter 16 – RICKETTSIA PROWAZEKII

Joseph C. Larsen
U.S. Department of Health and Human Services, Office of the Assistant Secretary for Preparedness and Response, Biomedical Advanced Research and Development Authority, Washington, DC
UNITED STATES

Corresponding Author (Larsen): joseph.larsen@hhs.gov

ABSTRACT

Rickettsia prowazekii, the etiological agent of epidemic typhus, is a Gram-negative obligate intracellular pathogen. The organism is normally transmitted via the feces of infected lice. The genus Rickettsia is divided into two groups, the Spotted Fever Group (SFG) containing 18 species including the human pathogens R. conorii and R. rickettsii, and the Typhus Group (TG) containing the human pathogens R. prowazekii and R. typhi.

It is estimated that nearly 30 million people were infected with epidemic typhus following the First and Second World Wars [29]. An outbreak in Burundi involved thousands of cases exhibiting a case mortality rate that exceeded 10% [51]. These outbreaks still occur today and often serve as reminders of the consequences of the deterioration of social conditions.

The incubation period for epidemic typhus is typically 10 – 14 days [5], [41]. Patients generally experience 1 – 3 days of malaise before the rapid onset of fever and headache. Central Nervous System (CNS) manifestations can occur in up to 80% of patients and generally include coma, delirium, and seizures. Rashes, arthralgias, myalgias, and other non-specific constitutional symptoms are common [5]. Rashes and skin lesions are distributed mostly on the trunk and may spread centrifugally to involve the extremities. Cough is reported in a high proportion of patients (38 – 70 %), highlighting the primary pulmonary involvement of the disease [5]. Mortality is variable and was estimated to be around 60% in the pre-antibiotic era. Given antibiotic treatment, the mortality is now approximately 4%.

Infrequently, vasculitis is observed in the extremities of infected patients, and the resulting vascular compromise can lead to shock to a small proportion of individuals. Ischemia, which results from the vasculitis, can lead to necrosis and gangrene infection of the extremities.

An additional manifestation of infection with R. prowazekii is the development of Brill-Zinsser disease [28]. Characterized as a mild form of epidemic typhus, it is a recrudescent form of the disease which occurs in a subset of patients that are unable to eradicate the organism. The disease course is typically shorter in duration and is milder in clinical presentation. The mechanism of organism retention remains to be identified; however data from murine models suggest that adipose tissue is the host reservoir for recrudescent infection [7].

16.1 USE AS A BIOLOGICAL WEAPON

R. prowazekii was weaponized and field tested by the United States, the former Soviet Union, and Japan [3]. A number of characteristics make R. prowazekii well-suited for weaponization and dissemination. These include the organism’s environmental stability, low infectious dose, high morbidity, and significant mortality [3]. Modeling estimates suggest that 50 kg of aerosolized R. prowazekii released in an attack would result in approximately 100,000 casualties, with 19,000 deaths [3], [33], [42]. The weaponization procedure is technically
demanding, however, and would require highly sophisticated procedures and skilled personnel, decreasing the probability that individuals could produce large quantities of weapons grade Rickettsial agent [3]. Therefore, *R. prowazekii* is currently designated as a Category B biological threat agent by the United States Centers for Disease Control and Prevention.

16.1.1 Genomics

The 1.1 Mb genome of *R. prowazekii* is highly derived and is the product of reductive evolution, reflecting the organism’s strict intracellular lifestyle [2]. Twenty-four percent of the genome is non-coding sequence, suggesting a high degree of genomic reduction. A number for the genes required for amino acid biosynthesis are missing. Upstream genes involved in lysine biosynthesis, specifically the synthesis of diaminopimelate, a component of the cell envelope, are present suggesting their role in maintenance of cell structure versus amino acid biosynthesis. No genes required for the *de novo* synthesis of nucleotides are present. However, genes needed for the inter-conversion of nucleoside monophosphates to all four nucleotides exist, suggesting that *R. prowazekii* imports nucleoside monophosphates from the host cell. Genes encoding proteins involved in ATP production, including the TCA cycle, ATP synthase complex and multiple ATP/ADP translocases are present, suggesting that in addition to the acquisition of ATP from the host cell, the bacterium is able to synthesize its own ATP, likely when cytosolic levels decrease at latter stages of the infection.

Phylogenetic analysis of the genome suggests that aerobic respiration in eukaryotes originated from a progenitor of *Rickettsia*. Further, mitochondria and *R. prowazekii* share proteins involved in ATP production and transport, indicative of a close evolutionary relationship [2]. It is important to note, however, that the metabolic similarities between mitochondria and *R. prowazekii* probably resulted from evolutionary convergence and not vertical inheritance [27]. However, it is likely that *Rickettsia* and the mitochondria were derived from a common ancestor.

The genomic sequence of the members of the *Rickettsia* genus revealed the presence of multiple genes predicted to be involved in transport or secretion. Specifically, genes homologous to the *vir* Type IV Secretion System (T4SS) of *Agrobacterium tumefaciens* were found to be strongly conserved across a number of *Rickettsia* species [24]. While a functional role has not been ascribed for the *Rickettsia* T4SS, its involvement in both virulence and transfer of genetic information in other bacterial pathogens has been established, suggesting that this system likely plays a similar role in *Rickettsia* [1].

There is a paucity of genetic tools available for the study of *Rickettsia*. While genetic transformation has been demonstrated, the process remains laborious and time consuming, primarily due to the obligate intracellular nature of the organism [35], [38], [39], [40]. Directed mutagenesis to inactivate a single gene product was described recently [13]. It is clear that the development of more robust genetic tools will facilitate the identification of bacterial factors responsible for intracellular survival, growth, and immune evasion.

16.1.2 Pathogenesis

Many of the molecular mechanisms of pathogenesis of *R. prowazekii* remain uncharacterized. Many of the studies conducted to date to characterize host-pathogen interactions were carried out in related organisms: *R. conorii* or *Rickettsia* sp. While phylogenetically similar, the level of conservation of pathogenic mechanisms remains unclear. Differences in proteins involved in cell binding as well as mechanisms of intracellular spread have been reported, suggesting that there may be significant divergence [26].

Cell surface adhesion of *R. prowazekii* is predicted to be mediated by a bacterial adhesin, designated OmpB. This protein serves as a primary adhesin in SFG *Rickettsia* by binding to a Ku70, a cell surface receptor
component of a DNA-dependent protein kinase [36]. OmpB is sufficient for invasion and the process occurs in an actin, c-Cbl, clathrin and caveolin-2 dependent manner [10]. Although these data have not been confirmed experimentally in *R. prowazekii*, the bacterium induces local cytoskeletal rearrangements to facilitate cellular invasion, suggesting a similar mechanism may be employed [48].

*R. prowazekii*, as with all members of the genus *Rickettsia*, is able to invade non-phagocytic cells and rapidly escape the phagosome to freely replicate in the cytosol [30], [47]. Hemolysin and phospholipase D were identified as factors expressed early in infection and shown to complement a *Salmonella* mutant that was incapable of phagosomal escape. A phospholipase mutant of *R. prowazekii* was attenuated in the guinea pig model using body temperature and change in body weight as evaluation criteria, suggesting phagosomal escape is a critical step in the lifecycle of the bacterium [13].

Following escape into the eukaryotic cytosol, the bacterium replicates to high levels, eventually rupturing the infected cell and releasing progeny to infected adjacent cells. This contrasts with other members of the *Rickettsia* genus, specifically the SFG, which use actin-based motility to spread inter- and intracellularly [31].

Database mining revealed the presence of a gene implicated in cellular invasion in other bacterial pathogens. The protein, designated InvA, catalyzes the hydrolysis of dinucleoside oligophosphates and contains a specific motif found in the Nudix hydrolase family [21]. Transcriptional analysis demonstrated that InvA mRNA was greatly increased early in the infection process (~24 hours post-infection); suggesting the protein plays a role in intracellular survival [23]. Using immunogold electron microscopy, InvA was determined to be localized in the cytoplasm [22]. However, none of these studies provided direct evidence of InvA’s role in invasion or intracellular survival. The development of genetic system to readily introduce mutations could aid in the elucidation of the role of invA in the pathogenesis of *R. prowazekii*.

A characteristic of rickettsial infection is increased vascular permeability. Infection with *R. prowazekii* increases the transendothelial migration of murine and human PBMCs [6]. This is an active process related to virulence, as infection with attenuated strains does not result in increased migration. Infection of endothelial cells with SFG *Rickettsia* results in the up regulation of a number of pro-coagulant and pro-inflammatory cytokines, reflecting the host’s attempt to control the increased vascular permeability that is a consequence of infection [14], [46], [45], [44].

### 16.1.3 Host Response

Interferon-γ (IFN-gamma) is critical to the host response to *Rickettsia* infection, primarily though the activation of target cells. Cytokine depletion experiments demonstrated that mice lacking IFN-γ or TNF-α succumbed to infection with sub-lethal doses of *R. conorii*, highlighting the critical importance of these cytokines in the control of infection [16]. *In vitro* studies using human endothelial cells, hepatocytes, and macrophages infected with *R. conorii* demonstrated that these cells are capable of intracellular bactericidal activity [17]. The killing was dependent on the presence of nitric oxide, tryptophan degradation, RANTES, and reactive oxygen species [17].

Additional research has identified dendritic cells and Natural Killer (NK) cells as important mediators of resistance to rickettsial infection [8]. Experimental studies in animals led to the identification of mouse strains with differential susceptibility to infection with *R. conorii*. C57BL/6 mice were identified as intrinsically resistant to infection, while C3H/HeN mice were found to be susceptible. Dendritic cells from resistant mice had higher bacterial loads, up-regulated expression of major histocompatibility complexes and co-stimulatory molecules [15]. Further, dendritic cells from resistant mice more effectively primed CD4+ T-cell to produce IFN-γ. NK cell activity was found to be markedly increased at early time points in mice infected with *R. conorii*.
and *R. typhi*. Depletion studies demonstrated that the normally-resistant mouse strain C57BL/6 was made susceptible by the reduction of NK cell populations. Both serum IL-12 and IFN-γ were increased in infected mice, reinforcing the importance of these factors in the early response to rickettsial infection.

The role of humoral immunity in protection from infection with *R. conorii* has also been experimentally evaluated. SCID mice were passively transfused with monoclonal antibodies to OmpA, OmpB, or LPS [18]. Antibodies to LPS were not protective. Mice treated with polyclonal antisera or monoclonal antibodies against OmpA or OmpB showed protective efficacy against lethal challenge. The Fc region was also critical for protection, as mice were not protected when administered Fab fragments. The effect of antibody on the bacterium’s interaction with the host cell was also assessed [19]. Polyclonal sera and anti-OmpB monoclonal antibodies protected against *R. conorii* infection of endothelial cells and macrophages by opsonization which blocked phagosomal escape. The killing was mediated by nitric oxide, reactive oxygen species, and tryptophan starvation. While conventional thought suggests that control of an obligate intracellular parasite would require primarily a cell mediated immune response, these studies suggest that the humoral response play an important role as well.

At present, very little is known about the initial host response or pathogenesis of aerosolized exposures to *R. prowazekii*. It is unclear how the route of exposure will affect the immunopathology of the disease, or if results derived from studies examining the host response to other rickettsial species are able to be extrapolated to aerosol exposures.

### 16.1.4 Animal Models

There are no suitable aerosol exposure animal models of *R. prowazekii* available to evaluate medical countermeasure candidates. Animal models employed to study the interaction of the bacterium with its vector are available [32]. An intravenous non-human primate model of epidemic typhus was developed, which replicates the clinical signs and symptoms of epidemic typhus [25]. There are a few reports which describe and characterize the pathology in a murine intranasal exposure model of *R. prowazekii* [34], [52]. To date, however, there are no published reports describing the exposure of animals by the aerosol route. To facilitate medical countermeasure development for *R. prowazekii*, animal models which recapitulate human disease following aerosol exposure will need to be developed and characterized to support regulatory approval.

A murine model was developed which recapitulates some aspects of human disease [2]. Pathological findings from infected mice were suggestive of the development of interstitial pneumonia, hemorrhages in the lungs and brains, and multi-focal granulomas, all consistent with human disease. Bacteria were present in the blood, liver, lungs and brains and persisted for 9 days. No analysis was conducted on vascular changes upon infection, and such analyses will be critical to determining the degree that this model reflects the human experience.

### 16.1.5 Vaccines

Currently, there are no available commercial vaccines for *R. prowazekii*. Beginning in the late 1800s research began on the development of a typhus vaccine. These vaccines were produced from the feces of infected lice and posed significant technical obstacles to large-scale production as well as safety considerations for the staff involved.

Additional early developmental attempts included vaccines derived from the contents of louse intestines or vaccines made from *R. prowazekii* cultured from the lungs of small animals. A chemically inactivated vaccine, produced in chicken eggs, was used to immunize both Allied and Axis forces during WWII [49].
A modern live vaccine, based upon the Madrid E strain of *R. prowazekii*, was produced and administered to humans [37], [20], [42]. The Madrid E vaccine strain was highly immunogenic and robust, with antigen-specific antibodies detected within 2 weeks and immunity to virulent *R. prowazekii* lasting at least 5 years [50], [20]. This vaccine is not in use today due to the adverse reactions, lot-to-lot variability in production, potential reversion of attenuated phenotype, potential for unrestricted growth in immunocompromised individuals, and the development of recrudescent infection (Brill-Zinsser disease) [42]. DNA vaccines have also been employed; however, they are in an extremely early developmental stage [11].

The development of sub-unit vaccines for *R. prowazekii* has identified some candidate antigens but has also highlighted the difficulty in working with this organism. Soluble antigen cell fractions were used to vaccinate guinea pigs and the heat liable portions determined to be immunogenic and protective [12]. A major immunodominant component of these fractions was determined to be a protein known as Outer membrane protein B (OmpB). This protein is responsible for the extracellular S-layer of *R. prowazekii* and vaccination was shown to provide protection in the mouse and guinea pig models [9], [42]. Purification of OmpB from *R. prowazekii* is not practical, due to technical challenges, expense, and occupational hazards associated with handling large culture volumes. Purification of OmpB from a heterologous expression system, like *Escherichia coli*, has not been successful due to the inability to induce, purify, and refold the protein into its native form [42].

### 16.1.6 Anti-Microbial Therapies

The treatment of choice for infection with *R. prowazekii* is the administration of doxycycline for 5 – 10 days [43]. Doxycycline, tetracycline, minocycline, demeclocycline, and chloramphenicol are USFDA-approved for the treatment of *R. prowazekii* infection.

Little has been done to examine additional therapeutic approaches for *R. prowazekii*. Given the absence of a detailed knowledge of the molecular mechanisms of pathogenesis, the development of next generation medical countermeasures for these agents would appear to be a long-term goal.

### 16.2 CONCLUSION

There are significant barriers to the development of state-of-the-art medical countermeasures for *R. prowazekii*. Foremost, is the prioritization of resources for the development of countermeasures for all biological threats. The technical difficulties inherent in the production of large quantities of weapons grade agent, coupled with the general susceptibility of *R. prowazekii* to a number of available antibiotics, suggests that the development of next generation countermeasures for this agent may not be necessary or should at a minimum be a significantly lesser priority than other threat agents for which there are no effective countermeasures.

If additional research and development for medical countermeasures is to be pursued, there are a number of important scientific questions which should be addressed to facilitate development. The lack of an identified sophisticated genetic system has significantly hampered our understanding of the bacterial factors involved in cellular invasion, growth, immune evasion, and persistence. An understanding of each of these areas would shed light on the molecular mechanisms underlying the rickettsial life cycle and significantly increase our ability to formulate novel vaccine and therapeutic strategies.

A determination of the contribution of protein secretion, particularly the role of the T4SS in virulence or DNA transfer, and the subsequent characterization of the secreted substrates will also enhance our understanding of the pathogenic mechanisms of *R. prowazekii*. 
If there is increased interest in the development of medical countermeasures for this agent, the appropriate aerosol animal challenge models must be developed. Animal models are required that recapitulate human disease in order to facilitate regulatory approval of products in the absence of the ability to perform efficacy studies in humans.

In summary, there has been limited interest in developing next generation medical countermeasures for \textit{R. prowazekii}. To spur innovation for novel countermeasure development, it may ultimately prove more fruitful to focus on basic research on the molecular mechanisms of the \textit{R. prowazekii} life cycle.

16.3 REFERENCES


RICKETTSIA PROWAZEKII


Chapter 17 – BOTULINUM NEUROTOXINS

Virginia I. Roxas-Duncan¹ and Leonard A. Smith²

¹: Chief, Select Agent Management Branch, Biosurety Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; ²: Senior Research Scientist (ST) for Medical Countermeasures Technology, Office of Chief Scientist, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

UNITED STATES

Corresponding Author (Smith): Leonard.A.Smith@comcast.net

17.1 INTRODUCTION

Botulinum Neurotoxins (BoNTs), produced by strains of *Clostridium botulinum*, are the most poisonous of all known biological toxins, and are the etiologic agents of the neuroparalytic disorder botulism. All forms of botulism manifest essentially the same distinct clinical syndrome of symmetrical cranial nerve palsies that may be followed by descending, symmetric flaccid paralysis, which may progress to respiratory compromise, and even death [1]. If left untreated, as may be the situation in a mass casualty scenario or in a battlefield exposure, fatality rates can be as high as 60% [2], [3].

BoNTs are recognized bioterrorism agents that present a distinct threat to U.S. and allied military forces and civilian populations worldwide [4], [5], [6]. BoNT has been classified as a Category A agent by the Centers for Disease Control and Prevention (CDC) because of ease of production, dissemination, and extreme lethality [4]. The most probable methods for use of BoNT as a terrorist weapon include either a deliberate contamination of food or beverages, or release as an aerosol [7]. BoNT has also been designated as Tier 1 BSAT, a sub-set of select agents and toxins that present the greatest risk of deliberate misuse with significant potential for mass casualties or devastating effect to the economy, critical infrastructure, or public confidence, and pose a severe threat to public health and safety [8]. As such, entities that possess, use, or transfer BoNTs must follow added requirements described in the select agent regulations [9].

17.2 BoNT AS BIOLOGICAL WEAPON

BoNT was among the first agents to be considered as a potential Biological Weapon (BW), and has been developed as such by various state-sponsored programs in countries including Germany, Iraq, Japan, Russia, and the U.S. [2]. BoNT was first used as a weapon in the early 1930s when General Shiro Ishii, head of the Japanese Unit 731, admitted to subjecting prisoners to lethal ingestion of *C. botulinum*. The U.S. Army initiated a BW program in 1943. In early 1944, Allied intelligence revealed that Germany was planning to use BoNT as a weapon against an invasion force. This perceived threat led to the initiation of BW research on BoNT [10]. In 1969, by the executive order of President Richard M. Nixon, the U.S. BW program ended, and all BoNT stockpiles and other stored BW agents were destroyed. In 1972, the Soviet Union signed the Biological and Toxin Weapons Convention, and agreed to halt all offensive BW development and also to destroy existing BW stockpiles [7]. However, despite the ratification of this agreement in 1975, the Soviet Union still continued and significantly expanded their BW program until the early 1990s. In 1992, after the Persian Gulf War, Iraq admitted to the United Nations producing 19,000 L of concentrated BoNT, some of which were loaded into military weapons [11]. Between 1990 and 1995, the Japanese cult Aum Shinrikyo tried to use aerosolized botulinum toxin in downtown Tokyo and U.S. military facilities in Japan on at least three occasions; fortunately, these attempts proved unsuccessful [7].
BoNT may be used militarily to immobilize an opponent. It has been estimated that as a point source aerosol, BoNT could be used against a civilian population in a densely populated area to incapacitate or kill 10% of population within 0.5 km downwind [4]. In addition, terrorism use of BoNT might be presented as intentional contamination of food. A mass casualty attack involving BoNT would probably overwhelm the health care system and community medical resources, and significantly impact the medical response to victims of the attack [7].

17.3 THE ORGANISM AND ITS TOXINS

The bacterium typically associated with producing BoNTs is \( C. \ botulinum \); however, BoNTs can also be produced by \( C. \ butyricum \), \( C. \ baratii \) and \( C. \ argentinense \) [12]. \( C. \ botulinum \) is ubiquitously found in soil and aquatic sediments worldwide. It is a Gram-positive, anaerobic bacillus capable of forming endospores [4]. The spores of \( C. \ botulinum \) are relatively heat-resistant and pressure sterilization is necessary to ensure their destruction. In contrast to the spores, the toxin is relatively heat-labile, and is completely inactivated at 100°C for 10 minutes.

There are four groups of \( C. \ botulinum \) strains (Groups I to IV) classified based on metabolic activity [13] and genetic composition [14], [15]. Group I includes Type A strains and proteolytic strains of types B and F; Group II includes Type E strains and non-proteolytic strains of types B and F; Group III includes non-proteolytic strains of types C and D; and Group IV includes only strains that produce Type G.

\( C. \ botulinum \) elicits seven structurally similar but immunologically distinct neurotoxins (~ 150 kDa in mass), identified as serotypes A-G [16], [17]. Recently, a new purported serotype, designated as Type H, has been reported [18], [19] but this requires experimental confirmation. Of the seven serotypes, BoNT/A is thought to be the most toxic; based on animal studies, lethal human dose values (LD₅₀) have been estimated [4], [20]. Human botulism is caused primarily by BoNT/A, /B and /E (Arnon et al., 2001), and rarely by /F [21], [22]. BoNT/C and /D cause botulism in animals. BoNT/G, produced by \( C. \ argentinense \), has been associated with sudden death but not neuroparalytic illness in a few patients in Switzerland [23]. All seven serotypes can cause inhalational botulism in primates [24].

Several sub-types have been identified for at least six of the seven BoNT serotypes [15], [25]. Although all BoNT serotypes exert similar toxic effects on nerve cells, their intracellular target protein, characteristic mode of action and potencies vary considerably.

17.4 PATHOGENESIS

All BoNT serotypes inhibit acetylcholine release; however, their mechanism of action is via different intracellular protein targets [26]. Upon exposure to BoNT, pathogenesis occurs through a series of events (for reviews, see [4], [26]-[29]):

1) BoNT is activated by proteolytic cleavage; the activated structure is a 150 kDa polypeptide composed of a heavy chain (HC, MW = 100 kDa) and a light chain (LC, MW = 50 kDa) linked by a disulfide bond. This interchain S-S bond plays a critical role in cell penetration. If this link is broken before the toxin is internalized in the cell, the LC cannot enter the axon terminal membrane and toxicity is abolished.

2) BoNT enters the circulation and is transported to the neuromuscular junction.

3) At the neuromuscular junction, the HC binds to the neuronal membrane on the pre-synaptic side of the peripheral synapse. The HC binding domain interacts with membrane protein receptors and a group of sialic-acid-containing glycolipids called gangliosides.
4) BoNT is then internalized through receptor-mediated endocytosis into vesicles.

5) The HC translocation domain facilitates the transport of the LC across the membrane of the endocytic vesicle.

6) Once inside the cytoplasm, the LC (a zinc-containing endopeptidase) targets some of the proteins that form the synaptic fusion complex. These proteins, referred to as SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptors) proteins include SNAP-25 (synaptosomal-associated protein of 25 kDa; cleaved at different sites by BoNT/A, /C, and /E), synaptobrevin (also referred to as VAMP [vesicle-associated membrane protein], cleaved at different sites by BoNT/B, /D, /F, and /G, and syntaxin (cleaved by BoNT/C). Proteolytic cleavage of SNARE proteins inhibits the release of acetylcholine at the neuromuscular junction, resulting in flaccid muscle paralysis which is the primary clinical sign of botulism.

17.5 EPIDEMIOLOGY

*C. botulinum* spores are found worldwide in the soil and sea sediments, and in low numbers in the gastrointestinal tracts of some birds, fish, and mammals. In the U.S., the most frequent type isolated is Type A, followed by B and E, with an occasional isolate of Type F [28]. In Europe, Type B is the most frequent isolate, and Type A is comparatively rare [28], [30].

The onset and severity of botulism are dependent on the rate and amount of toxin absorption [4]. There are five clinical categories of botulism:

1) *Foodborne botulism* is caused by consumption of foods contaminated with BoNT;

2) *Wound botulism* has been associated with non-invasive infection and *in vivo* toxin formation. Cases of wound botulism are often linked with illegal drug use, mostly due to BoNT/A;

3) *Infant botulism* results when ingested *C. botulinum* spores germinate and colonize the gastrointestinal tract of infants aged < 1 year. Infant botulism is usually associated with BoNT/A and /B;

4) *Adult toxico infection* occurs after compromise of the gastrointestinal tract following surgical interventions and/or broad spectrum antibiotic use. BoNT/E producing *C. butyricum* and BoNT/F-producing *C. baratii* have been associated with adult toxico infection; and

5) *Iatrogenic botulism*, acquired through misuse of toxin for therapeutic or cosmetic reasons.

There is a concern over a sixth category, inhalational botulism, which does not occur naturally, and can be acquired by inhalation of aerosolized pre-formed BoNT. It has been of interest in recent years as a potential weapon of bioterrorism [11], [24]. Inhalation botulism has been shown in animal studies [31], [32]. There is only one human case of BoNT inhalational exposure documented in 1962 involving three German laboratory workers who presented with symptoms of botulism three days after handling experimental animals exposed to dry, highly purified BoNT/A [33]. The patients were hospitalized five days after exposure, administered equine antitoxin, and discharged nine days after. It should be recognized that all kinds of botulism are potentially fatal and are considered medical emergencies.

17.6 CLINICAL SYMPTOMS AND LABORATORY DIAGNOSIS

Symptoms of botulism appear several hours to within a few days [4], [32]. This incubation period depends on the amount of toxin absorbed and the rate of absorption. Person-to-person transmission of botulism does not
The prominent bulbar palsies can be summarized in part as 4Ds: diplopia, dysarthria, dysphonia, and dysphagia [1]. Botulism is an immense public health concern, so any occurrence necessitates prompt notification of public health authorities and an epidemiological investigation.

The most reliable method for the detection of BoNTs and for diagnosing botulism is the mouse bioassay. This test can be performed by the Centers for Disease Control and Prevention (CDC) or state public health laboratories. The assay involves injecting mice with samples collected from patients displaying symptoms of botulism. Mice will typically begin showing signs of botulism within eight hours. The BoNT serotype of samples can also be determined with this test by neutralizing the toxin with serotype-specific antibodies prior to injecting the mice [34]. In cases of foodborne or infant botulism, stool samples can also be cultured to look for C. botulinum. Samples of the suspected food should also be cultured anaerobically with heat or alcohol treatment to select for spores. Diagnosis is usually established by the demonstration of toxin from the stool. Other BoNT diagnostic tests involving Polymerase Chain Reaction (PCR), endopeptidase activity, or Enzyme-Linked Immunosorbent Assays (ELISA) are also currently being developed.

### Animal Models

Though the basic action of BoNTs has been studied in rats, mice, chickens, frogs, goldfish, squid, and crayfish, research into the biomedical aspects of the toxin has utilized fewer animal species [35]. Most of this work has involved vaccine development in rodents; a number of vaccines based on inactivated toxin or recombinant toxin molecules have been evaluated in these models. The clinical signs of respiratory aerosol exposure have been reported in several animal species including mouse, rabbit, guinea pig, and non-human primates [36]. Rhesus monkeys exposed to an aerosol lethal dose of BoNT/A (2,000 MIPLD<sub>50</sub>) exhibited clinical signs and symptoms that closely mimicked human food-borne botulism [37]. The rhesus macaque aerosol challenge model was used to evaluate the efficacy of recombinant Botulinum Vaccine (rBV A/B) that is being developed for protection against inhalational intoxication with BoNT/A1 and BoNT/B1 [38].

It has been estimated that a total of 600,000 mice were used per year for testing BoNT products worldwide [39]. Due to the escalating list of off-label clinical indications for BoNT, along with its potential use as a biological weapon, the demand for testing is anticipated to increase. Efforts to develop alternative methods for BoNT testing to reduce, refine (cause less pain and distress), and ultimately replace animal use are ongoing [40].
17.7 CLINICAL USES OF BoNT

Though BoNT has been long associated with food poisoning, it is also being used in the clinical setting for treating neurological disorders and for cosmetic indications. Over the last 20 years, commercial production and therapeutic applications of BoNTs have increased steadily [41]. Only BoNT serotypes A and B have been approved by the Food and Drug Administration (FDA) for clinical use. Four formulations of BoNT are FDA-approved: onabotulinumtoxinA (BOTOX®, Allergan, Inc.), abobotulinumtoxinA (Dysport™, Ipsen Biopharm Ltd.), incobotulinumtoxinA (Xeomin®, Merz Pharmaceuticals, LLC), and rimabotulinumtoxinB (Myobloc®, Solstice Neurosciences, Inc.). BoNT has been used therapeutically to treat conditions such as spasmodic torticollis, strabismus, blepharospasm, laryngeal dystonia, focal dystonias of the hand, limb spasticity, hemifacial spasm, cerebral palsy, migraine headaches, hyperhydrosis, and post-stroke spasticity [42]. In addition, BoNT has been found to be useful in the clinical management of urological, musculoskeletal, dermatological, and secretory disorders, as well as pain and chronic migraine [43], [44].

17.8 HOST RESPONSE

Exposure to BoNT results in flaccid paralysis due to the blockage of acetylcholine release at the neuromuscular junction. The large size of both the neurotoxin and the non-toxin complex proteins would provide immunogenic targets; nonetheless, the low amount of toxin present in the serum coupled with its fast clearance rate impede the stimulation of an effective immune response.

Serum antibody response was reported in two cases of infant botulism infection with C. botulinum toxin [45]. Both patients had specific IgG and IgM antibody response to the toxin type to which they are exposed (BoNT/A and /B, for infants 1 and 2, respectively), but not to BoNT/E during convalescence. However, the levels obtained were much lower than in an immunized adult (positive control). It should be noted that infant botulism is a toxico infection, hence, colonization with C. botulinum and subsequent production of low levels of BoNT may persist for weeks, providing a continuing source of antigenic material.

To date, very limited information is available on host-cell/immune response associated with BoNT exposure. A recent microarray analysis reported on the differential regulation of genes in human neuronal (SH-SY5Y) and epithelial (HT-29) cell lines [46]; however, the massive amounts of BoNT/A complex used (6 nM for 96 hours and 500 nM for 6 hours, for SH-SY5Y and HT-29, respectively) argue against the definitive conclusion of the study.

Studies involving the induction of neuronal outgrowth, sometimes referred to as sprouting, upon intoxication with BoNT/A, /D, and /F have been reported [47], [48]. This phenomenon is generally considered a secondary response to the paralytic actions of BoNT. The nerve terminal sprouts produced after BoNT intoxication have the ability to intercede stimulated vesicle turnover, albeit at levels much lower than the untreated parent nerve terminals [48]. Intoxication with BoNT/E failed to produce sprouting, while sprouting due to BoNT/F occurred for shorter periods (7 to 28 days after intoxication) than BoNT/A (4 to 60 days). A recent study on the effects of BoNT/A on neurite outgrowth were examined using primary cultures enriched with motor neurons isolated from embryonic mouse spinal cord [49]. The neuritogenic effects of BoNT/A exposure were concentration dependent and inhibited by Triticum vulgaris lectin, a known competitive antagonist of BoNT. Similar findings were observed with the isolated BoNT/A binding domain, revealing that neuritogenesis could be initiated solely by the binding actions of BoNT/A. An examination of the cellular response to BoNT intoxication may provide valuable information that may aid in the development of novel therapies for botulism.
17.9 MEDICAL COUNTERMEASURES DEVELOPMENT

17.9.1 Current Therapies

Standard therapy for botulism consists of supportive care and passive immunization with anti-toxin [4]. In extreme cases where botulism has progressed to complete paralysis, adequate mechanical ventilation and long-term intensive care are required. Patients are frequently evaluated of neurologic and respiratory status, and special care is taken to prevent the risks of secondary infection, ventilator mishaps, and nutritional diminution. For infant botulism, special attention should also be focused on the patient’s nutrition and respiratory functions [50].

Antibiotics have no known direct effect on BoNT, though secondary infections acquired during botulism usually necessitates antibiotic therapy [4]. Aminoglycosides and clindamycin are contraindicated because of their ability to exacerbate neuromuscular blockade [51], [52]; tetracyclines may increase the degree of neuromuscular blockade by impairing neuronal calcium entry. Aminoglycoside also increases the chance of ventilator support in infant botulism [53]. For wound botulism, comprehensive surgical debridement may also be required in addition to respiratory support and anti-toxin administration.

17.9.2 Anti-Toxin Therapy

A botulinum Heptavalent equine-Based Anti-Toxin (HBAT, Cangene Corporation) composed of < 2% intact Immunoglobulin G (IgG) and ≥ 90% Fab and F(ab’)_2 immunoglobulin fragments [54], [55] is the only FDA-licensed therapeutic intervention to non-infant botulism. This anti-toxin replaced the previously available FDA-approved bivalent Botulinum Anti-Toxin AB and an investigational monovalent Anti-Toxin E (BAT-AB and BAT-E, Sanofi Pasteur). HBAT contains equine-derived antibody against all BoNT serotypes: 7,500 U anti-A; 5,500 U anti-B; 5,000 U anti-C; 1,000 U anti-D; 8,500 U anti-E; 5,000 U anti-F; and 1,000 U anti-G per vial. The recommended adult dosing is one 20 mL vial of HBAT [56].

An FDA-licensed human-derived Botulism Immune Globulin intravenous (BabyBIG®, California Department of Health Services) is available for the treatment of infant botulism caused by C. botulinum Type A or Type B. BabyBIG® is obtained from the pooled plasma of adults immunized with the pentavalent (A-E) botulinum toxoid, having subsequent development of high titers of neutralizing antibodies against BoNT/A and /B. Since BabyBIG® is of human origin, it does not have the risk for anaphylaxis that is inherent with products derived from equine sources, nor does BabyBIG® demonstrate a risk for possible lifelong hypersensitivity to equine antigens. BabyBIG® has been shown to significantly shorten the hospitalization period and reduce treatment costs [57]. An adverse effect could be a transient, blush-like rash.

While anti-toxin can neutralize toxin molecules that are not yet bound to nerve endings, it will not reverse existing paralysis, but may limit the progression of the disease and prevent further nerve damage by clearing them from circulation. Thus, the anti-toxin must be administrated immediately after intoxication has occurred, ideally < 24 hours after the onset of symptoms [58], [59]. Compared to standard care alone, immediate administration of anti-toxin has been shown to shorten time on respiratory support, and also reduced hospitalization period [34], [58]. Once damaged has occurred, it may take weeks to months for regrowth of the neuronal proteins. Recovery of neuromuscular function requires new synapses to form. There is a risk of hypersensitivity reactions associated with the use of equine anti-toxin, but not with the human product. Hence, skin testing is required prior to anti-toxin administration. Due to the risk of adverse reactions, prophylactic anti-toxin is not recommended in patients who are exposed to BoNT but have no symptoms. These patients may undergo gastric lavage or induced vomiting in an attempt to eliminate the toxin prior to absorption [60].
17.9.3 Development of Alternative Countermeasures

The limitations of current therapies against botulism have prompted a search for alternative therapeutics that could be administered pre- or post-exposure. Efforts have concentrated on:

a) Antibodies that neutralize the toxin, and specifically target the enzymatic activity of the LC.

b) Molecules that antagonize nearly every aspect of BoNT pathogenesis including:
   - Binding;
   - Internalization;
   - Translocation; and
   - Enzymatic activity [36], [61], [62].

Studies have also been conducted on compounds that restore neuronal function. Several examples of these approaches are mentioned below.

17.9.3.1 Human Recombinant Monoclonal Antibodies

Currently, immunotherapy is deemed as the most effective immediate response to BoNT exposure. However, human anti-BoNT antiserum (BabyBIG®) is exclusively approved for use in infants, and equine antisera can induce serum sickness and anaphylaxis [4], [63]. Monoclonal antibody combinations (oligoclonal antibodies) may be viable substitutes for polyclonal antisera [64], [65]. Construction of scFv phage Ab libraries has enabled the generation of large panels of high affinity antibodies. Mouse neutralization studies revealed that effective protection is only seen when combinations of three or more mAbs are used [63], [66]. Under NAIAD sponsorship, antibody combinations that effectively protect against multiple BoNT/A, /B, and /E sub-types are currently being produced and tested to support FDA licensure. Antibodies that protect against the four remaining toxin serotypes (BoNT/C, /D, /F, and /G) are also in development.

17.9.3.2 Antibodies Specific for the Catalytic Light Chain

While a majority of the BoNT immunotherapy research has been focused on antibodies that bind the HC, efforts were also directed to explore the potential for antibodies that inhibit the enzymatic activity of BoNT. Using a novel hybridoma method for cloning human antibodies [67], [68], a fully human antibody specific for the BoNT/A LC was cloned which potently inhibited BoNT/A in vitro and in vivo, via mechanisms not previously associated with BoNT-neutralizing antibodies [69]. In another study, Dong et al. [70] created a library of non-immune llama single-domain VHH (camelid heavy-chain variable regions) displayed on the surface of the yeast Saccharomyces cerevisiae. Library selections on BoNT/A LC yielded 15 yeast-displayed VHVs, eight of which inhibited the cleavage of substrate SNAP25 by BoNT/A LC. The most potent VHH (Aa1) had a solution K(d) of 1.47 x 10^{-10} M and an IC_{50} of 4.7 x 10^{-10} M. X-ray crystal structure of the BoNT/A LC-Aa1 VHH complex revealed that the Aa1 VHH binds the alpha-exosite region of BoNT/A LC. Recently, Tremblay et al. [71] reported on the selection of small (14 kDa) binding domains specific for the protease of BoNT serotypes A or B from libraries of VHVs or nanobodies cloned from immunized alpacas. Several VHVs were demonstrated to exhibit high affinity (K_{D} near 1 nM) and were potent inhibitors of BoNT/A LC (K_{I} near 1 nM); a VHH inhibitor of BoNT/A LC was able to protect BoNT/A-mediated SNAP25 cleavage.

17.9.3.3 Inhibitors of Toxin Binding

One of the strategies attempted for BoNT therapeutics was to target the toxin domain that participates in BoNT binding. Several naturally-occurring lectins, which are large glycoproteins that specifically recognize terminal
sialic acid residues, have been examined and were shown to prevent BoNT/A, /B, /C1, /D, /E, /F, and Tetanus Neurotoxin (TeNT) from binding to pre-synaptic terminals [72]. Additionally, using mouse hemidiaphragm and rat Extensor Digitorum Longus (EDL) assay, Adler et al. [73] reported that pre-incubation of muscles with the lectin from *Triticum vulgaris* (TVL) significantly delayed the induction of paralysis in muscles exposed to BoNT/A, /B, and /E. Pre-treatment with TVL also resulted in the retention of approximately 20% of control tension in EDL muscles injected with BoNT/A or /E, and such effect lasted approximately seven days.

Receptor mimics have also been regarded as valid targets for designing BoNT inhibitors [61]. Soluble versions of the BoNT/B and /G receptors have been demonstrated to function as anti-toxins in cells and mice [74], [75]. However, a major drawback to this approach is the short window treatment; once the toxin gets into the nerve cells, the effectiveness of the agent is very limited.

**17.9.3.4 Inhibitors of Internalization and Translocation**

Several compounds that inhibit the acidification process of endosomes by various mechanisms have been evaluated for toxicity and ability to inhibit BoNT-induced synaptic failure. Two protein ionophores, nigericin and monensin, were found to be the least toxic, and blocked vesicle acidification by acting as H⁺ shunts to neutralize pH gradients [76]. Treatment with nigericin, the least toxic of the two ionophores, was not able to reverse existing synaptic failure after BoNT/A poisoning. When applied in conjunction with BoNT/A or /B, the onset of paralysis was delayed by 2 – 3 fold. Ammonium chloride and methyamine hydrochloride have also been shown to antagonize the toxin internalization step [77]. However, these amines only function by inhibiting the acidification process of endosomes; they do not inactivate the toxins nor irreversibly modify tissue function at concentrations that inhibit the onset of BoNT-induced paralysis. Other compounds that have been examined were uncouplers of oxidative phosphorylation CCCP, FCCP, and vesicle H⁺-ATPase inhibitors including Bafilomycin A, which was shown to antagonize BoNTs A-G [78]. Some of these compounds were toxic or had low safety margins, hence they were deemed unsuitable as therapeutic candidates.

Anti-malarial compounds (4-amino and 8-amino quinolines) were also evaluated as potential BoNT/A inhibitors. Only 4-aminoquinolines and quinacrine delayed BoNT/A-induced neuromuscular block by more than 3-fold compared to the control (toxin only) values [79]. Maximum protection was solely achieved when the tissues were exposed to the compounds prior to or at the same time as the toxin treatment; a delay of > 20 min abolished the inhibitory capacity of these compounds.

**17.9.3.5 Compounds that Restore Neuronal Function**

A known potassium channel blocker, 3,4-diaminopyridine (3,4-DAP) was evaluated for its ability to antagonize BoNT-induced depression of tension in rat diaphragm muscles [80]. BoNT-induced paralysis was nearly completely inhibited after addition of 100 μM of 3,4-DAP, and this effect was sustained even after four hours of treatment. The antagonistic effects of 3,4-DAP was also demonstrated in vivo, provided its concentration in the plasma is maintained at ~ 30 μM during the course of intoxication [81]. However, 3,4-DAP is generally toxic, thus, its high drug concentration requirement prohibits routine therapeutic use [82].

Mastoparan, a phospholipase activator, was evaluated for its ability to attenuate BoNT intoxication. Addition of mastoparan and 80 mM K⁺ completely prevented BoNT inhibition of radiolabeled acetylcholine in PC12 cells, but this effect was blocked by either EGTA or the N-type calcium channel blocker ω-conotoxin [83]. These findings imply that the effects of mastoparan are dependent on Ca²⁺ influx via the neuronal type voltage-sensitive Ca²⁺ channels.
17.9.3.6 Natural Products BoNT Inhibitors

Some plant-derived natural products have been reported as possible inhibitors against BoNTs. Toosendanin, a triterpenoid derivative from the bark of *Melia toosendan* was reported to inhibit BoNT/A, /B and /E in monkeys in the early 1980s [84]. This molecule was hypothesized to act as a pre-synaptic blocking agent that modifies the acetylcholine release by modulating the activity of the calcium channel [85], [86]. The ability of toosendanin and a more potent tetrahydrofuran analog to selectively inhibit translocation of BoNT/A and /E LCs with subnanomolar potency has also been reported [87]. After completion of LC translocation, toosendanin was demonstrated to stabilize a channel-opened state apparent at higher concentrations than required to arrest translocation. Such bimodal modulation of the protein-conducting channel is determined by the conformation of cargo within the chaperone [87]. A Function-Oriented Synthesis (FOS) [88] has been applied to toosendanin analogs to discover the structural features that are important for the inhibitory effects.

Other natural product BoNT inhibitors include the aqueous extract from stinging nettle (*Urtica dioica*) leaf [89] that showed inhibitory effects against BoNT/A LC, and the thearubigin fraction of black tea (*Camellia sinensis*) that reduced the onset of paralysis in mice due to intoxication by BoNT/A, /B and /E [90]. Lomofungin, a secondary metabolite that was first isolated from the soil-dwelling Gram-positive bacteria *Streptomyces lomodensis* in the late 1960s, was identified as a BoNT/A LC inhibitor from a High Throughput Screening (HTS) of a drug library [91]. Lomofungin displays non-competitive inhibition kinetics against BoNT/A LC [92]. Another natural product, chicoric acid from *Echinacea* has been shown to bind to a BoNT/A exosite, and displayed partial non-competitive inhibition that can be synergistic with a competitive active site inhibitor [92]. Recently, chicoric acid was reported to be a cross-over inhibitor of BoNT/B LC [93].

17.9.3.7 Inhibitors of Enzymatic Activity

Over the past 10 years, the major focus of research efforts with respect to the development of candidate BoNT inhibitors has been the toxic reaction step involving the proteolysis of one of the SNARE proteins by the LC. Various approaches have been used, and studies primarily focused on the development of active site inhibitors.

17.9.3.7.1 A. Zinc Chelators

Initial studies involved testing the efficacy of metal chelators to block the action of BoNT using mouse phrenic-nerve hemidiaphragm assay [94], [95]. While several of these chelating compounds delayed the onset of BoNT/A poisoning, none completely blocked the action of the toxin. TPEN (N,N,N', N'-tetrakis-[2pyridylmethyl]ethylenediamine), the most effective chelator tested that delayed BoNT-induced paralysis by 5-fold, was also found to cause degeneration of neuritis, bleb formation at the cell membrane, and ultimately, cell lysis. These findings limit the usefulness of zinc chelators as BoNT/A therapeutics [95].

Three analogs of the metalloprotease inhibitor, phosphoramidon, were evaluated as a potential BoNT/B therapeutic in a cell-free assay using fluorescence following cleavage of a synaptobrevin peptide as the output measurement [96]. Analogs with methyl or ethyl substitutions were found inactive, while the phenyl substituted analog was only slightly more active than phosphoramidon in inhibiting BoNT/B.

17.9.3.7.2 B. peptide and pseudopeptide Inhibitors

Early studies have been conducted to investigate the catalytic requirements of BoNT as well as to understand the properties of the reactants for proteolysis; some of these efforts evolved into searches for peptide-based inhibitors. A number of peptide inhibitors against BoNT LCs have been reported, including Ac-CRATKML-NH₂ [97]. This peptide displayed a $K_i$ of 2 μM, and was hypothesized to bind to the BoNT/A active site by its
N-terminal cysteine sulphydryl group. In an attempt to improve the inhibitor binding of Ac-CRATKML-NH₂, Schmidt and Stafford [98] synthesized 10 additional peptides, including the 7-residue pseudopeptide, N-acetyl-2-mercapto-3-phenylpropionyl-RATKML-amide, which inhibited BoNT/A in vitro with a $K_i$ of 330 nM. Though CRATKML is a potent BoNT/A inhibitor in cell-free system, it was not found effective in the ex vivo mouse phrenic nerve hemidiaphragm assay [99]. The testing of four cell membrane permeable CRATKML-derived peptides (Cps) demonstrated the lack of efficacy of these Cps against in vitro and in vivo BoNT/A actions of motor nerve endings [100].

A long peptide with amino acid sequence Ac-EKADSNKTRIDEANCRATKMLGSG-NH₂ corresponding to SNAP-25 residues E183 to G206 but with a Q198C mutation, has been shown to inhibit BoNT/A LC protease activity ($IC_{50} = 3.4 \mu M$; [101]). This peptide was also demonstrated to exhibit very tight ($K_D = 24.9 \text{nM}$) but slow binding (second order rate constant ($k_{on}$) of 76 M$^{-1}$ s$^{-1}$) to BoNT/A LC, in contrast to the short peptide inhibitor Ac-CRATKML-NH₂ which did not show gradual binding.

Two alpha-thiol amide pseudopeptide inhibitors of BoNT/A were also reported to exhibit submicromolar $K_i$ values [102]. The structures and inhibitory properties of these two inhibitors corroborated previous reports that in substrate-based BoNT/A inhibitors, residues on the N-terminal side of the zinc ligand do not contribute to binding affinity, and that positioning the sulphydryl group on the alpha carbon of the N-terminal moiety provides stronger inhibition compared to positioning it on the beta carbon [103].

D-arginine, arginine hydroxamate and basic peptides were shown to effectively inhibit BoNT/A activity [104]. The 3-D structures of a few of these peptides bound at the active site [105] led to Arginine-Arginine-Glycine-Cysteine (RRGC) having a basic tetrapeptide structure as the most potent inhibitor ($K_i = 158 \text{nM}$; [106]). The basic tetrapeptides tested seem to be resistant to intracellular protease action. Crystal structures of BoNT/A light chain in complex with inhibitors L-arginine hydroxamate, 4-chlorocinnamic hydroxamate and 2,4-dichlorocinnamic hydroxamate have been also reported; at best, these molecules inhibit at 300 nM levels [107].

Studies of peptide inhibitors have inspired the development of substrate-based peptidomimetics as novel active site inhibitors. Several nanomolar-range Peptide-Like Molecule (PLM) inhibitors resembling the cleavage site sequence of SNAP-25 were reported, one of which has a $K_i$ value of 41 nM [108]. Using previous information from co-crystal structures, three related PLMs were synthesized to improve potency [109]. A co-crystal structure for one of these inhibitors revealed the PLM interactions, and also demonstrated the remarkable plasticity of the substrate binding cleft of BoNT/A LC.

Several peptides have been evaluated against BoNT/B. These peptides contain the synaptobrevin QF cleavage site but are not identical in primary structure to the sequence surrounding the QF bond [110]. Though Buforin I, a 39-amino acid peptide with the QF site at position 24-55, inhibited BoNT/B in vitro in a dose-dependent manner, and exhibited an $IC_{50}$ of 1 $\mu M$, it was not a substrate of BoNT/B. Two Buforin I deletion peptides, Peptide 24 (contains aa16-39) and Peptide 36 (contains aa1-36) were constructed and showed 25% and 50% as potent, respectively, than the parent peptide. These results suggest that the tertiary structure of buforin was critical for its antagonistic effect. Eswaramoorthy et al. [111] reported on a crystallographic study of BoNT/B in complex with bis(5-amindino-2-benzimidazolyl)methane, revealing that this molecule had rearranged the active site, and removed the zinc ion that, seemingly, caused the loss of proteolytic activity.

Oost et al. [112] synthesized several putative substrate transition state analog inhibitors and tested them against BoNT/B LC. Though the most effective analog was observed to exhibit a $K_i$ of 1.1 $\mu M$, the authors noted that it contained an additional methylene in the backbone chain compared to the native sequence P1 glutamine, thus may reflect the enzyme product structure instead of the transition state.
17.9.3.7.3 C. Aptamers

Aptamers have been recently reported to have strong potential as an antidote against botulism [113]. Three RNA aptamers have been identified through SELEX-process, and inhibition kinetic studies revealed low nM $K_i$ and non-competitive nature of their inhibition. Though this approach has some advantages, the major drawbacks of aptamer-based therapeutics include cost and stability.

17.9.3.7.4 D. Small Molecule BoNT Inhibitors

The drawbacks associated with the use of peptides as drug candidates (e.g. poor tissue penetration, serum resistance, oral bioavailability, and quick elimination), and the potential usefulness of small molecules as pre- and post-exposure therapeutic agents have led many laboratories instead to pursue small molecule approaches. Research has focused predominantly on developing small molecule inhibitors that target the BoNT/A LC protease, due to the serotype’s persistence and highly toxic nature (for reviews, see Refs. [114]-[116]). Utilizing a fragment-based approach, a recent study reported the discovery of benzoquinone BQ compounds as irreversible BoNT/A inhibitors [117]. The increasing number of $C. botulinum$ toxin “sub-types” or toxin genetic variants (currently ~ 35) [118], [119] and their sequence variability influence the efforts to develop medical countermeasures against BoNT [118].

17.9.4 Drug Delivery Vehicle Research

In combination with the discovery and development of BoNT small molecule inhibitors, continuing efforts are also geared to develop and evaluate methods for targeted delivery of drugs into Peripheral Cholinergic Nerve Cells (PCNC), which are the sites of action of BoNTs. Therapeutic targeting is critical to deliver an effective concentration of the drug specific to PCNC, and also to minimize potential systemic toxicity associated with the drug treatment. Several studies have examined the ability of recombinant BoNT HC (rHC) or a neutralized full length BoNT holoprotein as drug delivery systems. Goodnough et al. [120] observed that BoNT/A and unlabeled rHC were able to compete for binding, implicating specific neuronal targeting. The efficiency of cargo delivery into the cytosol was evaluated by coupling labeled dextran to the Delivery Vehicle (DV). The negligible dextran discharged into the cytosol suggested minimum separation of the cargo from the DV. In another study, a recombinant BoNT/A HC with an amino terminal fusion coupled to a dextran moiety with a fluorescent tracking dye was incubated with mouse spinal cord neurons [121]. The binding of the fluorescent tag and its internalization into the endosomes were observed, but minimal levels were detected in the cytosol. The movement of a small, membrane permeable dye from the endosome into the cytosol is hypothesized to be due to passive diffusion instead of an active translocation event. Ho et al. [122] reported on a recombinant BoNT/A HC with an amino terminal GFP fusion that was internalized into mouse neurons; nevertheless, the GFP cargo was observed to be almost exclusively limited to endocytotic vesicles.

Although the heavy chain of BoNT comprises the domains necessary for binding and internalization into endosomes, recent studies suggest that all or part of the light chain is essential to translocate a ligated cargo into the cytosol. Recombinant fusion proteins (full length BoNT/D fusion protein bearing amino terminal GFP, luciferase, dihydrofolate reductase or BoNT/A LC protein) were found to promote translocation of cargo proteins into the cytosol in an enzymatically active form [123]. A recombinant neutralized BoNT/A bearing an E224A E262A double mutation, labeled with Alexa-488, has been shown to specifically bind and internalize into human SH-SY5Y neuroblastoma cells [124]. Additionally, this protein was found to marginally bind to the surface of human rhabdomyosarcoma cells with a toxicity limit of 1 µg in mice. No additional information is available regarding the drug conjugation study on this protein. Moreover, recombinant versions of full-length BoNT/A holotoxin devoid of catalytic activity were developed [125]-[127] which could be potentially used to deliver therapeutic cargoes.
17.9.5 Challenges and Potential Therapeutic Strategies

An inhibitor must possess three important characteristics to serve as an effective anti-neurotoxin drug or model compound for development: a high degree of selectivity for one or more BoNTs, strong binding affinity, and low toxicity [103]. In re-examining the overall research and development strategy of BoNT therapeutics, Larsen [36], [128] presented some of the scientific challenges associated with the development of a botulism therapy which include:

1) BoNT serotypes require long substrates for efficient proteolysis;
2) The extensive interface between BoNT and its large substrate may pose a technical obstacle to inhibitor development;
3) The observed flexibility in the BoNT active site upon binding of candidate inhibitors may hinder inhibitor development; and
4) Many of the interactions between BoNTs and their substrates occur at exosites which are distant from the active site;
5) The non-correlation between in vitro and in vivo data [129] emphasizes an urgent need to develop in vitro assays that will enable accurate representation of in vivo conditions;
6) The stringent substrate requirements of BoNTs greatly increase the difficulties of developing multi-serotype inhibitors [103]; and
7) The necessity for intracellular delivery of candidate inhibitors underlines the complexity of anti-botulinum drug development.

Some research studies addressing these obstacles are underway. For instance, exosites have lately been targeted for inhibitor development [91], [70]. Structural data have also provided valuable information regarding the potential chemical properties of molecules capable of binding the BoNT active site [130]. Assays and model development are critical to the BoNT therapeutic effort. Though advances in the development of high throughput screening and cell-free biochemical assays have greatly enhanced the initial identification and testing of candidate BoNT inhibitors, caution should be used in extrapolating in vivo potency from these assays. Assays with an observed disconnect between in vitro and in vivo [100], [129] data contends that greater research is crucial toward the development of in vitro cellular systems that will allow prompt identification of BoNT inhibitors while also precisely depict the in vivo conditions.

Though some BoNT serotypes are known to persist intracellularly for prolonged periods of time, the mechanism for toxin persistence, and the identification of the cellular protein(s) involved in BoNT persistence or recovery remain largely uncharacterized. An understanding of these pathways will be critical to the elucidation of novel host-derived therapeutic targets for botulism treatment. Recent findings demonstrated a structural basis for the remarkable persistence of BoNT/A. Mutagenesis study involving a recombinant BoNT/A has revealed a C-terminal dileucine motif that was essential for the protease longevity [131]. In another report, the relative persistence of BoNT/A LC compared with BoNT/E LC has been attributed in part by the selective targeting of the latter to the ubiquitin-proteosome system by the RING finger protein TNF-Associated Factor 2 (TRAF-2; [132]). These findings pave way for attacking BoNT/A LC persistence either through delivery of designer ubiquitin ligases, or through the use of small molecules capable of directly entering cells and retargeting other ubiquitin ligases to BoNT/A [132].
17.10 CONCLUSION

BoNTs, the causative agents of botulism, are considered biological threats to both our military and the public. Effective medical countermeasures against BoNT intoxication are limited. Currently, the only available treatment other than supportive care is a botulinum heptavalent equine-based anti-toxin. Nonetheless, anti-toxin cannot intervene in the pathogenesis of the disease once the toxin enters the nerve cell, and cannot support all those infected in the event of a biological terrorist attack. Hence, there is a critical need for post-intoxication therapy that can be administered rapidly and effectively to a large infected population. Since small molecules provide an opportunity to treat botulism both before and after cellular intoxication has occurred, considerable research efforts have been devoted to the development of these types of inhibitors. A number of strong contributions to the field have been made, yet no small molecule inhibitor was identified that possesses the appropriate characteristics (safety, efficacy, solubility) required to be a pharmaceutical intervention. Several scientific challenges related to the development of BoNT therapeutic countermeasure exist. Research studies addressing these obstacles are underway. Additionally, potential novel strategies to therapeutic development, e.g. host directed therapeutics, and targeting pathways involved in the cellular response to BoNT intoxication, are being explored. An understanding of these mechanisms may allow for the development of effective, innovative therapeutic strategies to counteract BoNT intoxications.

17.11 REFERENCES


BOTULINUM NEUROTOXINS


[54] CDC. Investigational heptavalent botulinum antitoxin (HBAT) to replace licensed botulinum antitoxin AB and investigational botulinum antitoxin E. MMWR 2010; 59, 299.


[73] Adler M, Apland J, Hilmas C, Ternay A, Janda K and Deshpande SS. Meeting Abstract. 43rd Interagency Botulism Research Coordinating Committee Meeting. Nov. 14-17, 2006, Silver Spring, MD, USA.


[86] Ding J, Xu TH and Shi YL. Different effects of toosendanin on perineurally recorded Ca\textsuperscript{2+} currents in mouse and frog motor nerve terminals. J Neurosci Res 2001; 41:243-49.


Disclaimer: Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.
Chapter 18 – RICIN

Virginia I. Roxas-Duncan¹, Martha L. Hale², Jon M. Davis³, John C. Gorbet⁴, Patricia M. Legler⁵ and Leonard A. Smith⁶

1: Chief, Select Agent Management Branch, Biosurety Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; 2: Research Microbiologist, Integrated Toxicology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; 3: Lieutenant Colonel, Medical Service Corps, Office of the Assistant Secretary of Defense for Nuclear, Chemical and Biological Defense, Chemical and Biological Defense Program, Washington, DC; formerly, Chief, Division of Integrated Toxicology, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; 4: Captain, Medical Service Corps; Chief, Department of Aerosol Services, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; 5: Research Biologist, U.S. Naval Research Laboratory, Washington, DC; 6: Senior Research Scientist (ST) for Medical Countermeasures Technology, Office of Chief Scientist, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

UNITED STATES

Corresponding Author (Smith): Leonard.A.Smith@comcast.net

18.1 INTRODUCTION

Ricin is a potent toxin derived from the ornamental and widely cultivated castor plant, Ricinus communis L. (Euphorbiaceae). Ricin is mostly concentrated in the seed of the plant, popularly known as the castor bean, which despite its name, is not a true bean. The purified ricin toxin is a white powder that is water soluble; it inhibits protein synthesis leading to cell death. Ricin is stable under normal conditions, but can be inactivated by heat above 80°C. After oil extraction and inactivation of ricin, the defatted mash and seed husks are used as animal feed supplement and fertilizer, respectively [1].

In 1978, the lethality of ricin was overtly established after the high-profile assassination of Bulgarian dissident Georgi Markov [2]. Since then, numerous incidents involving ricin or castor seeds for nefarious purposes have been reported [3]-[9]. More recently, various extremists and terrorist groups have also experimented with ricin; some involved mailing ricin-tainted letters to the offices of United States (U.S.) politicians. These events have heightened concerns regarding ricin’s potential for urban bioterrorism, and thus prompted its constant inclusion in Weapons of Mass Destruction (WMD) investigations [10]. The wide availability of the castor plants, the relative ease of toxin production, the toxin’s lethality, stability, and media coverage fortify the appeal of ricin for those in quest of retribution and public attention.

In the U.S., the possession or transfer of ricin and genes encoding its functional form is regulated by the Centers for Disease Control and Prevention (CDC) Select Agents and Toxins Program. Ricin has been classified by the CDC as a Category B threat agent. Category B agents are the second highest priority agents and include those that are moderately easy to disseminate, result in moderate morbidity and low mortality rates, and require specific enhancements of CDC’s diagnostic capacity and enhanced disease surveillance [11], [12]. Investigators must register with the CDC prior to utilizing non-exempt quantities of ricin in their research. There are no federal regulations restricting the possession of castor plants, however, some states or cities (e.g. Hayward, CA, USA) prohibit possession of castor plants or seeds.
Ricin is listed as a Schedule 1 toxic chemical under both the 1972 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction, usually referred to as the Biological Weapons Convention (BWC), or Biological and Toxin Weapons Convention (BTWC), and the 1997 Chemical Weapons Convention (CWC) [13], [14].

18.2 HISTORY, BIOLOGICAL WARFARE, AND TERRORISM

18.2.1 History

The castor plant, also known as the Palm of Christ, was initially indigenous to the south-eastern Mediterranean region, eastern Africa, and India, but is now widespread throughout temperate and sub-tropical regions [15]-[17]. *Ricinus* is a Latin word for ‘tick’ to describe the castor seed’s appearance, and *communis* meaning ‘common’ to describe its worldwide distribution. For centuries, the castor plant has been cultivated for numerous economically important products, primarily castor oil [17]. In Ancient Egypt, Europe, India and China, castor oil was used for lighting, body ointments, purgative, cathartic and other ethnomedical systems. Castor oil was also reportedly used as an instrument of coercion by the Italian Squadristi, the Fascists armed squads of Benito Mussolini [18]. Political dissidents and regime opponents were forced to ingest large amounts of castor oil, triggering severe diarrhea and dehydration that oftentimes led to death [19]. Presently, castor oil has abundant commercial applications including medicinal and industrial purposes [20]-[24]. Because of its economic benefits and myriads of uses, castor seeds are being produced in more than 30 countries in the world. In 2013, world castor oil seed production totaled 1.86 metric tons; the leading producers include India, China (mainland), and Mozambique [25].

Ricin was discovered in 1888 by Hermann Stillmark, a student at the Dorpat University in Estonia (Stillmark, 1888, as cited in Franz and Jaax [5]). During Stillmark’s extensive research, he observed that ricin caused agglutination of erythrocytes and precipitation of serum proteins [17]. In 1891, Paul Ehrlich studied ricin and abrin in pioneering research that is now recognized as the foundation of immunology [17]. Ehrlich found that animals vaccinated with small oral doses of castor beans were protected against a lethal dose of the toxin. Additional experiments using abrin and ricin showed that the immunity was specific, was associated with serum proteins, and could be transferred to the offspring through milk. Further research on ricin showed that the toxin described by Stillmark was actually two proteins, one with an agglutinin with a molecular weight of 120 kDa (*R. communis agglutinin I*) possessing little toxicity and the other, *R. communis agglutinin II*, a smaller molecular weight protein (60 kDa) with little agglutinating capacity but found to be extremely toxic. Nearly a century after Stillmark’s original discovery, Olsnes and Phil [26] demonstrated that the 60 kDa toxic protein (ricin) inhibited protein synthesis and that the 60S ribosomal sub-unit is the toxin’s molecular target [27].

Although ricin is considered to be a possible biological threat (see below), its potential medical applications have been also explored. During the last decade, ricin has been used extensively in the design of therapeutic immunotoxins, often called “magic bullets”. In such, ricin, ricin A chain (RTA), or a related toxin is chemically or genetically linked to a binding ligand such as an antibody or used in other conjugates to specifically target and destroy cancer cells, and also as alternative therapies in AIDS and other illnesses [28]-[30]. Ricin-based immunotoxins conjugated to either the anti-CD22 antibody RFB4 [31], [32] or its Fab fragment [33] have been reported to provide enhanced therapeutic efficacy and improved anti-tumor activity [34]-[36]. However, the U.S. Food and Drug Administration (FDA) has placed a hold on the clinical testing of RTA-based immunotoxins because they caused Vascular Leak Syndrome (VLS) in humans, a condition in which fluids leak from blood vessels leading to hypoalbuminemia, weight gain, and pulmonary edema [37]. While progress has been made in understanding the mechanisms of immunotoxin-mediated VLS, significant effort is still required to generate
RTA-derived immunotoxins that exhibit reduced cytotoxicity and lesser ability to impair epithelial cells, but also preserve anti-tumor activity [38].

18.2.2 Ricin as a Biological Weapon

During WWI, the U.S., aware of the German biological warfare program, examined ricin for retaliatory intentions [39]. Two methods of ricin dissemination were described in a 1918 technical report:

1) Adhering ricin to shrapnel bullets for containment in an artillery shell; and
2) Production of a ricin dust cloud (Hunt et al., 1918, as cited in Smart [39]).

The thermal instability of ricin constrained its initial use in exploding shells, and ethical and treaty issues limited its use as a poison or blinding agents. WWI ended before the toxin could be weaponized and tested. During WWII, ricin was evidently never used in battle despite its mass production and being armed into ricin-containing bombs (also known as W bombs), because its toxicity was surpassed by the even more potent biological agents of the time [5]. Interest in ricin diminished with the production and weaponization of other chemical agents, e.g. sarin. During the Cold War, the Soviet Union studied ricin as a possible biological weapons agent. A former top Russian official who defected to the U.S. in 1991 asserted that Russia developed ricin as a weapon, and that the toxin used against the Bulgarian dissidents Georgi Markov and Vladimir Kostov was formulated in Russian laboratories [40]. Iraq reportedly manufactured and tested ricin in animals and used it as payload in artillery shells [41]-[44]. Syria and Iran were believed to have produced unknown quantities of the toxin [45]. Ricin was also found in Afghanistan in 2001 after the collapse of the Taliban Government [46], [47].

Even though ricin’s potential use as a military weapon was investigated, its utility over conventional weaponry remains ambiguous. Despite its toxicity, ricin is less potent than other agents such as Botulinum Neurotoxin (BoNT) or anthrax. It has been estimated that eight metric tons of ricin would have to be aerosolized over a 100 km² area to achieve about 50% casualty, whereas only kilogram quantities of anthrax spores would cause the same effect [48]. Furthermore, wide-scale dispersal of ricin is logistically impractical. Thus, while ricin is relatively easy to produce, it is not as likely to cause as many casualties as other agents [49].

18.2.3 Ricin as a Terrorist Weapon and Use in Biocrimes

The well-publicized “Umbrella Murder” of the Bulgarian writer and journalist Georgi Markov in 1978 [2] represents the first documented case of a modern assassination using a biological agent [50], though this remained unproven. Markov defected to the West in 1969 and was a vocal critic of the then Bulgarian communist regime. The Bulgarian secret police had previously attempted to kill Markov twice, but failed. However, on September 7, 1978, Markov was assaulted with an umbrella tip while waiting at a bus stop in London. He subsequently developed severe gastroenteritis, high fever, and died on September 11, 1978. The autopsy revealed a small platinum pellet with an X-shaped cavity. Further examination of the pellet showed the presence of ricin [2], [51]. Prosecutors have failed to identify, arrest, or charge anyone for the crime. On September 11, 2013, a news report disclosed that Bulgaria was abandoning its investigations of the notorious case 35 years after the cessation of the absolute statute of limitations [52].

Days before Markov’s assassination, an attempt was made to kill another Bulgarian defector, Vladimir Kostov [2]. However, the pellet lodged in the fatty tissue in Kostov’s back and prevented the toxin from being released from the sugar-coated pellet; he survived the incident. Since then, several cases involving the possession, experimentation, or planned misuse of ricin by bioterrorists and extremist groups have been investigated or prosecuted by law enforcement agencies worldwide [5]-[7]. Recent related incidents include the following:
In April 2013, letters containing ricin were mailed to Republican Senator Roger Wicker of Mississippi, and the White House [53], [54]. A Mississippi martial arts instructor, Everett Dutschke, was charged with sending those ricin-tainted letters [55].

In May 2013, five letters testing positive for ricin were mailed from Spokane, Washington to an Air Force base, a local judge, the Central Intelligence Agency, and two addressed to the White House [56].

On June 7, 2013, actress Shannon Richardson was arrested for allegedly sending ricin-tainted letters to New York Mayor Michael Bloomberg and President Barack Obama [57].

These reports further substantiate ricin’s image as an attractive lethal poison, and ostensibly, a biological weapon of choice by extremist groups and individuals.

18.3 DESCRIPTION OF THE AGENT

18.3.1 Biochemistry

While ricin is a well-known toxin that can be extracted from castor bean mash, most do not realize that it is related in structure and function to the bacterial Shiga toxins and Shiga-like toxin (also known as Verotoxin) of *Shigella dysenteriae* and *Escherichia coli*. Antibiotic-resistant Shiga toxin-producing *E. coli* was responsible for 54 deaths in Germany in 2011 [58]; the Shiga toxin gene encoding the toxin was carried by this infectious pathogen. Ricin is non-infectious; however, both the structure and enzymatic activities of ricin and Shiga toxins are similar (Figure 18-1). These protein toxins belong to a family of toxins known as Ribosome Inactivating Proteins (RIPs). There are over 60 different plant and bacterial species that produce RIPs [59]-[61]. Type I and II RIPs include the plant toxins ricin, abrin, mistletoe lectins, volkensin, modeccin, saporin, trichosanthin, luffin, and the bacterial Shiga toxin and Shiga-like toxin.

**Figure 18-1: Structural and Functional Similarities Among Ribosome Inactivating Proteins (RIPs).** The A-chains of plant RIPs such as ricin (PDB 3HIO) and abrin (PDB 1ABR) are structurally and functionally related to the bacterial Shiga toxin A-chains (PDB 1R4Q and 1R4P). The A-chains catalyze the same reaction to inactivate ribosomes and halt protein synthesis [190]-[192].

Ricin, a Type II RIP, consists of two glycoprotein sub-units: a catalytic A-chain (RTA) and a lectin B-chain (RTB) which binds cell surface oligosaccharides containing galactose [59], [62]-[64]. The RTA and the RTB are
of approximately equal molecular mass (~ 32 kDa) and are covalently linked by a single disulfide bond. The protein coding region of ricin consists of a 24 amino acid N-terminal signal sequence preceding a 267 amino acid RTA. The RTB has 262 amino acids. It consists of two major domains with identical folding topologies [62], each of which is comprised of three homologous sub-domains (α, β, γ) that probably arose by gene duplication from a primordial carbohydrate recognition domain [65]. RTB binds terminal β 1,4-linked galactose and N-acetyl galactosamine (Gal/GalNac) [66] that are on the surface of almost every cell type. A 12-amino acid linker in the pre-protein joins the two chains. The carboxyl-terminal end of the RTA folds into a domain that interacts with the two domains of the B chain [62]. A disulfide bond is formed between amino acid 259 of the RTA and amino acid 4 of the RTB [59], [63], [64]. Thirty percent of the RTA protein is helical. The RTA folds into three somewhat arbitrary domains. The active site cleft of the RTA is located at the interface between all three domains.

18.3.2 Pathogenesis

Pathogenesis resulting from ricin intoxication is a two-step process. The first phase occurs at the cellular level in which the toxin kills cells in a cell-cycle independent fashion; the second phase is primarily caused by systemic reactions that develop in response to cell death and tissue damage.

The cell binding component of the toxin (RTB) binds to cell surface lipids and proteins with exposed terminal β-1,4-linked galactose molecules that are found on most mammalian cells, permitting ricin to bind indiscriminately to most cells in the body [17], [66], [67]. In addition to binding to surface glycoproteins, ricin contains three mannose oligosaccharide chains, two in RTB and one in RTA that provides another route for ricin binding to the cell via mannose receptors located primarily on macrophages and dendritic cells [68].

Once RTB binds to the cell, it is endocytosed [69]. At this point, there are three possible fates for ricin:

1) Entry into endosomes and recycling to the cell surface;
2) Degradation via the late endosomes; or
3) Entry to the trans-Golgi network and entry to the Endoplasmic Reticulum (ER) via retrograde trafficking.

In the ER, a protein disulfide isomerase reduces the toxin into RTA and RTB [70]. The low lysine content of RTA probably enables the molecule to evade the ER-Associated protein Degradation (ERAD) pathway and chaperone proteins such as calreticulin, transport RTA from the Golgi apparatus to the ER; dislocation of RTA from the ER may involve the translocon component Sec61p [71]-[73]. Post-dislocation of RTA in the cytosol probably involves Hsc70 which may also aid the protein in binding to its ribosomal substrate [74]. Additionally, the ribosome itself may act as a suicidal chaperone by facilitating proper refolding of RTA that is required for the catalytic activity of the enzyme [73].

Extensive investigations on reactions controlling RTA’s binding to ribosomes provide detailed information on the RTA’s enzymatic functions [17], [75]. RTA catalyzes the hydrolysis of a specific adenine in the ricin-sarcin loop of the 28S ribosomal RNA (rRNA) (Figure 18-2). The ricin-sarcin loop interacts with eukaryotic elongation factor EF-2. The binding of EF-2 to the ricin-sarcin loop is required for the translocation of the peptidyl-tRNA from the A-site to the P-site on the ribosome during protein synthesis. The depurinated ricin-sarcin loop fails to bind EF-2 and the ribosome stalls with the peptidyl-tRNA stuck in the A-site [76], [77]. The overall effect is a halt in protein translation and cell death.
Figure 18-2: The Ricin A-Chain Catalyzes the Hydrolysis of an Adenine in the Ricin-Sarcin Loop (the Depurinated rRNA is shown as a dotted line). The aminoacyl-tRNA is delivered to the A-site by eukaryotic elongation factor eEF1A and peptidyl transfer follows. The binding of eukaryotic elongation factor eEF-2 carrying GTP is required for the peptidyl-tRNA to translocation from the A-site to the P-site, this movement requires eEF-2. The depurinated loop fails to bind eEF-2, and the ribosome stalls with the peptidyl-tRNA in the A-site. Figure adapted from Mansouri S, Nourollahzadeh E, Hudak, KA. Pokeweed anti-viral protein depurinates the sarcin/ricin loop of the rRNA prior to binding of aminoacyl-tRNA to the ribosomal A-site. RNA. 2006;12:1683-1692.

Analysis of reactions resulting from mixing purified rat ribosomes with RTA shows that RTA reaction follows classical Michaelis-Menten enzyme kinetics, and the enzymatic action has been calculated to be 0.1 μmol/L [78]. Furthermore, these studies predict that one RTA molecule would be able to depurinate 1,500 ribosomes/min., thus making one ricin molecule sufficient to kill the cell. Site-directed mutagenesis and the development of transition state mimics have yielded mechanistic information. The hydrolysis reaction catalyzed by RTA is thought to proceed via a dissociative mechanism with an oxocarbenium transition state [79]. Glu-177 in the active site stabilizes the developing positive charge on the ribosyl ring while Tyr-80 and Tyr-123 have been proposed to activate the leaving group by pi-stacking with the adenine [80] (Figure 18-3). The enzymatic activity of RTA is the primary source of toxicity and therefore must be attenuated in RTA sub-unit vaccines by incorporating the Y80A mutation [81] or removing the C-terminal residues (residues 199-267) [82]. The mutations interfere with rRNA binding.
Activation of apoptotic processes is one method by which RTA kills cells but the apoptotic pathways are somewhat cell-dependent [81]. There is evidence that some cells have novel ricin-specific pathways for activating apoptosis. Wu and colleagues [84] found that RTA binds to a novel binding protein (BAT3) that is found in the cytoplasm and nucleus of many cells. BAT3 possesses a canonical caspase-3 cleavage site that...
appears to be exposed when RTA binds to BAT3; apoptosis is then activated with caspase-3 cleavage. The finding that BAT3 may play a role in ricin-induced apoptosis could identify new targets for preventing ricin toxicity.

Ricin intoxication has been shown to activate numerous signaling pathways including Mitogen-Activated Protein (MAP) kinases and subsequent secondary signaling pathways such as the stress-activated protein kinase family [85]. MAP kinases regulate activation of cytokines such as IL-8, IL-1β, and TNF-α that, in turn, cause inflammatory reactions and tissue damage. Although inflammatory responses caused by ricin have been described previously, pathways and resulting cellular responses were only recently examined [86]. Korcheva et al., [87] demonstrated that intravenous administration of ricin in mice resulted in cellular signaling pathway activation and a significant increase in serum pro-inflammatory cytokine levels. Additional research in which ricin was instilled by an intracheal route showed similar signaling pathway activation as well as an increase in pro-inflammatory cytokine levels, although more inflammatory reactions and tissue damage were observed in the lungs [88]. While these studies have initiated the systemic pathogenesis characterization of ricin intoxication, further efforts aimed to determine the cellular responses induced by ricin will lead to a greater understanding of its pathogenesis and may also enable the development of new treatment strategies to combat the effects of intoxication.

18.4 CLINICAL SYMPTOMS, SIGNS, AND PATHOLOGY

Experimental animal studies reveal that clinical signs and pathological manifestations of ricin toxicity depend upon the dose as well as the route of exposure [5], [27], [89]. The common routes of entry are oral intoxication (ingestion), injection, and inhalation. The differences observed in pathology among various routes are likely due to the fact that RTB binds to a wide array of cell surface carbohydrates [90]. Once bound, RTA is internalized and results in death of intoxicated cells. Although symptoms may vary, in most cases, there is a time-to-death delay of approximately 10 hours, even with a high dose of toxin [91]. Additionally, in animals and humans intoxicated either by injection or oral ingestion, a transient leukocytosis is commonly observed, with leukocyte counts rising 2 to 5 times above their normal values. The LD50 and time to death for animals by various routes have been reported, and the values for humans were estimated based on animal experiments and accidental human exposures [5], [7], [40].

18.4.1 Oral Intoxication

Oral or intragastric delivery is the least effective and least toxic route, reportedly 1000 times less toxic than parenteral routes. The reduction in toxicity may be due to poor absorption of the toxin across the epithelium and slight enzymatic degradation of the toxin as it traverses the gastrointestinal tract. Ingestion of castor beans is the most common route of poisoning for humans and domestic animals. Worbes and colleagues [92] provide an updated list of ricin intoxication in humans worldwide. Since the late 1880s, 875 cases of accidental poisoning and 13 fatalities were reported in the literature (1.5% death rate); there were 11 intentional poisonings, five of which were fatal (45.5% death rate). In recent years, ricin intoxication resulting from intentional poisoning using mashed seeds or crude preparations of ricin has become a major bioterror weapon as well as a method of suicide (see Refs. [8], [9] for review).

There is great variability in effects from ingestion of seeds, probably related to the number of seeds, the degree of mastication that releases ricin from the seeds, the age of the individual, and to a lesser extent, the cultivar of the castor bean plant [92], [93]. In addition, an accurate description of ricin intoxication in humans is complicated by other factors including the presence of other somewhat toxic components such as the ricin agglutinin protein.
and the alkaloid ricinine that are found in castor seeds and crude preparations of ricin [92]. These substances can also cause tissue damage and contribute to pathological manifestations.

In spite of numerous differences that may play a role in oral toxicity, all fatal or serious cases appear to have a similar clinical history; a recent case study is presented in Table 18-1 [94]. Within a few hours, there is an onset of nausea, vomiting, and abdominal pain which is followed by diarrhea, hemorrhage from the anus, anuria, cramps, and dilation of the pupils [95]. Fever develops, followed by thirst, sore throat, and headache, leading to vascular collapse and shock. Death usually occurs by day three or thereafter. Common histopathological findings during autopsy are multi-focal ulcerations and hemorrhages in the stomach and small intestinal mucosa. There was significant lymphoid necrosis in intestinal associated lymph nodes, lymphoid tissue, and spleen. Necrosis was also observed in cells of the reticuloendothelial system resulting in liver damage and nephritis. Macrophages and macrophage-derived cells appear to be very susceptible, probably due to the large numbers of mannose receptors present in the cell membrane [68], [96].

Table 18-1: Case Report: Ricin Poisoning Causing Death After Ingestion of Herbal Medicine [200].

<table>
<thead>
<tr>
<th>Case Report: Ricin Poisoning Causing Death After Ingestion of Herbal Medicine [200].</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 42-year-old male Saudi patient presented to the emergency department with a 12-hour history of epigastric pain, nausea, repeated attacks of vomiting, chest tightness, and mild non-productive cough.</td>
</tr>
<tr>
<td>These symptoms were preceded by a 5-day history of constipation for which the patient ingested a large amount of a mixture of herbal medicine preparation 2 days prior to his admission. A review of systems was unremarkable. He had no history of any medical illnesses and medication use except for the herbal medicine. Initial examination showed a mild elevation of temperature (38°C), with generalized abdominal tenderness and hyperactive bowel sounds. His respiratory system examination showed equal bilateral air entry and no added sounds. The rest of his systemic examinations were unremarkable.</td>
</tr>
<tr>
<td>Laboratory investigations on admission showed mild leukocytosis of 14 x 10⁹/L, a normal platelet count of 200 x 10⁹/L, and normal hemoglobin level of 15.8 g/dL. Liver enzymes initially showed mild to moderate elevation of Alanine Transaminase (ALT) 86 U/L (normal range up to 37 U/L), Aspartate Transaminase (AST) 252 U/L (normal range up to 40 U/L), and serum lactate dehydrogenase 281 U/L (normal range 72 – 182 U/L), and the renal function was normal. The initial coagulation profile was impaired as documented by a prolonged prothrombin time (19 seconds, control 12 seconds) and a prolonged activated partial thromboplastin time (56 seconds, control 32 seconds). Electrocardiogram showed a right bundle branch block, and a chest radiograph was normal.</td>
</tr>
<tr>
<td>After 4 hours of admission, the abdominal pain became worse, and the patient started showing subcutaneous bleeding at the intravenous sites and upper gastrointestinal bleeding, manifested as hematemesis. The patient was managed by intravenous fluid therapy, fresh frozen plasma and platelet transfusion, and gastric decontamination with activated charcoal. A gastrointestinal consultation was requested in which endoscopy was planned after stabilization of the patient, but was not performed because of rapid deterioration of the patient.</td>
</tr>
<tr>
<td>In the second day after admission, his liver enzymes increased to a level of 5980 U/L for ALT and 7010 U/L for AST. Serum albumin was 31 g/L (normal range 38 – 50 g/L), total protein was 59 g/L (normal range 66 – 87 g/L), and the platelet count dropped to 85 x 10⁹/L. His renal function also deteriorated, elevating the creatinine level to 150 Umol/L (normal range up to 123 Umol/L), and urea...</td>
</tr>
</tbody>
</table>
to 110 mmol/L (normal range 1.7 – 83 mmol/L). His blood and sputum cultures and sensitivity were negative for bacterial pathogens, and an abdominal computerized tomography scan was normal. The patient was managed conservatively with supportive measures as maintained earlier; however, he remained persistently hypotensive necessitating inotropic support. On the 3rd day, he developed cardiopulmonary arrest and was resuscitated; however, he could not be revived. The sample of the herbal medicine powder was sent to the university lab. The chemical contents were extracted by the liquid chromatography/mass spectrometry technique, revealing the presence mainly of ricin powder which was further identified by the immuno-polymerase chain reaction assay that confirmed the presence mainly of ricin with no other significant contaminants. This finding could be the implicated as the cause for the patient’s fulminant clinical course.

18.4.2 Injection
Pathological damage caused by injection of ricin depends largely upon the dose. Results of a clinical trial in which 18 – 20 μg/m² of ricin were given intravenously to cancer patients indicated that the low dose was fairly well tolerated, with the main side effects being flu-like symptoms, fatigue, and muscular pain [97]. Some patients experienced nausea and vomiting, but after two days, they had recovered and experienced no further side effects. At low doses, intramuscular or subcutaneous injections may result in necrosis at the injection site possibly resulting in secondary infections [98]. High doses by either route cause severe local lymphoid necrosis, gastrointestinal hemorrhage, diffuse nephritis, and splenitis. Targosz and colleagues [99] describe a suicide case in which an individual injected himself subcutaneously with a large dose of ricin extracted from castor beans. The 20-year old male was admitted to the hospital 36 hours after injection. He suffered from severe weakness, nausea, dizziness, headache, and chest pain. Clinical exams showed hypotension, anuria, metabolic acidosis, and hematochezia. The patient was observed with hemorrhagic diathesis and liver, kidney, cardiovascular, and respiratory systems failure requiring endotracheal intubation and artificial ventilation. Although given maximal doses of pressor amines and hemorrhagic diathesis, treatments were ineffective and the patient developed symptoms of multi-organ failure followed by asystolic cardiac arrest. Resuscitation was not effective and the patient died shortly thereafter. A post-mortem examination revealed hemorrhagic foci in the brain, myocardium and pleura.

In the case of Georgi Markov [2], the lethal injected dose was estimated to be 500 μg. Markov experienced severe local pain after the injection. This was followed by a general weakness 5 hours later. Fifteen to 24 hours later, he had elevated temperature, nausea, and vomiting. He was admitted to the hospital with a high fever and signs of tachycardia. While his blood pressure remained normal, lymph nodes in the affected groin were swollen and sore, and a 6-cm diameter area of induration was observed at the injection site. Just over 2 days after the attack, he suddenly became hypotensive and tachycardic with a pulse rate of 160 beats/min and a white blood cell count of 26,300/mm³. He became anuric developing gastrointestinal hemorrhaging and complete atrioventricular conduction block. Shortly thereafter, Markov died from cardiac failure complicated by pulmonary edema; the time of death was 3 days after he was initially poisoned [2].

18.4.3 Inhalation
There are no reports in which humans have been subjected to ricin by accidental inhalation or premeditated aerosolized exposure. Most of the human data comes from descriptions of workers being exposed to castor bean dust in or around castor bean processing plants [100]. Allergic manifestations induced by ricin dust were first described in 1914 [101]. Symptoms and clinical signs of intoxication were later differentiated from the allergic
syndrome and further investigations showed that the allergens and toxin were two different molecules [102]-[104].

Because no data exist for human exposure, it is important to determine whether there is consistency between rodents and non-human primates (and other animal models) that can be used to extrapolate an accurate representation of inhalational ricin in humans. Unlike other routes of intoxication, damage caused by an aerosol exposure is greatly dependent upon particle size, and to a lesser extent upon the dose and cultivar from which ricin was obtained [86]. Ricin extracted from R. communis var. zanzibariensis was twice as lethal as ricin extracted from the Hale Queen variety [86]. The differences are more than likely related to variations in the isotoxins of ricin found in the seeds from different cultivars [86].

For ricin to reach the lung, the particles would need to be a size that could move around the nasal turbinates and flow with the airstream to the lung. Roy and co-workers [105] compared the outcome of 1 µm versus 5 µm particle size given to mice in an aerosol challenge. With the 1 µm particles, the majority of ricin was found in the lung and by 48 hours, lung tissue show significant lesions with alveolar edema, fibrin, and hemorrhage. Seventy-two hours post-exposure, all of the mice had died. Conversely, there were no deaths observed when mice were exposed to ricin with a 5 µm Mass Median Diameter (MMD). Most of the toxin was found in the trachea and there was little lung damage observed in histological sections of lung tissue taken 48 hours post-exposure.

When rats were exposed to a sub-lethal dose (LC₃₀) of ricin with particle sizes < 1 µm, damage was limited to the lung and there were no histological changes noted before 8 hours post-challenge [86], [106]. By 48 hours, pathological changes observed included necrosis and apoptosis in bronchial epithelium and macrophages present in the alveolar septae. Photographs (Figure 18-4) of tissue sections from CD1 mouse lungs 48 hours after exposure show perivascular edema and pulmonary epithelial cell necrosis (CL Wilhelmsen, unpublished data). Three days post-exposure, there was significant diffuse alveolar edema, and severe capillary congestion and macrophage infiltration or the alveolar interstitium. By day four, there was a rapidly resolving pulmonary edema, renewal of the bronchial epithelium even though there was severe passive venous congestion in all solid peripheral organs. Fourteen days post-exposure, all animals survived. Examination of tissue sections from sacrificed animals was similar to control tissues, except for focal areas of intra-alveolar macrophage infiltration [106]. Additionally, when rats and mice were given lethal doses of ricin by aerosol, no indication of lung damage was observed during the first 4 – 6 hours [14], [106], [107]. By 12 hours, there was an increase in total protein and polymorphonuclear cells in the bronchial lavage, indicating damage to the epithelial cell barrier. Thirty hours after challenge, alveolar flooding was apparent, along with arterial hypoxemia and acidosis. Histopathology showed lesions throughout the respiratory tract, spleen and thymus. A median lethal dose of ricin by inhalation was determined to be 1 µg/kg body weight for both Sprague Dawley rats and BALB/c mice [107]. Further characterization of inhaled ricin exposure was performed by examining lung tissue sections for the presence of ricin [5]. Immunohistochemical studies showed that ricin binds to the ciliated bronchiolar lining, alveolar macrophages and alveolar lining cells [14]. This finding further substantiates the importance of the lung epithelium and alveolar macrophages in the inhaled ricin intoxication process.
Figure 18-4: Histological Sections of Lungs from CD1 Mice Exposed to Ricin by Aerosol Showing
(A) Perivascular Edema and Pulmonary Epithelial Necrosis, H&E Stain; 25X Original Mag;
(B) Pulmonary Epithelial Cell Necrosis, H&E Stain, 100X Original Mag.
Photographs courtesy of LTC Catherine L. Wilhelmsen, DVM, Ph.D, Pathology Division, USAMRIID.

As with other laboratory animal models, investigations in which Non-Human Primates (NHPs) were challenged with an aerosolized dose of ricin indicate that disease progression is proportional to particle size [107], [109]. Inhalational challenge with a particle size of 8 µm was not lethal and did not cause lung damage, suggesting that the upper airways can effectively remove the toxin before it reaches the lung. Inhalational challenge with a particle size of 1 µm presented an entirely different picture with histopathologic changes beginning as early as 4 – 6 hours post-exposure. By 8 hours, pulmonary changes included alveolar edema, perivascular interstitial edema and lymphangiectasis, alveolar septal necrosis, and hemorrhage. At 16 hours, progression of pulmonary tissue damage continued, and by 24 hours, there was edema, pulmonary congestion, necrotic alveolar septa, and necrotic bronchiolar epithelium (Figure 18-5). Thirty-two hours later, alveoli contained fluid (edema) mixed with fibrin and viable or degenerate neutrophils, and macrophages. The bronchiolar epithelium was necrotic and often sloughed into the lumen while lymphatics surrounding the airways were moderately dilated and the endothelium of many small vessels had atrophied. There was also epithelial cell degeneration in the trachea and subacute inflammation. The cortex of adrenal glands showed mild degeneration and necrosis and there was lymphoid depletion and lymphocytolysis in the mediastinal lymph nodes. A similar course of disease was observed in an earlier study in which NHPs were challenged with ricin (~ 1 µm particle size), but the pre-clinical period varied between 8 and 24 hours in relation to the size of the original challenge dose [109]. This stage was followed by anorexia and decrease in physical activity. The time of death was also dose dependent and occurred between 36 and 48 hours.
18.4.4 Cause of Death

While the exact cause of death from ricin toxicity is not known, clinical symptoms of individuals exposed to lethal doses of the toxin suggest that death results from a severe inflammatory response and multi-organ failure [94], [95], [99]. A lethal dose of ricin given to mice by intravenous injection or intratracheal instillation results in a systemic inflammatory response, thrombocytopenia, hemolytic anemia, renal failure, and microvascular thrombosis, pathologies that are similar to those observed in man [87], [88]. Initially, the fact that macrophages are extremely sensitive to ricin led investigators to believe that macrophages might play a significant role in ricin intoxication [110]. Recent findings demonstrated that pulmonary inflammation caused by ricin required the presence of both macrophages and Interleukin-1 (IL-1) signaling pathways [111]. Also, studies using bone marrow derived macrophages showed that ricin inhibition of protein translation led to activation of IL-1β-dependent inflammation by activating innate immune signaling through the Nod-Like Receptor (NLR) family member, NLRP3 [112]. NLRP3 is an innate immune pattern recognition receptor found in the cytosol that is activated by molecular patterns found on many pathogens or other danger-associated proteins. Activation of NLRP3 stimulates IL-1β processing via a multi-protein complex, the inflammasome. Further investigations are necessary to understand how ricin activates severe inflammatory responses that lead to multi-organ failure, shock, and death.

18.5 DETECTION AND DIAGNOSIS

Early clinical symptoms of ricin intoxication may resemble symptoms caused by other biothreat agents and, therefore, it is essential to identify the etiological agent in order to provide the best treatment for exposed patients. The cellular uptake of ricin is rapid and thus limits the diagnosis of ricin in blood and other fluid samples. Additionally, the ricin concentration may be below the current levels of detection, making diagnosis more difficult [113]. Because of the inability to detect ricin in patients, identifying the toxin in environmental or forensic samples associated with the exposure remains the most reliable method for determining the presence of ricin and the possibility of intoxication. Ricin does not replicate and therefore, detection relies upon the ability to identify physical attributes of the toxin within the sample. The most common method for toxin identification utilizes anti-ricin antibodies to which ricin would bind. In recent years, several variations of antigen (toxin)-antibody assays have been developed [114]. Physical characterization using liquid chromatography and mass
spectroscopy complements the antibody-based methods and permits development of signatures of the toxin preparation [115].

18.5.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA provides an economical and straightforward method for detecting the presence of ricin in environmental and forensic samples. A capture antibody ELISA is a common method of detection. Ricin is initially “captured” onto the matrix via an anti-ricin Monoclonal Antibody (Mab) recognizing RTB. A second anti-ricin Mab, usually recognizing RTA, binds to the immobilized ricin, and the second Mab is then detected by an anti-mouse IgG conjugated to an enzyme such as horse radish peroxidase that forms a colometric reaction upon the addition of its substrate solution [86]. The Limit Of Detection (LOD) for these assays has been greatly improved by using methods that amplify the detection signal or that use a more sensitive signal such as those generated by Electrochemiluminescence (ECL). Using slight modifications of these assays, Poli and colleagues [116] reported LODs of 100 pg/100 µL in human serum and urine that had been spiked with various concentrations of ricin. Other studies such as those by Roy et al. [105] detected ricin in lungs, stomach, trachea, and nares using an ELISA based on time-resolved fluorescence. While these colorimetric and ECL methods permit detection of high pg or low ng concentrations, there are still sensitivity issues, particularly when assessing foods or biological tissues. Recently, an immuno-PCR assay that uses a Polymerase Chain Reaction (PCR) to amplify a DNA-labeled reporter system bound to the anti-mouse IgG permits accurate detection of ricin in these biologic samples ranging from 1 pg/mL to 100 pg/mL [117]. The immuno-PCR may offer a method that greatly enhances the ability to detect ricin in environmental samples, and more importantly, provides a technique that will accurately determine ricin in tissues from individuals exposed to ricin.

18.5.2 Hand-Held Assay Detection Devices (HHAs)

While routine capture ELISA provides accurate diagnostic tools, these assays require a laboratory setting and instruments to measure the signal. Antibody-based Hand-Held Assay (HHA) devices were developed to enable first responders to assess the situation in the field [118]. HHAs were initially developed for detection of the anthrax in the letters sent through the mail to office of Senator Tom Daschle in 2001. The success of anthrax spore identification initiated development of HHAs for ricin and other biothreat agents. In 2004, HHAs identified ricin in letters sent to the Dirksen Senate Office building [118]. For ricin, HHAs have an anti-ricin Mab bound as a single line on the matrix bed. The sample is added to one end of the bed, and capillary action causes the sample to flow across the matrix. The toxin binds to the antibody and then another detection antibody is added. If ricin is present, then the detecting antibody causes color development at the line. If samples are positive using a HHA, samples are sent to a laboratory for confirmation and further analysis.

18.5.3 Sample Verification Platforms

Laboratories such as USAMRIID and the National Biodefense Analysis and Countermeasure Center (NBACC) need capabilities that will accurately identify ricin. Multiple instrumental platforms with ELISA-based formats have been developed, including the following:

a) M1M ECL-based ricin immunoassay (LOD, 0.05 ng/mL);
b) Luminex MAGPIX multiplex (LOD, 0.001 ng/mL); and
c) MesoScale Discovery (MSD) PR2 Model 1900 ECL (LOD, 0.2 ng/mL).

Both the M1M ECL-based ricin immunoassay and the Luminex MAGPIX utilize magnetic beads that are labeled with anti-ricin antibodies [118]. Once ricin binds to the magnetic beads via the antibody, the sample is
sent through the instrument where the magnetic beads are captured by an internal magnet. The magnet is set on an electrode that delivers the proper amount of electrical potential resulting in the emission of light identifying that the sample contains ricin. The MSD PR2 is a highly sensitive ELISA, and has the advantage of using less sample amount (25 µL). The detection antibody is conjugated to a chemiluminescent label that allows for the detection of the toxin by ECL.

On May 30, 2013, a multiplexed rapid ricin detection assay was launched by Radix BioSolutions Ltd. (Austin, TX, USA) through the CDC’s Laboratory Response Network [120]. This assay was developed using Luminex xMAP® technology that permits concurrent detection of several agents in an adaptable, multiplexed assay architecture. Following this report, Tetracore, Inc (Rockville, MD) publicized the successful study completion and validation of its BioThreat Alert Lateral Flow Assay and BioThreat Alert Lateral Flow Assay Reader by the Department of Homeland Security for ricin detection [121].

18.5.4 Liquid Chromatography / Mass Spectrometry (LC/MS)
Another method for ricin detection includes identification by LC/MS. The combination of liquid chromatography and mass spectrometry allows for the separation of mixtures in a sample while being able to identify specific substances based on their molecular mass via their mass to charge ratio (m/z) [122]. The ionization of the molecules in the sample can either be protonated or deprotonated depending on the characteristics of the analyte and the mode of detection [122], [124]. The advantage of using this technique allows for the detection of ricin when very little sample is available. Picogram amounts of ricin can be detected within a 5-hour time frame allowing for fast, reliable detection [125]. LC/MS can also be used to characterize other components within the sample because they may provide “signatures” that suggest the origin of the agent. For example, a highly pure form of the toxin might indicate that the ricin had been used by an organized terrorist group such as Al Qaeda, while a less pure form may indicate less organized groups or individuals acting alone [86].

18.5.5 Ricin Activity Assay
When the presence of ricin is detected using an ELISA or other physical types of assays, the ability to determine whether the toxin is active becomes important for forensic evidence. The assay itself needs to accurately detect ricin’s biological activity in samples of limited size (about 50 mL) and low toxin concentration (about 10 ng/mL), and preferably, with an assay time less than 6 hours. To meet these criteria, a Cell-Free Translation (CFT) assay was developed at USAMRIID [126]. The CFT assay measures luminescence generated by the enzyme luciferase produced from the translation of luciferase m-RNA in a rabbit reticulocyte lysate system. The amount of luminescence, produced when the luciferin substrate is added to luciferase, is proportional to the amount of luciferase produced in the \emph{in vitro} translation system. When ricin is added to the mixture, translation of luciferase mRNA is reduced which decreases the amount of luciferase produced. Since the amount of luminescence developed is proportional to the amount of luciferase present in the CFT mixture, a reduction in luminescence, as compared to a ricin standard control, provides a quantitative assessment of active ricin in the sample [126].

Table 18-2 summarizes the most commonly used techniques for ricin detection and their sensitivity limits.
### Table 18-2: Biochemical Methods for Ricin Detection and Their Limits of Sensitivity.

<table>
<thead>
<tr>
<th>Method</th>
<th>LOD (ng/mL)</th>
<th>Time (hr)</th>
<th>Detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA-based</td>
<td>0.01 – 10</td>
<td>5 – 7</td>
<td>Ricin</td>
<td>[86], [105], [114], [116]</td>
</tr>
<tr>
<td>Hand-held</td>
<td>10 – 50</td>
<td>90</td>
<td>Ricin</td>
<td>[118]</td>
</tr>
<tr>
<td>ECL-based ELISA</td>
<td>0.001 – 10</td>
<td>4 – 7</td>
<td>Ricin</td>
<td>[118]-[121]</td>
</tr>
<tr>
<td>Immuno-PCR</td>
<td>0.01 – 0.1</td>
<td>3 – 5</td>
<td>Ricin/Ricinine</td>
<td>[114], [117]</td>
</tr>
<tr>
<td>LC/MS</td>
<td>0.1 – 8</td>
<td>5</td>
<td>Biological Activity</td>
<td>[122]-[125]</td>
</tr>
<tr>
<td>CFT</td>
<td>10 – 50</td>
<td>4 – 5</td>
<td>Biological Activity</td>
<td>[126]</td>
</tr>
</tbody>
</table>

*a Each method may include several different assays using similar principles and formats.

*b The Limit Of Detection (LOD) is the lowest amount of ricin detected.

*c The time required to perform the assay.

*d The assays detect either the physical form of ricin or determine the biological activity.

### 18.5.6 Diagnosis

Diagnosis of ricin intoxication is challenging in that the cellular uptake of ricin is extremely rapid and limits the availability of ricin for diagnosis in blood and other fluid samples to 24 hours post-intoxication [113]. Experimental data suggests that the plasma half-life of ricin is bi-phasic with the early α phase half-life lasting approximately 4 minutes; the longer β phased half-life was determined to be approximately 83 minutes long. The bi-phasic half-life suggests rapid distribution and uptake of the toxin followed by the slow clearance of excess toxin [113]. Additional LC/MS assessment of urine samples for metabolites, particularly alkaloids such as ricinine that is commonly found in ricin preparations would indicate ricin intoxication, particularly if the individual has symptoms associated with ricin intoxication [86]. Individuals who survive ricin intoxication would develop circulating antibodies in their blood and could be used to confirm intoxication. However, these antibodies would not be present until approximately two weeks post-intoxication, and therefore could not be used in the initial diagnosis.

### 18.6 MEDICAL MANAGEMENT

Despite the history of ricin’s use as a weapon, and unlike other toxin-mediated illnesses such as botulism, there is no FDA-approved therapeutic for ricin exposure. Due to the fact that ricin does not have cell-specific selectivity, treatment of ricin intoxication is dependent on the site or route of entry, is largely symptomatic, and basically supportive to minimize the poisoning effects of the toxin. Medical countermeasures that have demonstrated capability to disrupt the ricin intoxication process include vaccines and antibody therapy. Both rely on the ability of antibody to prevent the binding of ricin to cell receptors. To ensure maximum protection, the vaccine must be given before exposure, and sufficient antibody must be produced.

### 18.6.1 Ricin Vaccines

Development of a ricin vaccine has previously focused on either a deglycosylated ricin A chain (dgRTA) or formalin-inactivated toxoid [127]. Although both preparations conferred protection against aerosolized ricin,
the proteins aggregated and precipitated over time. Additionally, ricin is not completely inactivated by formalin and may retain some of its enzymatic activity (albeit approximately 1,000-fold lower than native ricin). Thus, other approaches to vaccine development have been investigated to develop a safe and efficacious candidate.

Recent research has focused on developing recombinant RTA sub-unit vaccines to eliminate cytotoxicity and improve the stability of the vaccine (reviewed by [13]; Figure 18-6). Researchers at the University of Texas developed RiVax that contains the Y80A mutation to inactivate catalysis, and the V76M mutation to ensure the removal of any trace VLS activity from the immunogen [128], [129]. RiVax is at least 10,000-fold less active than wild-type RTA but has also been shown to protect rodents against aerosol challenge [129]. In 2006, RiVax was tested in Phase I clinical trials. Results of these studies showed that RiVax appeared to be immunogenic and well tolerated in humans [130], [131]. However, while such findings were encouraging, vaccine formulation and stability remain problematic. Hence, a lyophilized formulation that retained immunogenicity when stored at 4°C was developed [132], [133]. RiVax has been out-licensed to Soligenix for more advanced clinical trials [134], [135].

To overcome both safety and stability issues simultaneously, researchers at USAMRIID structurally modified the RIP-protein fold of RTA to create a non-functional scaffold for presentation of a specific protective epitope [82]. The engineered RTA 1-33/44-198 (RVEc<sup>TM</sup>), was produced in <i>E. coli</i>, and lacks the C-terminal residues 199-276 as well as a loop between residues 34 – 43 (Figure 18-6). RVEc<sup>TM</sup> contains a number of well-characterized protective B cell epitopes, but is more stable and less prone to aggregation. Based on pre-clinical studies, this product was determined to have a reasonable safety profile for use in human studies; it demonstrated no detectable RIP activity or evidence of VLS [136]-[139]. In April 2011, USAMRIID launched a Phase I
escalating, multiple-dose study to evaluate the safety and immunogenicity of RVEc™ in healthy adults, and was completed in November 2012 [138]. The vaccine was well-tolerated and immunogenic [138], [139]. In June 2013, a Phase 1a (Version 2.0) protocol was implemented as a single-dose, single-center clinical study to allow for the administration and evaluation of a fourth boost vaccination [139]. The ELISA and TNA anti-ricin IgG endpoint titers for the four boosted subjects indicated a robust response very soon after a boost vaccine. In conjunction with this study, another protocol was also started in June 2013 for the collection of plasma from previously RVEc™ vaccinated subjects for passive transfer studies in animal models to demonstrate IgG as a surrogate marker for clinical efficacy. No adverse events have been reported on this study [139].

The RVEc™ Final Drug Product (FDP) passed stability testing through the 48 months [139]. In addition, the potency assay results confirmed the vaccine elicited protective immunity in mice against 5X the lethal ricin toxin dose, and were capable of inducing anti-ricin neutralizing antibodies. An End Of Clinical Use (EOCU) stability testing to include the 54-month time point was initiated in October 2013 for both the FDP and the diluent [139].

A comparative immunogenicity and efficacy study between RVEc™ and RiVax has been conducted in mice [140]. Both candidate RTA vaccines were found equally effective in eliciting protective immunity, however, there were quantitative differences observed at the serologic level. RVEc™ was slightly more effective than RiVax in eliciting ricin-neutralizing antibodies. Further, the antisera elicited by RVEc™ were toward an immunodominant neutralizing linear epitope on RTA (Y91 to F108), while those of RiVax were confined to residues 1 – 198 [140].

18.6.2 Antibody Treatment

Passive protection with aerosolized anti-ricin Immunoglobulin (IgG) has been evaluated as prophylaxis before aerosol challenge. In mice, pre-treatment of nebulized anti-ricin IgG protected against aerosol exposure to ricin [141]. Pre-clinical studies also have shown the protection afforded by neutralizing monoclonal antibodies against a lethal dose challenge of ricin [142]-[144]. Researchers at Defence Science and Technology Laboratory (Dstl) in Porton Down, UK have developed polyclonal anti-ricin antibodies that were raised in sheep immunized with ricin toxoid plus Incomplete Freund’s Adjuvant [145]. The protective efficacy of both IgG and F(ab’)2 were demonstrated in mice against ricin intoxication when administered 2 hours following either systemic or inhalational ricin challenge, while the smaller Fab’ fragment did not prevent death from ricin intoxication [145], [146]. This demonstrates the feasibility of producing an effective ovine anti-ricin antibody product. In a recent study, four chimeric toxin-neutralizing monoclonal antibodies were produced and evaluated for their ability to passively protect mice from a lethal-dose ricin challenge [147]. The most effective antibody, c-PB10, had the lowest IC50 in a cell-based toxin-neutralizing assay and was sufficient to passively protect mice against systemic and aerosol toxin challenge [147].

It should be noted that the use of anti-toxins as therapies for toxin exposure has limitations including:

1) Anaphylactoid or anaphylactic reactions;
2) Requirement of timely detection of exposure; and
3) The therapeutic window is dependent on the toxin and the dose received [145].

18.6.3 Supportive and Specific Therapy

The route of exposure for any agent is an important consideration in determining prophylaxis and therapy. For oral intoxication, supportive therapy includes intravenous fluid and electrolyte replacement and monitoring
of liver and renal functions. Standard intoxication principles should be followed. Because of the necrotizing action of ricin, gastric lavage or induced emesis should be used cautiously. Aerosol-exposed patient may require the use of positive-pressure ventilator therapy, fluid and electrolyte replacement, anti-inflammatory agents, and analgesics [148]. Percutaneous exposures would necessitate judicious use of intravenous fluids and monitoring for symptoms associated with VLS.

18.6.4 Development of Ricin Small Molecule Inhibitors

Reaching intracellular space with a ricin inhibitor would provide an ideal pre- and post-exposure therapeutic. At a minimum, small molecule inhibitors must possess sufficient safety and efficacy to enable a pathway to licensure. A strong safety profile is critical since no diagnostic capability exists to identify personnel who have received a clinically significant dose of ricin. Ideally, the inhibitor would also be self-administered, which would greatly reduce the burden on the healthcare system allowing the provider to focus on patients who require more intensive care and medical resources.

A variety of approaches have been used to identify suitable small molecule ricin therapeutics. Potential compounds fall into three broad mechanisms of action:

1) Those that target the RTA;
2) Those that target the retrograde transport pathway used by ricin to gain access to the cytosol; and
3) A group that alters the cellular stress response following ricin intoxication.

A notable absence among published work is molecules directed against the RTB that might prevent ricin from entering cells. However, the molecular structure of RTB makes it an extremely difficult drug target. X-ray studies show that RTB is composed of two domains with each domain possessing three sub-domains that bind to sugars [62], [149]. Selected mutations of RTB suggest that three of the six sites must be inactivated in order to prevent cellular intoxication [62]. Since these three sites are widely separated on RTB, it would present a formidable challenge in the design of inhibitors that still possess drug-like characteristics. Furthermore, the RTB carbohydrate binding regions are small and shallow; these features present yet another hurdle for the design of drug-like molecules [150]. Similar approaches have been used to identify small molecule inhibitors of shigatoxin, a prokaryotic enzyme with related enzymatic activity but limited structural homology to ricin, suggesting pharmacophore discovery is broadly applicable [151]-[164].

In contrast, RTA presents a much more tractable drug target. Even though the large, open, and polar nature of the active site makes it a difficult drug target [155], [156], there are high-resolution X-ray structures of the active site that can aid in the design of inhibitors [157]. Furthermore, the mechanism of action for ricin is well-described and provides additional criteria for the design of drugs that target the active site. Drug discovery approaches for ricin therapeutics have typically relied on Virtual Screening (VS), or high throughput cell-based assays. Virtual screening uses computational methods to evaluate large numbers of compounds for possible activity against ricin but requires careful consideration of molecular parameters to ensure optimal results, access to libraries of appropriate chemicals [158], [159], and structural data such as high resolution crystal structures of the target molecule [160]. No single VS software is ideal as each produces different results [160]. There are several excellent recent in-depth reviews that provide additional background on VS [161]-[163]. While it allows for evaluation of a large number of chemicals, VS hasn’t always been able to identify appropriate candidates for drug development [156], [160], [164]. Regions of proteins, such as the active site of ricin that have large and polar pockets are difficult drug targets [155], but there are successes seen in discovering RTA inhibitors [151], [165], [166].
Another screening technique, cell-based High Throughput Screening (HTS), requires an appropriate cellular model of intoxication and a method to identify compounds that have activity against ricin. For cell-based assays the tested compounds should be soluble in cell culture media or with an excipient compatible with cellular growth. The solubility requirement significantly reduces the number of compounds that can be tested in cell-based assays. Furthermore, poor solubility may mask an otherwise useful molecule because it cannot be delivered to the cells in a high enough concentration to have an observable effect. More comprehensive discussion on cell-based HTS can be found in several recent reviews [167]-[170]. In spite of these limitations, the complimentary methods of VS and cell-based HTS assays have identified a variety of active compounds. Some of these successes will be highlighted in the next sections.

### 18.6.5 RTA Inhibitors

Several research groups used RTA as a target to identify potential lead compounds from chemical libraries. One of the most potent active site inhibitors was developed by a rational drug design process [171] and built upon an earlier observation that Pteroic Acid (PTA) bound to the ricin active site with a modest IC₅₀ of 600 µm [166]. While PTA is not a suitable candidate because of its limited solubility, it served as a platform for designing derivatives. Several derivatives of PTA were found to have increased solubility, and when a linker was included that enabled additional contacts between RTA and the inhibitor, RTA inhibition was enhanced [172]. Saito et al., [171] built on this observation by adding di- and tri-peptide linkers to PTA that allowed binding both the specificity pocket and a distant secondary pocket within the ricin active site. The addition of these linkers and the resulting interaction with the secondary pocket provided a 100-fold improvement in the IC₅₀ [171].

Additional successes in identifying RTA inhibitors through virtual screening have been reported by Pang [151] and Bai [163]. Although both groups’ compounds target RTA, Pang’s [151] molecules targeted a site distant from the active cleft yielding inhibition of ricin enzymatic activity, while Bai’s [165] are active site inhibitors. Pang’s [151] deliberate choice was based on the recognition that the large size and polar features as well as the multiple electrostatic interactions between rRNA and the active site made it an unattractive and difficult drug target. Pang [151] also took advantage of a structural change that occurs in the ricin active site upon binding of the toxin to the α-Sarcin-Ricin Loop (SRL) in the 28S ribosome which causes Tyr80 in the active cleft to move to a new position where it participates in the depurination of the ribosome by packing with the bases of rRNA [157], [173], [174]. Thus, if the movement of Tyr80 is blocked ricin is rendered inactive. Preventing the movement of the Tyr80 is a novel approach to developing RTA inhibitors by avoiding the complication of designing drugs for the ricin active site. This approach to inhibitor design was designated as the “door-stop” approach because it prevents Tyr80 from undergoing the necessary conformational change for enzymatic activity. The Pang group [151] screened over 200,000 molecules with molecular weights under 300 Da and 226 were predicted to block the movement of Tyr80. When evaluated in a cell-free translation assay using firefly luciferase, several of these compounds inhibited ricin. Unexpectedly, several compounds enhanced the firefly luciferase assay, but was the result of the compounds directly interacting with the firefly luciferase and not the RTA. This interaction precluded the determination of the IC₅₀ of the compounds and serves to underscore that appropriate controls need to be present when screening chemicals for activity in this reporter assay [175]-[178]. Notably however, functional studies revealed that the Pang compounds [151] protected cells exposed to ricin, suggesting that ricin inhibitors using the “door-stop” approach is a validated model. Furthermore, these results demonstrated that direct competition with the ricin active site, a difficult target, was not essential to achieve inhibition of the ricin catalytic activity.

The VS approach conducted by Bai [165] identified several new classes of inhibitors. Bai [165] used two different virtual screening programs, one to identify molecules that could bind to RTA in which the Tyr80 has been displaced and a separate program that identified candidate compounds that bound to the RTA form in
which Tyr80 was not displaced. Compounds ranked highly by both programs were selected for further study, and revealed a variety of new chemical entities for further development [165]. In vitro kinetic studies showed that these compounds possess a potency similar to PTA. While many of the compounds were cytotoxic, two were identified that protected Vero cells exposed to ricin. The best performing compound showed little cytotoxicity and protected about 90% of cells exposed to ricin [165]. Nevertheless, the cytotoxic compounds can still serve as starting points to improve their binding to RTA while reducing their toxicity.

18.6.6 Transport Inhibitors
The second category of inhibitors, transport inhibitors, blocks the retrograde movement of ricin through the cell and may have their greatest utility as pre-exposure treatments. Indeed, compounds that inhibit the retrograde transport of ricin have substantial efficacy in animal models when used in a pre-exposure setting [179]. However, due to the retrograde pathway taken by ricin to arrive at its cellular target, inhibitors of this normal cellular process also have a potential to exhibit significant toxicity. For example, Ilimiquinone (IQ), a marine sponge metabolite, inhibited ricin in a dose-dependent manner in a Vero cell assay [180]. However, IQ also caused the Golgi apparatus to fragment into smaller vesicles, although this effect was reversible when IQ was removed [181]. Additional molecules have been identified that alter retrograde transport and protect cells from ricin challenge; however, the utility of these molecules for continued development is questionable because they also disrupt the Golgi architecture [182]. In spite of the potential toxicity of retrograde transport inhibitors, several groups identified inhibitors of ricin transport, some of which show limited toxicity in cellular- and animal-based assays of efficacy. Stechmann and colleagues [179] used a protein synthesis cell-based high throughput screening assay to identify compounds that restored normal levels of protein synthesis after ricin exposure. Of more than 16,000 compounds, they identified two that were inhibitors of retrograde transport. Despite functionally blocking retrograde movement, these compounds exhibited no effect on the architecture of the Golgi complex or on cellular transport pathways such as endocytosis, vesicle recycling, degradation, or secretion [179]. These two compounds were further examined in an animal model of intranasal ricin challenge. The compounds completely protected challenged animals when treatment was given one hour prior to ricin exposure; no acute toxicity was observed in animals that received only the test compounds [179]. However, these compounds may not be ideal candidates for further development due to instability [183].

18.6.7 Inhibitors of Cellular Stress Response
Rather than targeting the ricin molecule or the retrograde transport pathway described previously, another target is the cellular response to ricin. When ricin depurinates ribosomes in target cells, these cells enter a condition known as ribotoxic stress response [184], [185]. The ribotoxic stress response leads to activation of Stress Associated Protein Kinases (SAPK) and other cellular changes [184]. Activation of SAPKs including p38 Mitogen-Activated Protein Kinase (p38MAPK) can lead to the release of pro-inflammatory cytokines and the induction of apoptosis in cells [186]-[188]. A screen of molecules identified two compounds that protected cells from ricin challenge but did not act on ricin itself or on the retrograde pathway. These molecules were further studied to determine their mechanism of action [189]. One compound acted to reduce the activation of the SAPK p38MAPK by acting upstream of p38MAPK activation. The other compound acted as an inhibitor of caspase 3 and 7 activation thus blocking a critical step in the induction of apoptosis [189].

18.7 SUMMARY
Ricin is a potent toxin derived from the ornamental castor plant, *R. communis* L. which has been cultivated worldwide for its oil since ancient times. Because of its potency, stability, wide availability of its source plants, and popularity in the internet, ricin is considered a significant biological warfare or terrorism threat. Ricin was
developed as an aerosol biological weapon during WWII, but was not used in combat or in mass casualty attacks. As a biological weapon, ricin has not been considered as useful in comparison with other agents such as anthrax or BoNT. Nevertheless, its popularity as well as its track record in actually being exploited by extremists groups and individuals accentuates the need to be vigilant of its surreptitious misuse. Despite ricin’s notoriety as a potential biological agent, its medical applications have been also explored. Ricin has contributed to early immunology, the understanding of cell biology, treatment of cancer, AIDS, and other illnesses. Clinical manifestations of ricin poisoning vary depending on the routes of exposure. Aerosol exposure represents the greatest threat to forces posed by ricin and can lead to death via hypoxia. Diagnosis of ricin exposure is based upon both epidemiological and clinical parameters. Currently, there is no U.S. FDA-approved drug or vaccine against ricin intoxication; treatment is mainly symptomatic and supportive. Since vaccination offers a practical prophylactic strategy against ricin exposure, considerable efforts have been devoted to develop a safe and effective ricin vaccine to protect humans, in particular soldiers and first responders. Recombinant candidate ricin vaccines are currently in advanced development in clinical trials. Efforts are also underway to develop small molecule inhibitors for the treatment of ricin intoxication. Recent findings suggest that refinement of the newly-identified ricin inhibitors will yield improved compounds suitable for continued evaluation in clinical trials.

18.8 REFERENCES


Lord JM, Roberts LM and Robertus, JD. Ricin: structure, mode of action, and some current applications. FASEBJ. 1994;8:201-208.


[129] Smallshaw JE, Richardson JA and Vitetta ES. (2007) RiVax, a recombinant ricin subunit vaccine, protects mice against ricin delivered by gavage or aerosol, *Vaccine* 25, 7459-7469.


RICIN


Chapter 19 – MEDICAL INTERVENTIONS AGAINST STAPHYLOCOCCAL ENTEROTOXIN B

Robert P. Webb\(^1\) and Leonard A. Smith\(^2\)

1: Molecular and Translational Sciences, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD; 2: Senior Research Scientist (ST) for Medical Countermeasures Technology, Office of Chief Scientist, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD

UNITED STATES

Corresponding Author (Smith): Leonard.A.Smith@comcast.net

19.0 EXECUTIVE SUMMARY

*Staphylococcus aureus* is globally recognized as a major pathogen that causes food poisoning and various infections in both humans and animals. This bacterium is normally found in about one-third of the population and frequently acts as an opportunistic pathogen. *S. aureus* secretes an astonishing variety of enzymatic and non-enzymatic virulence factors that contribute to its ability to cause disease in human hosts. Staphylococcal Enterotoxins (SEs) are members of the larger bacterial Superantigen (SAg) family and are potent activators of the immune system. Staphylococcal Enterotoxin B (SEB) has been extensively studied since the early 1960s when it was investigated as a potential battlefield incapacitant in U.S. biological offense programs and it is currently classified as a category B threat agent by the Centers for Disease Control and Prevention (CDC).

SAgs are virulence factors that target the adaptive immune response through a modified immunological synapse. These protein toxins bypass the conventional antigen processing mechanisms in the immune system and instead cross-link Major Histocompatibility Complex (MHC) Class II molecules on Antigen Presenting Cells (APC) and T-Cell Receptors (TCR). This irregular antigen presentation causes a T-cell proliferation which elicits an increase cytokine levels followed by a subsequent transient T-cell hypoactivation. Systemic intoxication by SAgs such as SEB can lead to the life-threatening Toxic Shock Syndrome (TSS). The binding and immunomodulatory influence of these SAgs are variable across different animal species and has had a direct impact on the development of animal models utilized to evaluate medical interventions against SEB.

Both *S. aureus* and its derived SEs warrant the development of medical interventions. The increasing incidence of *S. aureus* Bacteremia (SAB) caused by Methicillin-Resistant (MRSA) strains of the bacteria has become a significant health issue throughout the world. The potential application of SEB as an agent of bioterrorism has increased efforts to produce pharmaceutical countermeasures against the toxin. While there are currently no licensed prophylactic or post-intoxication treatments for SEB, a number of different potential medical interventions are being pursued. A promising vaccine based on a recombinant, attenuated form of the SEB protein is currently in advanced development. Immunotherapy, peptide mimetics that competitively bind native binding targets of SAgs to block the hyperstimulated immune response, as well as a number of protein and small molecule drugs that reduce the pro-inflammatory response are still in the early stages of research and development.

19.1 INTRODUCTION

In the past century, the medical relevance of *Staphylococcus aureus* has increased as a result of both the rise in incidence of human pathogenicity and development of resistance to anti-microbials. The extensive use of clinical
and surgical procedures, such as the use of intravascular catheters, increases the direct contact of the external environment with intravascular regions and may disseminate the bacteria directly into the bloodstream, potentially causing *S. aureus* Bacteremia (SAB). The incidence of SAB has increased significantly throughout the world [1]. There has also been a rise in the reported incidence of antibiotic-resistant strains of *S. aureus*, increasing the ability of these pathogens to spread in a hospital setting [1]. There is a high mortality rate associated with SAB and the increased duration of hospital stays and treatments associated with this type of infection are a significant burden on the health care systems [1], [2].

*S. aureus* produces a wide variety of exoproteins that facilitate the pathogen’s ability to colonize and produce diseases in mammalian hosts [3], [4]. The primary function of these proteins is presumably the conversion of local host tissues into the nutrients required for bacterial growth and immunomodulation. The Staphylococcal Enterotoxins (SEs) secreted from *S. aureus* are part of a larger family of Bacterial Superantigens (BSAg), including Toxic Shock Syndrome Toxin-1 (TSST-1), that exert a profound detrimental effect on the immune system [5], [6]. Staphylococcal Enterotoxin B (SEB) and related SAg toxins are potent stimulators of the immune system that induce a wide variety of human disease symptoms ranging from classic food poisoning to toxic shock [7], [8]. SEB binds directly to Major Histocompatibility Complex (MHC) Class II receptors on Antigen Presenting Cells (APC) and specific regions of T-Cell Receptors (TCRs) in an atypical fashion [9]. The resulting hyperactivation of monocytes, macrophages and T lymphocytes produces a pro-inflammatory response followed by T-cell anergy; a tolerance mechanism in which the lymphocyte is functionally inactivated following an antigen encounter, but remains alive for an extended period of time in a hyporesponsive state.

In addition to *S. aureus* itself, a great deal of research has focused on the individual SEs produced by the bacterium as well. During the 1960s, the U.S. sponsored an offensive Biological Warfare (BW) program which included SEB, then referred to as PG, as a potential agent that could be employed to incapacitate soldiers in a battlefield setting [10]. The 1972 Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (biological) and Toxin Weapons and on their Destruction signed by President Richard M. Nixon went into effect in March of 1975, effectively terminating the U.S. BW efforts. However, because of its ease of production and dispersion, its high morbidity rate, as well as delay of symptom onset and the difficulties distinguishing an intentional release of the toxin from a naturally occurring intoxication, SEB is still considered to be a potential agent of bioterrorism. The relative stability of the toxin makes it suitable for dispersion as an air, food, or water-borne agent [10]. As such, staphylococcal enterotoxin B is currently classified as a category B threat agent by the Centers for Disease Control and Prevention (CDC).

The complex nature of the *S. aureus* induced disease states makes the development of effective medical interventions a significant challenge. The recent characterization of a number of SAg structures, as well as that of their interactions with the individual components of the immune system, has allowed the production of new classes of vaccine and therapeutic products. This review summarizes the current advances in the development of medical countermeasures against SEB.

### 19.2 MICROBIOLOGY

#### 19.2.1 The Organism and its Toxins

*S. aureus* is a globally recognized opportunistic bacterial pathogen that is responsible for a diverse spectrum of diseases including food poisoning in humans as well as various infections in both animals and humans. The bacterium is found primarily on the mucosal membranes of humans and animals but can also be found in the environment and contaminated food. Approximately 30 – 50 % of the human population carries *S. aureus* as a
commensal, primarily in the nasopharynx where the bacteria can reside asymptomatically as a transient or asymptomatic persistent member of the mucosal microbiotic environment [11]. Most newborns are colonized during their first week of life [12]. *S. aureus* is ubiquitous in foods that have been either handled by humans or contaminated with animal matter including fruits, vegetables, meats, as well as pastrY and dairy products [13]. A 2011 study reported that anywhere from 37 – 77 % of all U.S. meat and poultry samples were contaminated with *S. aureus*, 52% of which were multi-drug-resistant isolates [14]. *S. aureus* are non-motile, non-spore forming, Gram-positive, facultative anaerobic, chemoroganotrophic bacteria with a respiratory and fermentative optimal of 37°C (Figure 19-1). When cultured, *S. aureus* generally produces medium to large size beta-hemolytic colonies that are light tan to golden in color, and the Latin-derived genus name means “golden cluster seed”. Alexander Ogston is credited with the discovery of *S. aureus* in 1880 by the microscopic examination of pustulent abscesses on a patient. Like most cocci, *S. aureus* is extremely resistant to drying (may remain viable for months) and other adverse environmental conditions including pH, temperature, and many disinfectants. Interestingly, staphylococci can grow in high saline conditions and most strains grow in media supplemented with 10% NaCl.

![Figure 19-1: Colorized Scanning Electron Micrograph of Staphylococcus aureus. Photo in the public domain from PHIL (ID#:10046 CDC’s Public Health Image Library).](image)

Most isolates of *S. aureus* produce cytotoxins and enzymes (α-, β-, γ-, γ-variant and δ-hemolysins, nucleases, lipases, collagenase and hyaluronidase) that aid in the conversion of local host tissue into nutrients required for bacterial growth and facilitate spread of the pathogen throughout the host [15]. Some isolates also secrete leukotoxins (Luk) and Pyrogenic Toxin Superantigens (PTSAgs) that includes TSST-1, staphylococcal enterotoxins and exfoliative toxins [4]. The SEs are low-molecular weight proteins that range in size from 27 to 30 kDa. Until the 1980s, SEA-SEE were the only *S. aureus* enterotoxins described in the literature. However, advances in biotechnology, concerns over the increasing number of antibiotic-resistant clinical isolates, the potential use of SEs as bioterrorism agents and the parallel interest in the development of prophylactic and
therapeutic interventions has dramatically expanded the field of study and facilitated the discovery of new SEs. Proposed nomenclature suggests that SEs be classified as superantigens that induce emesis upon oral administration in a primate model; while those without emetic properties and those not yet tested to be designated as Staphylococcal Enterotoxin-Like toxins (SELS). The SEs and SELs, while structurally and functionally similar, have been divided into homology groups based on their amino acid sequences [16]-[19]. The well-characterized SEA, SED, and SEE and the more recently characterized SELJ, SELN, SELO and SELP share 51 – 81 % homology and comprise Group 1. Group 2 is composed of the classic SEB, SEC1, SEC2 and SEC3 which share 66 – 98 % homology as well as the new classes SEG, SER and SELU. Group 3 is made up of only the newly characterized SEs and SELs including SEI, SELK, SELL, SELM and SELQ. Group 4 consists solely of the TSST-1 that was originally designated as SEF but was re-named when it was found not to display in-vivo biological activity consistent with an enterotoxin and group 5 consists of the SEH toxin. All of the toxin encoding Open Reading Frames (ORFs) are located on mobile genetic elements including bacteriophages, pathogenecity islets, genomic islets and plasmids [20]-[22].

19.2.2 Toxin Structure

The genetic identity of the SEs was initially characterized by Bergdoll and collaborators in the 1970s [23]-[25]. SEB is a 28.4 kDa protein, consisting of 239 amino acids that display considerable thermal stability compared to other protein toxins such as ricin and botulimum neurotoxins. The toxin remains biologically active after 3 minutes in boiling water. X-ray crystallography of SEB reveals a structure very similar to that of other superantigens and consists of two distinct domains connected by a 6 amino acid residue extended loop (Figure 19-2) [26], [27].

![Figure 19-2: Staphylococcal Enterotoxin B Structure. Domain I (in pink) is the amino-terminal region. Domain II (in blue) is the carboxy-terminal region of the protein. The 6 residue extended loop bridging the domain I and II is depicted in yellow. Structure generated from Cn3D using MMDB ID: 58370PDB ID: 3SEB from the NCBI structural database.](image_url)
Domain I contains the amino terminal region of the protein, consists of residues 30 to 120 and contains a β-barrel and 3 α-helices. The β-barrel has a disulphide bridge containing 18 amino acids between the participating cysteines and forms a highly mobile, solvent exposed loop. This structure is a conserved feature in all of the characterized SEs, although the sequence homology and the distance between the functional cysteines varies [27]. This loop is reportedly responsible for the emetic properties of SEs in primate models [28]. Substitution of the Cys residues of the SEC1 solvent loop with Ala ablates emetic activity [28]. However, mutating these residues to Ser does not block emesis; suggesting a specific conformation may be required for the disulfide loop to elicit emesis [28]. The β-barrel structure is highly similar to the well-characterized oligosaccharide/oligonucleotide fold (OB-fold) and exclusively binds to MHC Class II molecules [29], [30]. The OB-fold is found in several toxin and non-toxin proteins where it is thought to play a role in DNA or carbohydrate binding. Domain II contains amino acid residues 127 – 239, consists of anti-parallel β-sheets aligned directly adjacent to a central α-helix and is considered to be unique to microbial superantigens. The amino terminal tail comprised of residues 1 – 20 is found on the surface of domain II. Analysis of the high resolution crystal structure of SEB provided important supporting evidence for the proposed interaction of the toxin with components of the immune system that result in the characteristic inflammatory response.

19.2.3 Mechanism of Toxicity

The term superantigen was initially used in 1989 upon the observation that the mitogenic activity of these toxins resulted in a significant proliferation of T-cells [31]. Normally, antigens are internalized, digested into smaller peptide fragments and presented by the MHC Class II receptors on the surface of APCs. The MHC Class II receptor is an αβ heterodimer with α1/α2 and β1/β2 sub-domains [32]. The antigen presented on the MHC Class II receptor creates a molecular synapse with the TCR which is also an αβ heterodimer; but with immunoglobulin-like variable Vα/β and constant Cα/β domains. The processed antigen comes into contact with all of the α/β domains on both the MHC Class II molecule and TCR and on average, induces proliferation in 1 of every 1000 T-cells through clonal expansion of antigen-specific cells [33], [34]. Superantigens are believed to be the most mitogenic substances ever characterized [35] and levels as low as 0.1 pg/ml are sufficient to stimulate an uncontrolled T-cell response, resulting in adverse physiological reactions [36], [37]. SEB, like all SAgs, is not processed into smaller peptide fragments but binds as an intact protein on the α1 sub-domain (or DRα) of the MHC II molecule, outside of the conventional peptide binding groove, and interact directly with the TCR Variable β (Vβ) region [38]; not the Vα and Vβ combination of TCRs utilized by conventional antigens. Stimulation of cells through external receptors makes the development of interventions easier than toxins that actually enter the cell. Arad reported that CD28, an immune regulator constitutively expressed on the surface of T-cells is believed to be a third target receptor for superantigen toxins and contributes to the high affinity with which they bind [39]. Superantigens essentially cross-link the APC MHC II with a TCR; eliciting an exaggerated immune response including T-cell proliferation, anergy, and a cytokine storm of chemokines and pro-inflammatory cytokines including interleukins IL-6, IL-8, II-12, IL-1B, Interferon gamma, (IFNγ), IFNγ-Induced Protein 10 (IP-10); Macrophage Inflammatory Protein 1 alpha (MIP 1α), Monocyte Chemotactic Protein 1(MCP-1), Tumour Necrosis Factor alpha (TNFα) and beta (TNFβ) [17]. A majority of the pathophysiology relative to SAg exposure is attributed to T-cell cytokines IL-2, INF-γ, and particularly TNF-α [37], [40]. These protein toxins can activate anywhere from 5 – 30 % of the T-cells [16], [31], [41]-[44] where a more conventional antigen will stimulate less than 0.01% of available T-cells. Furthermore, this reaction is particularly remarkable since T-cells are not directly involved in the immediate immune response against bacteria [45].
19.3 EPIDEMIOLOGY AND CLINICAL SYMPTOMS

19.3.1 S. aureus Epidemiology

*S. aureus* produces an array of virulence factors and a single strain capable of producing multiple SEs can cause the most prevalent form of food poisoning [46]-[48]. In addition to the gastrointestinal form of the disease, the incidence of systemic blood-borne infections has been increasing and been complicated by the advent of antibiotic-resistant strains of the bacteria. *S. aureus* can produce a diverse arsenal of products that contribute to the pathogenesis of infection and this bacterium has also become an increasingly prevalent source of blood-borne infections associated with health care systems around the world. *S. aureus* is the most common bacterial pathogen found in clinical isolates from hospital inpatients in the USA [49]. The SENTRY Anti-microbial Surveillance Program reported that *S. aureus* was the primary cause of nosocomial bacteremia in North America and Latin America and the second most common cause in Europe [1]. In the period from 2002 – 2003, *S. aureus* was found to be the most prevalent cause of early-onset bacteremia in 6697 patients from 59 different hospitals in the USA [50]. There has been an increase in the prevalence of *S. aureus* antibiotic-resistant strains [51] that has allowed these pathogens to spread in both hospital and community settings. The increased frequency of invasive surgeries, intravascular devices and immunocompromised patients has also contributed to a sharp rise in *S. aureus* Bacteremia (SAB) over the last 30 years [52]. Methicillin-Resistant *S. aureus* (MRSA) was first characterized in 1960 [53] with incidental increases occurring until the early 1970s. The first gentamicin-resistant outbreak occurred in Australia in 1979 and extensive outbreaks occurred in other countries by 1981 [54]. *S. aureus* strains resistant to penicillin, cephalosporin, methicillin, vancomycin and linezolid have also been reported [1]. Management of antibiotic-resistant strains of *S. aureus* in hospitals represents a significant burden on the health care system. In a survey of 150 patients, MRSA-related Surgical Site Infection (SSI) resulted in a 7-fold increased risk of death, more than 3 weeks of additional hospitalization and more than $60,000 of additional charges per patient as compared to non-infected control surgical procedures [2]. There are numerous management and prevention guidelines for SAB but few uniform recommendations except applying a combination of aggressive antibiotic therapy and removal of the source infection. Many cases of SAB originate from colonization of the nasal mucosa; thus, elimination of nasal carriage by locally applied or systemic antibiotics is a useful prevention strategy [55].

A more comprehensive understanding of the virulence factors involved in the pathology of *S. aureus* may provide information for integrated management strategies that combine different treatment methods capable of targeting both the pathogen and its interactions with the immune system.

19.3.2 Clinical Symptoms of SEB Intoxication

Clinical symptoms of SEB intoxication can be variable and are dependent on the dosage and route of exposure [56], [57]. The actual incidence of SEB-related food poisoning is unknown; many cases are so mild that patients do not seek treatment. Additionally, diagnoses in the emergency department are usually presumptive, and a number of other diseases may mimic SEB-induced gastroenteritis. The gastrointestinal form of SEB toxicity, while potentially debilitating for short durations, is rarely fatal with adequate hydration [58]. The initial presentation of SEB intoxication may be unremarkable but patients generally display acute onset with complaints of significant abdominal distress or dyspnea (shortness of breath) [58]. Oral ingestion of SEB presents with what is considered to be classic symptoms associated with food poisoning including diarrhea, violent vomiting, fever of 40 – 41 °C, headache, myalgia (muscle pain), dehydration, and in severe instances, lethal shock [57]. Patients may also display hypotension, tachycardia, hyperperistalsis, and diffuse non-localizing pain [57], [58]. Symptomatic onset occurs within 6 – 12 hours after toxin ingestion, most cases are self-limiting with full recovery observed at about 48 hours. Humans are not naturally exposed to SEB through
inhalation of aerosolized toxin and so the clinical symptoms from this route of exposure must be inferred from animal studies. However, there have been historical examples of inhalational SEB intoxication that occurred in laboratory workers in 1963 – 1964 during the U.S. offensive biological warfare program [59]. Two individuals exposed to an aerosol exposure of a crude filtrate of SEB both developed fever, myalgias, non-productive cough, nausea, vomiting, and diarrhea within 2 hours with resolution of symptoms occurring by the third day. Five individuals that came into contact with highly purified SEB transferred from the fur of monkeys exposed to aerolized SEB toxin developed fever, cough, tightness in the chest, anorexia, nausea and vomiting anywhere from 3 to 24 hours post-exposure. A leak in a pressurized tube carrying aerosolized SEB used in a monkey study resulted in an exposure of 15 laboratory workers. Ten individuals became symptomatic within 1.5 to 24 hours (most within 12 hours) of exposure; nine of the individuals had to be hospitalized. In addition to the symptoms described above, these individuals displayed previously unreported upper respiratory symptoms as well as pharyngeal and tympanic inflammation. Rusnak reported that three laboratory workers that had inadvertent ocular transfer exposures developed conjunctivitis and localized swelling within 16 hours of exposure; symptoms not previously reported [59].

The toxicity of SEB varies between different animal species due to the difference in MHC II HLA receptor binding affinities and exposure route. Because humans are not naturally exposed to SEB by a respiratory exposure, the amount of the toxin needed to elicit symptoms must be inferred from animal studies [10], [57]. The estimated human Lethal Dose (LD50) is 0.02 µg/kg and the 50% Effective Dose (ED50) is 0.0004 µg/kg for an aerosol exposure. Non-Human Primate (NHP) studies indicate the intraperitoneal or intravenous delivered estimated ED50 is 0.03 – 0.26 µg/kg for monkeys and 12 – 40 µg/kg for chimpanzees. Extrapolations of these data suggest that a 70-kg human would require a cutaneous dose of 840 µg of SEB to elicit clinical symptoms; an ocular route would require an estimated 2 µg [59].

19.4 MEDICAL MANAGEMENT

Currently, there are no FDA-licensed vaccines or therapeutics against SEB. However, the potential of SEs to be used as an offensive biological threat agent has accelerated research efforts to develop medical interventions against both pre- and post-intoxication events. Characterization of the specific, multiple interactions of the SAg toxins with immune system components has resulted in a variety of new therapeutic interventions. The lack of a standardized animal model to evaluate these products has somewhat hampered efforts to accurately ascertain the potential of these interventions in a human host system.

19.4.1 Small Animal Models

The use of the appropriate animal model that responds to SEB in a fashion that closely mimics the response observed in humans is essential when testing vaccines and therapeutics. Humans and other primates are more susceptible to SAg than most other species because their MHC II bind these toxins with a higher affinity [60]. Rhesus monkeys (Macaca mulatta) have been historically used as a model system to study the effects of SEB due to the toxin’s strong emetic response when delivered orally [61], [62]. A comparison of the human, rhesus monkey and chimpanzee (Pan troglodytes) T-cell response to four different SAggs indicated the rhesus lymphocytes responded to 10,000 times lower concentrations of SEB than human cells [63]. Because of their enhanced sensitivity to SEB, the rhesus monkey is considered the most relevant animal system to evaluate prophylactic and therapeutic products designed for eventual use in humans [64]. However, the cost of obtaining and maintaining non-human primates in compliance with the required regulatory guidelines has warranted the evaluation of alternative animals systems to perform initial testing of medical interventions against the effects of SEB.
Mice are not inherently sensitive to SAg-induced lethal shock because of the lower affinity of these endotoxins to the mouse MHC-II molecules [65], [66]. In order to elicit SEB-Induced Lethality (SEBIL), and to permit the handling of lower toxins amounts, potentiating agents such as lipopolysaccharides [67], [68], D-galactosamine [69], actinomycin D [70] and viruses [71] are administered. These agents act synergistically with SAgs to either elicit a cytokine response or to increase the cytokines half-life to mimic the toxic effects of SAgs in a susceptible host animal. However, because it can be potentially difficult to ascertain the specific contribution of the potentiator molecules to the overall toxicity response, it can difficult to accurately determine the effectiveness therapeutic interventions designed to treat BSag-induced shock in humans. An alternative mouse model that does not require potentiators instead uses a dual-dosing of SEB administered intranasally and intraperitoneally (i.p.) [72], [73]. This “double tap” model allowed the use of relatively low amounts of toxin to induce systemic cytokine release and death in mice that was attributed exclusively to SEB, without the contribution of potentiator molecules. Parsonet described a rabbit TSS model using a subcutaneous infusion pump to administer a total of 150 μg of TSST-1 over a period of seven days that resulted a characteristic Toxic Shock Syndrome (TSS) physiological response [74], [75]. Intra-bronchiole inoculation of SAgs TSST-1, SEB and SEC was reported to induce lethal pulmonary disease in rabbits [76]. Although this study did not include any specific pro-inflammatory molecule profiles during the infection, administration of soluble high-affinity Vβ-TCR G5-8 (an inhibitor described in Section 4.3.2) was found to dramatically increase survival. Mateu described a porcine model in which PBMC from miniature pigs treated with 1 μg/ml SEB elicited lymphoproliferation and a strong IFN-γ response [77]. Yorkshire piglets given lethal doses 150 μg/kg of SEB i.v. reportedly developed a biphasic clinical response highly similar to that described in humans [78] and which was characterized by lymphoproliferation and increases in cytokine production [79].

Transgenic mice with modified MHC-II profiles have been increasingly utilized as an alternative model of SAg-induced toxicity in the development of therapeutic interventions. Mice expressing Human Leukocyte Antigen (HLA) DR3 and human CD4 molecules, in place of their native murine analogues, were reported to respond to several log lower concentrations of SEB and produced higher levels of pro-inflammatory cytokines IL-6 and IFN-γ than observed in wild-type BALB/c mice in a splenic cell proliferation assay [80]. Vaccination of transgenic mice with a recombinant, attenuated SEB antigen induced significant levels of anti-SEB antibodies that protected the mice from a surge in pro-inflammatory cytokine secretion after a challenge of 10 μg of SEB. Transgenic mice expressing human HLA-DQ8 and CD4 were likewise found to be susceptible to SAg-induced toxic shock using Streptococcal pyrogenic exotoxin A (SpeA) [81]. When administered 5 μg of SpeA, the HLA DQ8 transgenic mice displayed a robust T-cell proliferation and a significant increase in the production of TNF-α, IFN-γ and IL-6 that correlated with mortality. These findings suggest that these types of transgenic mice may be useful for modelling vaccine and therapeutics against bacterial SAgs for eventual human application.

19.4.2 Vaccines

Berdoll reported that a partially purified SEB vaccine consisting of an acid precipitated cell culture supernatant, toxoided with 0.7% formalin and adsorbed onto aluminium hydroxide, protected Rhesus monkeys against 200 Minimal Emetic Doses (MED) of SEB [82]. A highly purified form of SEB [83] delivered either as an untreated enterotoxin or a toxoided product by intracutaneous injection protected monkeys against 25 LD₅₀ of SEB delivered i.v. [84]. Parallel studies optimized the formaldehyde-inactivation conditions believed to promote intramolecular bond formation that produces high molecular weight polymers that elicit the production of protective antibodies [85]. The SEB toxoid is typically prepared by a formalin incubation of 30 days at pH 7.5 [86]. The SEB toxoid adjuvanted with alum was evaluated in monkeys by intracutaneous [84] and intramuscular administration [87]-[89]. In general, three i.m. inoculations of the toxided antigen was sufficient to elicit protective immunity against an aerosol challenge of the toxins. Oral administration of the SEB toxoid by intratracheal [88], [89] or intragastric inoculation [84], [87], [88] indicated the toxoided SEB alone was a poor
mucosal immunogen. Marginally improved responses to oral administration was observed when the toxoided SEB was encapsulated in either proteosome [89] or poly (DL-lactide-co-glycolide) microcarriers [87], [88].

The toxoid vaccine candidate has been largely replaced by a recombinant, attenuated SEB produced by modifying conserved receptor-binding amino acid residues common to all bacterial superantigens [90]. Molecular modelling of existing SAgs indicated a conserved Lysine (L45) within the SEB hydrophobic binding loop that is critical for the molecular recognition of the DRα sub-unit of MHC II HLA molecules [91]. A recombinant SEB L45R mutant was found to be non-toxic in mice yet elicited a robust immunological response [92]. Other structural binding motifs in the SEB protein include a polar cleft (E67, Y89, Y115) that accommodates L39 of the DRα sub-unit [91] and Y94 of the disulfide loop has extensive hydrophobic interactions with the HLA-DR1 molecule [93]. Three 10 μg inoculations of a recombinant SEB with L45R/Y89A/Y94A mutations administered i.m. were found to be well-tolerated and highly immunogenic in mice. The vaccine protected against a challenge of up to 30 LD50 and no evidence of cytokine induction was noted [90]. In the same study, monkeys given 2 inoculations of 20 μg of the recombinant mutant SEB (rmSEB) vaccine were completely protected against an aerosol challenge of 30 LD50 of wild-type SEB. Mice inoculated orally or nasally with the same rmSEB vaccine administered with a cholera toxin immunoadjuvant elicited comparable SEB-specific IgA and IgG levels in their saliva [94]. Nasal or oral inoculations also generated SEB-specific serum IgA, IgG, and IgM levels. However, the nasal route yielded higher specific IgG titers and offered superior protection against an i.p. challenge of 30 LD50. Rhesus monkeys vaccinated 3 times intramuscularly (i.m.) with 20 μg of the rmSEB vaccine developed significant antibody titers and were fully protected from an aerosol challenge of 75 LCT50 of SEB and no inflammatory cytokine or thermogenic responses were noted [64]. Seven day old piglets orally immunized 4 times with 1 mg of rmSEB with, or without, 100 μg of cholera toxin adjuvant elicited significant SEB-specific serum IgG and fecal IgA levels at 36 days post-immunization [95]. Interestingly, vaccine formulations with the cholera toxin adjuvant did not result in an increased antibody response as compared to rmSEB alone.

The recombinant triple mutant SEB antigen, now denoted STEBVax, was scheduled to begin a National Institute of Allergic and Infectious Diseases (NIAID) sponsored FDA Phase I clinical trial (Phase I Study of the Safety and Immunogenicity of Recombinant Staphylococcal Enterotoxin B Vaccine (STEBVax) in Healthy Adults) in January 2011. The study was suspended in December 2011 due to stability issues present in the aging production lots. A new cGMP production lot of the STEBVax is being developed at the Walter Reed Army Institute of Research in anticipation of a new NIAID sponsored clinical trial scheduled for the summer of 2012 [96].

19.4.3 Therapeutic Interventions

19.4.3.1 Immunotherapy

While vaccination with attenuated SEB mutants has been demonstrated to elicit protective immunity, immunization of the general public is not currently warranted, would be largely cost-prohibitive and not necessary for individuals that possess natural immunity [97], [98]. A number of studies have shown that Abs can protect against SEBILS in a variety of animal models and that passive immunization can potentially be an effective post-exposure therapeutic intervention. Furthermore, recent reports suggest that the synergistic effects observed when using two or more anti-SEB Monoclonal Antibodies (MAbs) could potentially lower the therapeutic indices of the antibodies. The crystal structure of SEB in complex with MHC-II and TCR regions indicates these two binding sites are spatially distinct and that their binding interface regions possess multiple, potentially immunogenic epitopes that might facilitate the effectiveness of combinatorial MAbs for therapeutic purposes.
There are currently no FDA-approved therapies available for treating enterotoxin-induced shock. Clinical studies suggest administration of Intravenous polyspecific Immunoglobulin G (IVIG) as adjunctive therapy [99], [100]. However, there are limited supply of this therapeutic and there is little comprehensive clinical trial data [101], [102]. Leclaire reported that 150 µg of an immunopurified anti-SEB Human Globin incubated with either 2 or 20 mouse LD₅₀s of SEB and injected i.p. into mice afforded 100% and 25% protection after 10 hours, respectively [103]. Passive immunization using immunopurified chicken anti-SEB IgY was evaluated in Rhesus monkeys [104]. Four animals each were given the anti-SEB IgY 20 min prior to, or 4 hours after, an aerosol challenge of 5 LD₅₀ of SEB. Both sets of animals survived the challenge, indicating that passive immunization against SEB is efficacious in the rhesus monkey model and that a post-exposure window of opportunity for therapeutic interventions exists. Passive immunization with pooled sera from mice vaccinated with recombinant SEB Q43P, F44P, or L45R mutants was shown to completely protect mice against a 2.5 µg challenge of SEB [92]. The potential therapeutic applications of human MAbs derived from a phage display library screened with STEBVax was determined using a mouse antibody neutralization assay in an LPS potentiated toxic shock model [105]. Approximately 2.5 µg of SEB was incubated with 10 µg of either HuMAb, or their derived Fabs, injected i.p. into BALB/c mice and observed for 72 hours; one of the antibodies was found to offer 68% protection. There are increasing reports of two or more antibodies being used in combination to attain a synergistic therapeutic effect. Tilahun generated a pair of non-cross reacting chimeric antibodies consisting of murine VL and VH segments and human Igk and IgG1 that when administered together displayed a synergistic effect in an HLA-DR3 mouse spleenocyte neutralization assay [106]. In a follow up study, the human-mouse chimeric MAbs in were tested in a more robust in vitro mouse model [107]. Approximately 1 mg of MAbs 82 M or Ch 63 with 50 µg of SEB delivered i.p. were found to confer 66% protection in HLA-DR3 mice; however, co-administration with equivalent amounts of the combined MAbs provided 100% protection. Human MAbs derived from B cells of individuals with anti-SEB titers were tested in an LPS-potentiated mouse toxin neutralization assays [108]. Approximately 500 µg of HuMAb-154 pre-mixed with SEB injected i.p. was found to confer complete protection against a challenge of 10 µg of i.p. SEB in an LPS potentiated mouse model. Varsheny reported a combinatorial effect of murine IgG1MAbs in two different mouse models [109]. Approximately 100 µg of MAb 20B was shown to confer 100% protection when incubated with 20 µg of SEB and injected i.p. in BALB/C mice potentiated with 25 mg D-Galactosamine. However, when used in combination with either MAbs 6D3 or 14G8, the total equivalent effective dose was lowered to 50 µg. Interestingly, the combination of two non-protective MAbs provided 60 – 100 % protection from SEB-induced lethal shock in HLA-DR3 mice where monotherapy with either MAb alone provided little or no protection.

19.4.3.2 Receptor-Toxin Interaction Inhibitors

The well-characterized structural information describing the interactions between SEB, MHC II and TCR has allowed the development of peptide antagonists to block the initial step of receptor-toxin interaction. Lehnhert reported that a DRα1-(GSTAPPA)₂ linker-TCRVβ3 chimeric receptor mimic competes with cell surface receptors for SEB, binds the toxin at micromolar affinity, and prevents SEB-induced IL-2 and T-cell proliferation [110]. A follow up study compared the SEB-receptor mimic with two additional mimics designed to be specific for TSST-1 and SEC3 by substituting TCRVβ3 domain with TCRVβ2 and Vβ8.2, respectively [111], [112]. Each receptor mimic was found to block SAg IL-2 and T-cell proliferation responses in a toxin-specific manner, suggesting the DRα1-linker-TcRVβ chimeras may be developed as a therapeutic intervention against staphylococcal SAgs. One potential drawback of this approach is that individual chimeras have to be constructed as TCR Vβ domains are different for each SE. Buopane reported that a mutant T-cell receptor β sub-unit, denoted G5-8, produced by several rounds of directed evolution, had an affinity of 48 pM to SEB as determined by Surface Plasmon Resonance (SPR), representing a 3-million-fold increase relative to the wild-type sub-unit [113]. Rabbits administered an i.v. combination 5 µg SEB per kg body weight and 500 µg/kg G5-8 followed 4 hours later with 0.15 µg/kg LPS (100 times the rabbit LD₅₀) displayed no adverse physiological
effects. Additionally, the G5-8 protein delivered two hours post-intoxication with 5 μg/kg SEB was shown to ameliorate fever and provide complete protection.

Short peptides encoding conserved SEB domains have also been developed to target the toxin-receptor interaction to ameliorate T-cell activation. A variant of the peptide antagonist encoding SEB residues 150 – 161, denoted p12, displayed potential broad-spectrum anti-toxin activity via reducing the induction of IL-2 mRNA in human PBMCs by SEB, SEA, SPEA or TSST-1 [114]. The p12 peptide delivered i.v. 30 min prior to a lethal challenge with 20 μg SEB provided 100% protection in BALB/C mice potentiated with D-galactosamine. Survivors were also cross-protected against SPEA and TSST-1. In a follow up study, it was shown that peptide antagonist p12 derivative, denoted p14, administered 30 min prior to an SEB challenge in mice also offered complete protection from a lethal challenge of SEB. Interestingly, p14 was not only protective when delivered prior to an SEB challenge, but was able to rescue a partial, but significant, number of mice undergoing lethal shock even when injected as late as 7 hours after the toxin delivery [115]. A subsequent study repeated the SEB challenge and characterized the p12 variant survivor’s antibody IgG and IgM responses and reported that even prior to cross-challenges with other BASgs, the mice displayed antibodies specific to SEA and TSST-1 [116]. Blocking induction of a cellular immune response leading to lethal toxic shock is believed to allow the superantigen to induce a vigorous humoral immune response that results in protective immunity.

A dodecapeptide encoding highly conserved regions of SEA/SEB/SEC amino acid residues 72 – 86 used at 750 μg/ml was shown to inhibit IL-2, INF-γ and TNF-β responses by 87%, 84% and 65%, respectively, in human PBMCs [117]. Two inoculations of the peptide administered in mice potentiated with LPS and D-galactosamine provided 90% protection against 0.2 μg of SEB. Interestingly, this peptide also provided 100% protection against the same amounts of SEA and SEC as well.

19.4.3.3 Cytokine Induction Inhibitors

In addition to the toxin-receptor binding antagonists, research efforts aimed at reducing or eliminating the AGs pro-inflammatory response have also yielded promising results and that perhaps include a post-intoxication intervention. The myeloid differentiation primary response gene 88, or MyD88, is an adaptor protein that is part of a complex signalling pathway that eventually induces transcription factors and cytokines that are part of the SE pro-inflammatory response. The conserved BB loop of the MyD88’s Toll Interleukin-1 Receptor (TIR) domain was used as a template to design three mimetic compounds evaluated for their ability to ameliorate an SEB-induced inflammatory response [118]. Compound 1 inhibited SEB-induced TNF-α, IFN-γ, IL-6 and IL-1β in a dose-dependent manner in PBMCs. Administration of 6 μg of this same compound at 30 min prior to a challenge of 0.25 μg of SEB in an LPS potentiated mouse challenge study provided up to 88% protection. A different approach to ameliorating the inflammatory response targeted the nuclear import of Stress-Responsive Transcription Factors (SRTFs) [119]. Many of the genes that encode the inflammatory response are under control of the SRTFs, such as Nuclear Factor κB (NFκB) [120], which act in concert to increase the transcriptional activity of multiple genes, including cytokines, chemokines and other inflammatory-related mediators. A chimeric peptide consisting of three individual domains:

a) An SRTF nuclear import inhibitor;

b) A membrane translocating motif; and

c) A Nuclear Localizing Signal (NLS) localized to T-cells inhibited the expression of inflammatory cytokines with 50% Effective Concentration (EC50) of 20 μM in SEB-stimulated primary splenocyte cultures.
The tripeptide administered i.p. into wild-type BALB/c mice before and after a D-Galactoasmine primed challenge of 5 μg of SEB significantly decreased serum TNFα, IFNγ and IL-6 levels. The protocol was replicated using transgenic C57BL/6 mice expressing IkB.DN in the T-cell lineage that inhibits NFκB and was also found to significantly attenuate inflammatory cytokine responses. However, 67% of the transgenic mice survived the challenge while only 17% of the wild-type mice survived re-enforcing the role of NFκB in the SEB-mediated inflammatory response.

Development of drugs to ameliorate the effects of SEB has largely been focused on immunomodulatory compounds that can reduce the pro-inflammatory response. Rapamycin is an FDA-approved drug that is an immunosuppressant used to prevent rejection in organ transplant surgeries. Krakauer reported that 6.9 μg/ml rapamycin blocked production of TNF-α (77%) IL1β (97%), IL-6 (67%), IL-2 (100%) and IFN-γ (100%) in human PBMCs exposed to 200 ng/ml SEB [121]. Using a murine “double tap” in vivo model, rapamycin was demonstrated to protect mice completely from an SEB challenge, even when delivered 5 hours post-exposure. Analysis of murine serum cytokines in rapamycin-SEB treated mice indicated a reduction of peak levels of MCP-1 and IL-6 by 90% and 80%, respectively, over control animals. Lactoferrin is a multi-functional glycoprotein with bacteriostatic and bactericidal activities that has been reported to protect against septic shock induced by LPS or Gram-negative infection in several different animal models [122]-[124]. Hayworth reported that bovine Lactoferrin (bLF)-inhibited SEB-induced T-cell proliferation and IL-2 production in HLA-DE4-IE splenocyte cultures. Additionally, bLF inhibited cytokine secretion in human PBMCs as well as human Jurkat cells, an SEB non-responsive cell line, engineered to express the Vβ17 chain of human TCR [125]. Tigecycline, a potent glycycline, shown to be effective against a wide range of bacteria, was also shown to suppress a wide range of cytokines and chemokines [122]. Human PBMCs incubated simultaneously with 200 ng/ml of SEB and 0.5 mM tigecycline were shown to have ≥ 95% reduction in IL-1β, TNF-α and IL-6 levels and also produced a 99% reduction in chemokines MIP-1α and MIP-1β. Tigecycline was also shown to inhibit T-cell proliferation in PBMC by 85% when used at a concentration of 0.1 mM [126]. In an effort to predict and effectively target convergence points in the multiple signal transduction pathways initiated by SEB, 5-Lipoxygenase (5-LO) was identified as a potential pathway interconnector [127]. It was shown that 5-LO inhibitor MK591 at 20 μM inhibited SEB-induced T-cell proliferation in human PMBCs by 85%. Monkey PMBCs incubated with 15 μg/kg SEB and 20 mM/kg MK591 were shown to have a 55% decrease in T-cell proliferation rates and a 62% decrease in TNF-α levels compared to sham control subjects. Pirfenidone [5-methyl-1-phenyl-2-(1H)-pyridone], an anti-fibrotic agent, was shown to protect mice from endotoxin-induced shock and dramatically decreases TNF-α concentrations in endotoxin-treated macrophage cultures [128]. Pirfenidone at 520 μg/ml was found to reduce levels of IFN-γ, IL-1β, and IL-6 by 67%, 100% and 50%, respectively in human PBMCs [129]. In the same study, 200 mg/kg pirfenidone completely protected mice up to 3 hours after either an i.p. challenge of 3 MLD50 of SEB in an LPS potentiated model or 3.5 hours after an aerosol challenge of 7 MLD50.

19.5 CONCLUSIONS

*S. aureus* is recognized as a particularly complex, virulent and successful pathogen. The bacterial superantigens produced by *S. aureus* represent some of the most powerful mitogens ever characterized. Picomolar concentrations of these protein toxins are capable of inducing an uncontrolled immunological response characterized by T-cell proliferation and significant increases in cytokine concentrations. SEB, a toxin often associated with food poisoning, was investigated by the US as an incapacitating agent during the 1960s and is currently a category B select agent. The potential of SEB to be employed in a biological terrorism event by food, water or aerosol dispersion has created a need for effective medical countermeasures. There are currently no licensed prophylactic or therapeutics for use against SEB. A recombinant, SEB antigen, devoid of native activity by the mutation of key amino acid residues, has shown promise in small animal and NHP studies and is currently
in Phase I clinical trials. The use of high resolution structural analysis has allowed the characterization of SEB binding interactions with receptors in the immune system and aided in the design of therapeutic molecules. This has made possible the design of specific peptide-based mimics that interrupt the normal SEB-immune receptor binding and block the inflammatory disease response.

19.6 REFERENCES


MEDICAL INTERVENTIONS
AGAINST STAPHYLOCOCCAL ENTEROTOXIN B

toxin superantigen gene profiles of Staphylococcus aureus associated with subclinical mastitis in dairy
 cows and relationships with macrorestriction genomic profiles, J Clin Microbiol, Vol. 43 (2005) 1278-
1284.

705-711.

[17] Larkin EA, Carman RJ, Krakauer T and Stiles BG. Staphylococcus aureus: the toxic presence of a

[18] Vasconcelos NG and Cunha ML. Staphylococcal enterotoxins: Molecular aspects and detection methods,


14-56.


[24] Huang IY and Bergdoll MS. The Primary Structure of Staphylococcal Enterotoxin B, J Biol Chem,

[25] Huang IY, Hughes JL, Bergdoll MS and Schantz EJ. Complete Amino Acid Sequence of Staphylococcal

[26] Swaminathan S, Furey W, Pletcher J and Sax M. Crystal structure of staphylococcal enterotoxin B,

[27] Papageorgiou A, Tranter HS and Acharya KR. Crystal structure of microbial superantigen staphylococcal
enterotoxin B at 1.5 Å resolution: Implications for superantigen recognition by MHC class II molecules

[28] Hovde CJ, Marr JC, Hoffmann ML, Hackett SP, Chi Y, Crum KK, Stevens DL, Stauffacher CV and
Bohach GA. Investigation of the role of the disulphide bond in the activity and structure of staphylococcal

MEDICAL INTERVENTIONS AGAINST STAPHYLOCOCCAL ENTEROTOXIN B


MEDICAL INTERVENTIONS AGAINST STAPHYLOCOCCAL ENTEROTOXIN B


Disclaimer: The views and opinions expressed in this paper are those of the author(s) and do not reflect official policy or position of the Department of the Army, Department of Defense, or the U.S. Government.
Chapter 20 – BIOLOGICAL SURETY

Samuel S. Edwin¹, Virginia I. Roxas-Duncan², America M. Ceralde³, Shelley C. Jorgensen⁴ and Neal E. Woollen⁵

1: Responsible Official and Surety Officer; Chief, Biosurety Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; 2: Alternate Responsible Official and Chief, Select Agent Management Branch, Biosurety Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; 3: Alternate Surety Officer and Chief, Personnel Reliability Program Branch, Biosurety Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; 4: Lieutenant Colonel, Medical Services; Chief, Surety Branch, U.S. Army Office of The Surgeon General/U.S. Army Medical Command, G-34 Protection Division, Falls Church, VA; 5: Lieutenant Colonel, Veterinary Corps; Director, Biosecurity Directorate, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

UNITED STATES

Corresponding Author (Edwin): samuel.s.edwin.civ@mail.mil

20.1 INTRODUCTION

Evidence of human engagement in chemical and biological warfare to terrorize individuals or opposing armies and concurrent efforts to reduce these threats date back to the dawn of civilization. Some of the more prominent reports of possible biological warfare from the past millennium include the poisoning of enemy water wells with rye ergot fungus, a hallucinating agent, by the Assyrians; the use of hellebore roots to poison the drinking water of Kirrh by Solon of Athens (600 BCE); the use of poison arrows dipped in gangrene- and tetanus-causing agents by the Scythian archers of the Trojan war (400 BCE); tossing of venomous snakes onto the opponent ships of Pergamus by Hannibal at Eurymedon (190 BCE); hurling decomposing human bodies into enemy water wells by Emperor Barbarossa at the battle of Tortona (1155); catapulting the cadavers of plague victims over the city walls of Caffa (now Feodosia, Ukraine) by the Tartars (1346); and sale of clothing from yellow fever and smallpox-infected patients by Confederate soldiers to unsuspecting Union troops during the American Civil War [1]-[5]. Causative agents were linked to infectious diseases by 19th-century scientists Louis Pasteur and Robert Koch. Advances in the field of microbiology soon led to the isolation of microbial agents from diseased humans and animals. Moreover, the development of in vitro methods to grow these pathogens in large scale gave those interested in biological weapons a new perspective in selecting an agent based on its ability to cause fear, disease, and mass casualties.

Recognizing the destructive powers of war, especially the devastation caused by chemical and biological weapons, developed nations of the world have attempted to establish international rules of engagement by drafting treaties and declarations that primarily focused on disarmament, laws of war, and war crimes (Table 20-1). The 1st International Peace Conference in 1899 at The Hague, Netherlands, produced the Prohibition of the Use of Projectiles with the Sole Object to Spread Asphyxiating Poisonous Gases [6]. Ratified by all major powers except the United States of America, this declaration states that in any war between signatory powers, the parties will abstain from using projectiles, “the sole object of which is the diffusion of asphyxiating or deleterious gases.” The 2nd International Peace Conference held in 1907 prohibited the use of poisons and weapons with poisons [7]. The major accomplishment of these peace conferences was the establishment of an international court for mandatory arbitration and dispute settlement between nations.
Table 20-1: Timeline of International Rules and Treaties to Limit or Ban Chemical and Biological Weapons Use.

<table>
<thead>
<tr>
<th>Year</th>
<th>Significant Event</th>
</tr>
</thead>
</table>
| 1899 | 1st International Peace Conference  
Prohibition of the Use of Projectiles to Spread Asphyxiating Poisonous Gases |
| 1907 | 2nd International Peace Conference  
Prohibition of the Use of Poisons and Weapons With Poisons |
| 1925 | The Geneva Protocol  
Prohibition of Germ (Biological) and Chemical Warfare |
| 1972 | Biological Weapons Convention  
Prohibition of Development, Production, and Stockpiling of Biological Weapons |
| 1986 | The Second Review Conference  
Establishment of Confidence Building Measures |

Despite the declaration prohibiting projectiles that spread poisonous gases, biological weapons were not unequivocally prohibited. The advent of World War I (WWI) led to rapid progression of chemical and biological weapons, particularly those that were developed and used by the German Army. Various chemical weapons were used extensively during WWI primarily to demoralize, injure, and kill entrenched enemies indiscriminately. These ranged from disabling tear gas to deadly phosgene and chlorine gases. Due to the widespread use of chemical weapons and rapid development of high-explosive agents during this war, WWI is often referred to as “The Chemists’ War.” With advances in the understanding of bacterial agents during the 19th century, the German Army launched a massive biological weapons campaign against the Allied Forces during WWI. However, instead of targeting humans, they concentrated on infecting livestock (horses and mules) with *Bacillus anthracis* and *Burkholderia mallei*. Several animals died from these infections, but these biological tactics failed to match the success of the chemical warfare efforts [8].

After the end of WWI and with no lasting peace in sight, the Biological Weapons Convention developed the “Protocol for the Prohibition of the Use in War of Asphyxiating, Poison or Other Gases and the Bacteriological Methods of Warfare,” signed in 1925 at Geneva, Switzerland, as an extension of the international peace conferences of 1899 and 1907. Also known as the “Geneva Protocol,” this treaty permanently bans the use of all forms of chemical and biological warfare. However, it did not prohibit the use of biological or chemical agents for research and development, storage, and transfer. Many countries that signed on to the Geneva protocol retained the right to retaliate against biological or chemical weapon attacks with their own arsenals. Treaties, declarations, and protocols produced by the international community continued to lack robust verification methods, leading to distrust among nations and reinvigoration of chemical and biological weapons programs prior to World War II (WWII). Several countries initiated biological warfare programs between the World Wars. The first scientifically informed use of biological agents as weapons began when the Japanese military conducted human experimentation with several infectious agents during combat, targeting military personnel and civilians in Manchuria and China [1], [2], [9]. During WWII, many countries, including the United States, Canada, United Kingdom, Germany, Japan, and the Soviet Union had active bioweapons programs with stockpiles of military significance. The Japanese military used biological weapons, killing tens of thousands of civilians and military [1], [2], [9]-[12].
In 1972, U.S. President Richard M. Nixon made the decision to abandon biological weapons research and signed the Biological Weapons Convention, the first multi-lateral disarmament treaty banning development, production, and stockpiling of biological weapons. The U.S. destroyed all biological weapon stockpiles and made the facilities that produced these weapons inoperable. Participant Nations in the 2nd Review Conference in 1986 agreed to implement a number of confidence-building measures to prevent ambiguities, doubts, and suspicions and to improve international collaboration toward peaceful biological research [13].

In 1995, an extremist microbiologist was arrested for obtaining *Yersinia pestis* by mail order in the United States. Concern about the ease with which disease-causing agents could be obtained led the U.S. Congress to pass the Antiterrorism and Effective Death Penalty Act of 1996 [14]. This act directed the U.S. Department of Health and Human Services (HHS) to establish:

a) A list of biological agents and toxins (“select agents”) that pose significant threat to public health and safety;

b) Procedures for regulating the transfer of these agents; and

c) Training requirements for entities working with these agents.

HHS delegated this authority to the Centers for Disease Control and Prevention (CDC) to establish the Laboratory Registration and Select Agent Transfer Program in 1996. Congress significantly increased the oversight of Biological Select Agents and Toxins (BSAT) following the anthrax attacks of 2001 by passing the USA PATRIOT Act (Uniting and Strengthening America by Providing Appropriate Tools Required to Intercept and Obstruct Terrorism Act of 2001) [15], which restricted access to BSAT, and the Bioterrorism Act (Public Health Security and Bioterrorism Preparedness and Response Act of 2002) [16], which included increased safeguards, security measures, and oversight of the possession and use of BSAT. The Bioterrorism Act also granted similar regulatory authority to the U.S. Department of Agriculture (USDA) over select agents that pose severe threat to animal and plant health or products [17]. This led to the establishment of the Federal Select Agent Program (FSAP).

The FSAP consists of the CDC Division of Select Agents and Toxins (CDC-DSAT) and the Animal and Plant Health Inspection Services (APHIS) Agricultural Select Agent Program that oversee the possession, use, transfer, and destruction of BSAT that has the potential to pose severe threat to public, animal, or plant health or to animal or plant products within the United States. This chapter details the key concepts of the FSAP and U.S. Department of the Army’s (DA’s) Biological Surety Program (BSP) and highlights how implementation protects the worker, the community, and the environment.

### 20.2 BIOLOGICAL SURETY

Biological surety, or “biosurety,” is a Department of Defense (DoD) program for Commanders and directors to implement and monitor judicious application of core principles pertaining to control of BSAT, biosafety and occupational health, personnel reliability, biosecurity, and emergency response in all military laboratories involved in developing medical countermeasures to BSAT for service members and the public. The principles of safety, security, agent accountability, personnel reliability, and incident response plans formulated by chemical and nuclear surety programs were instrumental during the development of the DA’s biological surety regulations [18]. Certain infectious agents and toxins, designated as BSAT, have the potential to pose a severe threat to public health and safety, animal or plant health, or animal or plant products, and their possession, use, and transfer are regulated by the HHS and the USDA under the Select Agent Regulations. In addition, research involving recombinant or synthetic nucleic acid molecules, including the creation and use of organisms and
viruses containing recombinant or synthetic nucleic acid molecules, is regulated by National Institutes of Health (NIH) Office of Biotechnology Activities. The intent of the DoD BSP is to properly safeguard BSAT that is in the possession or custody of DoD facilities against theft, loss, diversion, or unauthorized access or use, and to ensure that operations involving such agents are conducted in a safe, secure, and reliable manner per regulatory requirements.

The CDC-DSAT and APHIS Agriculture Select Agent Services monitor compliance of registered entities to HHS- and USDA-published final rules, outlined in 42 CFR Part 73 [19], 7 CFR Part 331 [20], and 9 CFR Part 121 [21]. One of the key components of the BSP that was unique to the DoD is the Biological Personnel Reliability Program (BPRP), which ensures that individuals with access to BSAT meet high standards of reliability and suitability. Recent updates to FSAP regulations require individuals with access to Tier 1 BSAT (Exhibit 1) be enrolled in a “suitability” program similar to the DA’s BPRP program. With this change, the FSAP and the BSP correspondingly enhance the safety of individuals working with BSAT, protect and safeguard communities with biocontainment laboratories, and monitor the security of BSAT in entities registered and authorized to work with these agents and toxins (Figure 20-1).

<table>
<thead>
<tr>
<th>Exhibit 1: List of Tier 1 Biological Select Agents and Toxins.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botulinum neurotoxins</td>
</tr>
<tr>
<td>Botulinum neurotoxin producing species of Clostridium</td>
</tr>
<tr>
<td>Ebola virus</td>
</tr>
<tr>
<td>Francisella tularensis</td>
</tr>
<tr>
<td>Marburg virus</td>
</tr>
<tr>
<td>Variola major virus (smallpox virus)</td>
</tr>
<tr>
<td>Variola minor virus (alastrim)</td>
</tr>
<tr>
<td>Yersinia pestis</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
</tr>
<tr>
<td>Burkholderia mallei</td>
</tr>
<tr>
<td>Burkholderia pseudomallei</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus</td>
</tr>
<tr>
<td>Rinderpest virus</td>
</tr>
</tbody>
</table>

20.2.1 Control of Biological Select Agents and Toxins

In accordance with 42 CFR Part 73 [19], the CDC-DSAT regulates agents and toxins that pose a severe threat to public health and safety. The APHIS Agriculture Select Agent Services regulates biological agents that pose a significant threat to plant and plant products in accordance with 7 CFR Part 331 [20]. Agents that cause severe threat to humans, animals, and animal products are known as the “overlap agents” and are regulated by the CDC-DSAT and APHIS Agriculture Select Agent Services in accordance with 9 CFR Part 121 [21]. In 2010, U.S. President Barack Obama, through Executive Order 13546 [22] directed HHS and USDA to:

a) Designate a sub-set of BSAT (Tier 1 [23], see Exhibit 1) that presents the greatest risk of deliberate misuse with the most significant potential to cause mass casualties or devastating effects to the economy, critical infrastructure, or public confidence;

b) Explore options for graded protection of Tier 1 BSAT to permit tailored risk management practices based on relevant contextual factors; and

c) Consider reducing the overall number of agents and toxins on the select agents list.

Federal BSAT regulations (42 CFR Part 73, 7 CFR Part 331, and 9 CFR Part 121) have been revised in accordance with Executive Order 13546 [20]-[22].
The FSAP mandates the appointment of a Responsible Official (RO) and an Alternate Responsible Official (ARO) within each registered entity to monitor compliance with the regulations governing Select Agents and Toxins (SATs). Entities are authorized to appoint multiple AROs. The RO is granted authority and control to ensure compliance with FSAP regulations. In the absence of the RO, the ARO monitors entity compliance to FSAP regulations. In the DoD, a unit Commander with a mission to conduct BSAT work (e.g. development of diagnostics, medical countermeasures) appoints an RO to monitor compliance of the entity to DoD, Army, federal, state, and local regulations governing BSAT. Regulatory oversight on entities that have a need to possess, use, and transfer BSAT is initiated by submission of various CDC APHIS forms that are specific for each regulatory component (Exhibit 2) [24].

Exhibit 2: Animal and Plant Health Inspection Service/Centers for Disease Control and Prevention Forms.

- APHIS/CDC Form 1: Application for Registration for Possession, Use, and Transfer of Select Agents and Toxins
- APHIS/CDC Form 2: Request to Transfer Select Agents and Toxins
- APHIS/CDC Form 3: Report of Theft, Loss, or Release of Select Agents or Toxins
- APHIS/CDC Form 4: Report of the Identification of a Select Agent or Toxin
- APHIS/CDC Form 5: Request for Exemption of Select Agents and Toxins for an Investigational Product

CDC: Centers for Disease Control and Prevention.

20.2.2 Registration for Possession, Use, and Transfer of Biological Select Agents and Toxins

The FSAP requires all individuals, laboratories, and entities to register for possession, use, and transfer of BSAT. The first step in this process involves providing information through the completion of APHIS/CDC Form-1, Registration for Possession, Use, and Transfer of Select Agents and Toxins [25]; as described in 7 CFR 331 [20], 9 CFR 121 [21], and 42 CFR 73 [19]. This form consists of several sections targeted to provide the regulatory agency with critical information on the biocontainment facility, safety, security, personnel, training, and research plans using SAT.

The entity is physically inspected by the FSAP following submission of the completed APHIS/CDC Form 1. The primary focus of this inspection is compliance with applicable federal regulations governing BSAT (7 CFR 331 [20], 9 CFR 121 [21], and 42 CFR 73 [19]). During this visit, the inspectors verify the information provided in the submitted APHIS/CDC Form 1; evaluate personnel training, including mentorship programs; conduct interviews of personnel to identify issues related to biosafety, biosecurity, and training programs; check the engineering controls supporting the containment suites; and corroborate the commissioning or service records of all supporting machinery, including air-handling units, breathing-air systems, validation data for autoclaves, and all inactivation procedures to ensure that proper parameters are met and the methods used are determined to be efficacious with respect to producing non-viable waste. Ideally, entity registration is granted for 3 years after all inspection observations are satisfactorily resolved. However, a “conditional” registration may be granted...
under special circumstances (e.g. during the interim when the entity needs to be operational to generate the data to satisfy a requirement). The FSAP inspectors ensure that the workers, communities, and the environment are not harmed by the operation of a containment or high containment laboratory.

APHIS/CDC Form 1 is also used to request changes to an approved registration. The entity must submit a letter to the FSAP requesting amendment to its registration and furnish the revised sections of the APHIS/CDC Form 1 related to the modifications. Most common amendments to registration involve addition and removal of personnel, name changes, addition or removal of agents or toxins, and changes in statement of work, including changes in project design, agent strains, animal models, modes of agent administration, and new laboratory projects.

### 20.2.3 Security Risk Assessment

Security Risk Assessment (SRA) is the method used to approve an individual for access to select agents or toxins in accordance with the USA PATRIOT Act of 2001 and the Public Health Security and Bioterrorism Preparedness and Response Act of 2002. The Federal Bureau of Investigation Criminal Justice Information Services division determines if an individual who has been identified by a registered entity as having a legitimate need to access a select agent or toxin meets one of the statutory restrictors that would restrict access.

A “restricted person” under 18 USC 175b (USA PATRIOT Act) is an individual [26] who:

- Is under indictment for a crime punishable by imprisonment for a term exceeding 1 year or who has been convicted in any court of a crime punishable by imprisonment for a term exceeding 1 year;
- Is a fugitive from justice;
- Is an unlawful user of any controlled substance;
- Is an alien illegally or unlawfully in the United States;
- Has been adjudicated as a mental defective or has been committed to any mental institution;
- Is an alien (other than an alien lawfully admitted for permanent residence) who is a national of a country as to which the secretary of state has made a determination (that remains in effect) that such country has repeatedly provided support for acts of international terrorism; or
- Has been discharged from the armed services of the United States under dishonorable conditions.

All individuals, including the RO, AROs, laboratory research staff, and animal-care workers requesting unescorted access to CDC- or APHIS-registered spaces containing BSAT require an approved SRA. Escorted individuals, such as inspectors and visitors with no access to BSAT, do not require an approved SRA. FSAP works closely with the Federal Bureau of Investigation Criminal Justice Information Services division to identify individuals who are prohibited to access BSAT based on the restrictions identified in the USA PATRIOT Act of 2001 [15]. This process involves submitting an amendment to the lead agency (CDC or APHIS) and adding the individual to the entity registration to obtain a unique Department of Justice number, which is recorded on a Bioterrorism Security Risk Assessment Form (FD-961). The completed FD-961 is reviewed, certified by the RO, and submitted to CJIS with two sets of fingerprints [26]. The FSAP authorizes individual access to BSAT based on the results of the SRA. The SRA is renewed every 3 years. All individuals with approved SRA undergo a general initial training, which provides site-specific information on biosafety, security, incident response, and insider threat awareness. Refresher training is provided annually to all SRA-approved individuals.
20.2.4 Biological Select Agents and Toxins Inventory and Accountability

FSAP regulations require complete, current, and accurate inventory of all Long-Term (LT) BSAT. Materials that contain or have been exposed to infectious select agents, including (but not limited to) laboratory cultures, animals, animal tissues, confirmed clinical specimens, plants, and plant tissues, are subject to FSAP regulations. Select toxins and recombinant or synthetic nucleic acids encoding functional forms of select toxins are also regulated. Animals inoculated with select toxins and their tissues are exempt from FSAP regulations. Inventory records are not required for BSAT that the FSAP has excluded from the provisions of the Select Agent Regulations, nor for inactivated BSAT materials as long as an approved method for inactivation is used.

CDC-DSAT defines LT storage as placement in a system designed to ensure viability for future use. As a rule, LT BSAT materials are not part of an ongoing experiment and have not been accessed for a significant period of time (e.g. 30 calendar days) [27]. SAT are considered Working Stock (WS) if the materials are:

a) A part of an ongoing experiment;

b) Accessed frequently; or

c) Not stored for an extended period of time.

FSAP regulations do not require inventory records for BSAT classified as WS; however, all WS must be kept and used in secure locations by approved individuals (i.e. those with current SRAs enrolled in a suitability program, if accessing Tier 1 agents). The DA’s interim guidance on BSAT inventory management allows BSAT to remain in WS status for up to 180 days; however, the DA guidance document requires individuals to maintain detailed records of all BSAT WS materials at all times.

Significant amounts of BSAT WS can be generated in a containment laboratory on any given day. Accounting for these materials can be challenging, as they are continuously used or consumed in various experiments. Entities with large BSAT inventories must establish procedures to retain only “valuable” BSAT. Establishing peer-reviewed and accepted criteria for retention and destruction of LT BSAT materials can be beneficial to the investigators and the host entity. An example of criteria developed for retention and destruction of LT BSAT materials is shown in Exhibit 3.

<table>
<thead>
<tr>
<th>Retention Criteria</th>
<th>Destruction Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Unique materials (serotypes, strains, etc.).</td>
<td>1) Potentially contaminated and/or degraded materials (e.g. samples that have been subjected to multiple freeze/thaw cycles).</td>
</tr>
<tr>
<td>2) Support ongoing research activities and all existing agreements.</td>
<td>2) Excess quantities from a specific microbe or toxin.</td>
</tr>
<tr>
<td>3) High scientific value for future scientific investigations.</td>
<td>3) No anticipated future scientific value with the understanding that projected future mission requirements can be difficult.</td>
</tr>
<tr>
<td>4) Deemed evidence material by law enforcement.</td>
<td>4) Materials that lack expected bioactivity.</td>
</tr>
<tr>
<td>5) Materials retained from published studies.</td>
<td></td>
</tr>
</tbody>
</table>
Specimen boxes containing LT BSAT materials can be wrapped with tamper-evident materials after verification by two BPRP-certified individuals. Follow-up tube-by-tube inventory is not needed as long as the tamper-evident seals remain intact. Reducing access and repeated contact with LT BSAT materials will preserve specimen integrity and will also allow for accurate real-time inventory of these materials. Additionally, uniform labeling of LT BSAT specimens should remain a priority for research staff in order to have well-labeled research materials for all current and future investigations. Advances in labeling technologies permit for human-readable information, barcodes, and radiofrequency identification tags to be incorporated on any size of specimen labels. Specimen tags that adhere to frozen tubes are also available, making it possible to label archival materials.

20.2.5 Centralized Management of Long-Term Biological Select Agents and Toxins

Maintaining accurate and current inventory of LT BSAT materials at all times can be burdensome to Principal Investigators (PIs) and research staff who are focused on meeting timelines for deliverables and project goals. BSAT inventory discrepancies identified during internal audits or announced and unannounced inspections by regulatory agencies can result in serious consequences to the registered entity with respect to continuation of research and loss of public trust. One proposed solution to this dilemma is to establish centralized management of LT BSAT materials under the care of the RO and the AROs to alleviate considerable inventory and accountability burden from the PIs and research staff (Exhibit 4). Under this model, LT BSAT materials that have been verified by a third party would be labeled with PI-specific information, wrapped with tamper-evident materials, and centrally stored within the registered laboratory space in dedicated storage containers with restricted access.

Exhibit 4: Centralized Biological Select Agents and Toxins Inventory Management Considerations.

<table>
<thead>
<tr>
<th>Reduced inventory burden on PI/researcher:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Transfer long-term BSAT accountability responsibility to RO/ARO and dedicated biological surety staff (select agent managers).</td>
</tr>
<tr>
<td>• Limit principal investigator/researcher responsibility to working stock BSAT materials.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enhanced accountability and security:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Manage long-term BSAT materials with dedicated staff:</td>
</tr>
<tr>
<td>• 100% long-term BSAT inventory verification and tamper-evident wrapping.</td>
</tr>
<tr>
<td>• Long-term BSAT consolidated within registered space.</td>
</tr>
<tr>
<td>• Eliminate variability in record keeping from multiple PIs/researchers.</td>
</tr>
<tr>
<td>• Enhance security of BSAT materials:</td>
</tr>
<tr>
<td>• Long-term BSAT in dedicated and locked freezers within registered spaces.</td>
</tr>
<tr>
<td>• Limit physical access to long-term BSAT materials.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Manageable process with economy of space and personnel:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Enhance real-time inventory awareness for long-term BSAT.</td>
</tr>
<tr>
<td>• Consolidate long-term BSAT within containment spaces.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maintained mission capability with enhanced flexibility:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Retain all unique and critical BSAT materials.</td>
</tr>
<tr>
<td>• Capture all essential characterization and experimental data (e.g. DoD BSAT database).</td>
</tr>
<tr>
<td>• Prepared to receive or send BSAT to other DoD entities at all times.</td>
</tr>
</tbody>
</table>
Centralized LT BSAT inventory management would enhance readiness for unannounced regulatory compliance inspections that include BSAT inventory verification, and would simplify the transition of BSAT inventory when a PI retires or leaves the institution. Verification of LT BSAT inventory by the PI or researcher and a third party would also allow for identification of archival specimens requiring new uniform labels. The PI or researcher will identify BSAT specimens no longer needed for current and future investigations, including potentially contaminated specimens, specimens with reduced or no bioactivity, and excess specimens.

20.2.6 Biological Select Agents and Toxins Inventory Audits

Registered entities are required to conduct complete inventory audits of a PI’s BSAT holdings in LT storage during physical relocation of a collection or inventory upon the departure of a registered PI with BSAT holdings, or in the event of a theft or loss of BSAT. In addition to the FSAP requirements, Army Regulation (AR) 50-1 requires annual 100% physical inventory of all BSAT holdings by each PI [28]. If the LT BSAT materials are verified and wrapped, the inventory burden is dramatically reduced, as long as the tamper-evident seals are intact. Army regulation also requires BSAT inventory audits of each registered PI at least once annually by the biological surety program staff [29]. These inventory audits include inspection of laboratory records of BSAT usage, physical inventory verification of both LT storage and WS BSAT, verification of the SAP registration of the PIs, BSAT transfer documentation, and BSAT destruction records. The annual BSAT inventory audits provide a great opportunity to interact with the registered PI and his or her technical staff and to identify areas where additional training may be warranted.

20.2.7 Biological Select Agent and Toxin Transfers

The Select Agent Regulations require entities to develop provisions and policies for shipping, receiving, and storing SAT, including documented procedures for receiving, monitoring, and shipping all SAT. There are primarily two types of BSAT transfers: intra-entity and inter-entity. BSAT material must be packaged by individuals approved by the HHS secretary or APHIS administrator for access to SAT. If the transfer involves Tier 1 BSAT, the approved individuals must be certified in the entity’s suitability program or personnel reliability program.

Intra-entity transfers of SAT are performed between two registered PIs with a complete chain of custody document. The sender and receiver must be registered with the SAP for the BSAT being transferred. These transfers are physically performed by approved individuals in accordance with entity-specific standard operating procedures. An approved APHIS/CDC Form 2 is not required for intra-entity transfer of BSAT materials.

Inter-entity transfers of SAT require an approved APHIS/CDC Form 2 prior to physical transfer of these materials. Once issued, an approved APHIS/CDC Form 2 is valid for 30 days. These transfers are governed by
the U.S. Department of Transportation Hazardous Material Regulations found in 49 CFR, parts 100 to 185 [30].
The approved individual packaging SAT must ensure compliance with all applicable laws concerning packaging
and shipping. DA uses approved BSP personnel trained and certified in shipping procedures to verify the
contents of the SAT shipments inside the containment laboratories prior to packaging. A completed chain of
custody form is retained with copies of shipping documents for at least 5 years (DoD standard). The individual
who witnesses packaging inside the containment laboratory also verifies the approved APHIS/CDC Form 2 and
the shipping documents. The FSAP has amended the Select Agent Regulations to accept and promote the
recommendation of the report of the Defense Science Board Task Force, Department of Defense Biological
Safety and Security Program [31], regarding the “lost in crowd” approach for all SAT shipments. However,
registered DoD laboratories are currently required to use a carrier that maintains positive control, ensures chain
of custody, is certified to handle HAZMAT (Hazardous Materials) Standards 6.1 (poisons) and 6.2 (infectious
substances), and requires two qualified drivers possessing current secret clearance, with at least one driver in the
truck or within 25 feet of the truck at all times [32]. Harmonization of DoD regulations with the FSAP is being
discussed to standardize the select agent and toxin shipping practices.

Exempt quantity (permissible amount) transfers of select toxins (Table 20-2) are not regulated by the FSAP [33].
The “toxin due diligence” provision was developed by FSAP to address concern that someone might stockpile
toxins by receiving multiple orders below the excluded amount. It requires a person transferring toxins in
amounts which would otherwise be excluded from the provisions to:

a) Use due diligence to ensure that the recipient has a legitimate need to handle or use such toxins; and

b) Report to FSAP if they detect a known or suspected violation of federal law or become aware of
   suspicious activity related to the toxin [34].

<table>
<thead>
<tr>
<th>Health and Human Services Toxins</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abrin</td>
<td>100 mg</td>
</tr>
<tr>
<td>Botulinum neurotoxins</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Short, paralytic alpha conotoxins</td>
<td>100 mg</td>
</tr>
<tr>
<td>Diacetoxyscirpenol</td>
<td>1,000 mg</td>
</tr>
<tr>
<td>Ricin</td>
<td>100 mg</td>
</tr>
<tr>
<td>Saxitoxin</td>
<td>100 mg</td>
</tr>
<tr>
<td>Staphylococcal enterotoxins (sub-types A, B, C, D, and E)</td>
<td>5 mg</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>1,000 mg</td>
</tr>
</tbody>
</table>

Centers for Disease Control and Prevention; Animal and Plant Health Inspection Services. Permissible Toxin Amounts.

Most “exempt” toxin transfers are to a non-registered PI or a collaborator who demonstrates a legitimate need to
handle or use the toxin being transferred. Due diligence must precede the transfer to ensure that the recipient
does not exceed the exempt quantity limit established by the FSAP with any existing remnant quantities in their
laboratories from previous investigations. The person initiating the transfer can require the recipient to complete
documentation stating the intended use of the toxins and a statement indicating that receiving the requested amount of the toxin will not put them over the limits established for the select toxins by the FSAP. Tracking “exempt” select toxin transfers and monitoring their use must be an integral part of a due diligence effort at the entity (sending and receiving) level to avoid investigators accumulating quantities of select toxins above the permissible amounts at any time.

20.2.8 Reporting Theft, Loss, or Release of Biological Select Agents and Toxins

FSAP requires an entity to immediately notify CDC or APHIS and appropriate federal, state, or local law enforcement agencies (by e-mail, facsimile, or telephone) of incidents involving theft, loss, or release (occupational exposure or release of an agent or toxin outside of the primary barriers of the bio-containment area) of SAT [35]. Thefts or losses also must be reported even if the SAT is subsequently recovered or the responsible parties are identified. A completed APHIS/CDC Form 3 must be submitted within 7 calendar days.

A BSAT inventory deficiency investigation may involve:

a) Immediate notification to the physical security office;

b) 100% physical inventory of all of the registered PI’s BSAT holdings by the RO or ARO;

c) Complete inspection of the PI’s BSAT usage records (laboratory notes); and

d) A complete database records check of the BSAT inventory holdings of the PI.

If theft of BSAT is suspected, appropriate law enforcement agencies must be informed.

Release of BSAT from “primary containment” or release resulting in “potential exposure” to individuals requires immediate notification to the FSAP. Spills of SAT in Biological Safety Level (BSL)-4 laboratories (sealed laboratories with personnel wearing positive pressure encapsulated suits) can be safely cleaned up without potential human exposure; no FSAP reporting is necessary because the entire BSL-4 laboratory is considered “primary containment.” However, if an individual experiences a breach in his or her positive pressure encapsulating suit at the same time as a spill or work done with animals outside of primary containment, initial notification to FSAP reporting is required, followed by the completion of APHIS/CDC Form 3. In contrast, SAT spills in BSL-2 and BSL-3 laboratories (unsealed directional airflow laboratories with personnel not wearing positive pressure encapsulated suits) outside of a functioning biological safety cabinet are reportable to FSAP, as these laboratory spaces are considered “secondary containment.” The data collected and analyzed by the CDC on theft, loss, or release reporting from 2004 to 2010 indicate that the risk of exposure from BSAT managed by U.S. laboratories to the general population is low [36].

20.2.9 Identifying Select Agents and Toxins

Identifying BSAT as a result of diagnosis, verification, and proficiency testing, and final disposition of the identified agent or toxin must be reported to FSAP within 7 calendar days by completing APHIS/CDC Form 4. Identifying Tier 1 BSAT (see Exhibit 2) from diagnostic samples requires immediate (i.e. within 24 hours) reporting to FSAP via e-mail, facsimile, or telephone. BSAT identified from proficiency testing specimens must be reported within 90 days of receipt of the sample. Any amount of select toxin identified must be reported to FSAP. Entities not registered with the FSAP are also required to report BSAT that have been identified from diagnostic specimens. Unregistered entities have 7 calendar days to transfer to a registered entity or destroy the identified SAT to remain in compliance with current federal regulations.
20.2.10 Restricted Experiments

An individual or an entity approved by the FSAP may not conduct restricted experiments without prior approval by the HHS secretary or APHIS administrator. Restricted experiments are:

a) Experiments that involve the deliberate transfer of, or selection for, a drug resistance trait to select agents that are not known to acquire the trait naturally, if such acquisition could compromise the control of disease agents in humans, veterinary medicine, or agriculture; and

b) Experiments involving the deliberate formation of synthetic or recombinant nucleic acids containing genes for the biosynthesis of select toxin lethal for vertebrates at an LD₅₀ (the amount necessary to kill 50% of the subject population) that is less than 100 ng/kg body weight [19].

Additional guidance on restricted experiments involving recombinant or synthetic nucleic acids is outlined in the NIH’s *Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules* [37]. This guidance is mandated for research that is conducted at or sponsored by an entity that receives any support for recombinant or synthetic nucleic acid research from the NIH, including research performed directly by the NIH.

Most registered entities designate the responsibility of identifying, reviewing, and approving restricted experiments to their Institutional Biosafety Committee (IBC). The biosafety officer and the RO are members of the IBC. Entity-specific IBC-approved research proposals with restricted experiments are forwarded to FSAP for review and approval. Restricted experiments containing HHS and overlap select agents will be further reviewed by the Intra-governmental Select Agents and Toxins Technical Advisory Committee. Restricted experiments involving USDA select agents will be further reviewed by subject-matter experts from APHIS.

A typical request to FSAP to review a restricted experiment includes, but is not limited to, description of:

- The proposed experiment, including intended objectives;
- Nucleic acid insert and the intended biological characteristics of the recombinant gene product;
- Cloning/expression vector;
- Host organism used for molecular cloning;
- Selection methods (recombinant or passive);
- Antimicrobial markers use;
- BSL considerations;
- Estimated amount of toxin (recombinant or synthetic) to be produced (if applicable); and
- Any planned animal or plant experiments [38].

Restricted experiments using recombinant and passive selection methods and all select agent products resulting from these experiments are also subject to FSAP regulations. Transfer of any products of restricted experiments must be coordinated through the FSAP. The DA and the DoD require all of their research laboratories to remain in full compliance with all federal regulations governing BSAT.

20.2.11 Biosafety

Biosafety in microbiological and biomedical laboratories is based on two key principles: “containment” and “risk assessment.” Core concepts of containment include microbiological practices, safety equipment, and facility safeguards that protect laboratory workers, the environment, and the public from exposure to
infectious organisms. Risk assessment is a process that enables the appropriate selection of microbiological practices, safety equipment, and facility safeguards that can prevent laboratory-associated infections. Modern biosafety practices described in the 5th edition of *Biosafety in Microbiological and Biomedical Laboratories* [39] are accepted as standards of practice by all CDC-registered entities to conduct work with SAT. The DA mandates the use of the current version of the manual and DA pamphlet 385-69, *Safety Standards for Microbiological and Biomedical Laboratories* [40], in all U.S. Army activities and facilities in which infectious agents or toxins are used, produced, stored, handled, transported, transferred, or disposed, including the Army National Guard, the U.S. Army Reserve, and contractors and consultants conducting microbiological and biomedical activities for the Army. The detailed principles and practices of biosafety are covered in a separate chapter of this textbook.

It is critically important to thoroughly train individuals in biosafety practices prior to providing access to the containment laboratories to handle, manipulate, and store BSAT. Training must include:

- Microbiological laboratory techniques;
- Use of Personal Protective Equipment (PPE), safety equipment, and containment laboratory equipment;
- Information on bloodborne pathogens;
- An entity-specific chemical hygiene plan;
- BSAT-specific information;
- Emergency exit operations;
- Immediate first aid; and
- Reporting requirements for potential exposures to infectious agents and toxins.

Some of this initial training can be structured into mentorship programs in which individuals approved as mentors ensure new laboratory workers are able to work safely within the containment laboratories before they are granted independent access. In addition to project-specific training, the worker requesting access to the containment laboratories with BSAT is trained to recognize biohazards, understand potential health risks associated with exposures, provide appropriate first aid, and carry out follow-up reporting procedures.

FSAP regulations require individuals with access to Tier 1 BSAT to be enrolled in an occupational health program [19]-[21]. AR 50-1 [28] also requires Commanders and directors of the entities with a biological surety mission to establish and implement an occupational health program. Core elements of an occupational health program include:

- a) Risk assessment;
- b) Medical surveillance;
- c) Access to clinical occupational health services and management; and
- d) Hazard communication.

Select agent risk assessments should consider:

- Route of exposure;
- Infectious dose;
- Agent virulence;
• Incubation period;
• Environmental stability;
• Communicability;
• Genetic modification;
• Available resources for pre- and post-exposure prophylaxis;
• Available vaccine options; and
• PPE use and biocontainment requirements [41].

Occupational health plans are required to comply with U.S. Department of Labor and Occupational Safety and Health Administration regulations, as well as patient confidentiality laws. Promoting a safe and healthy work environment requires limiting exposures to infectious agents and toxins, promptly detecting and treating exposures, and using information gained from incidents to further improve safety measures and worker training. Occupational health and safety is a shared responsibility among the individual workers, their supervisors, PIs, biosafety specialists, healthcare providers, and the employer.

20.2.12 Personnel Reliability

Personnel reliability programs existed for decades in the U.S. military. The BPRP, modeled after the military’s nuclear and chemical personnel reliability programs, ensures that individuals with access to BSAT meet the highest standards of reliability [18] [42]-[44]. The concept of personnel reliability was implemented over a decade ago in DoD laboratories working with BSAT [45]. AR 50-1, *Biological Surety*, outlines the BPRP described herein [28].

Individuals with access to BSAT in DA and DoD laboratories are required to be enrolled in a BPRP. The FSAP added the “suitability” requirement for individuals with access to Tier 1 SAT in October 2012. The FSAP’s suitability assessment of personnel is based in part on the DoD’s personnel reliability programs. The BPRP and the suitability assessment of personnel are primarily designed to reduce the risk of SAT misuse by an individual who has access to these agents (insider threat). The intent of the U.S. Army’s BPRP [18] and the FSAP’s suitability assessment of personnel [46] is the same; however, there are significant differences between the two programs.

The Commander or director is the head of the organization’s BPRP and can serve as the reviewing official. The reviewing official appoints Certifying Officials (COs) who determine the reliability and suitability of individuals requiring access to SAT and ensure they are appropriately qualified and trained to perform their duties. Commanders and directors may appoint BPRP monitors to assist COs in administering day-to-day activities; however, COs are responsible for continuous monitoring of individuals enrolled in the personnel reliability program. The reviewing official monitors the CO’s decisions to disqualify individuals and may overturn them when procedures are unfair, inconsistent, or incorrectly applied. AR 50-1 requires the reviewing official to review all individual disqualification actions submitted by the CO [28]. The FSAP recommends suitability decisions on individuals requesting access to Tier 1 BSAT be a combined decision of the CO, RO, and the entity leadership (e.g. Commander, director, or reviewing official) [46].

To begin enrollment in the BPRP, supervisors of individuals who need to access BSAT in the CDC-registered containment spaces contact the designated CO. The CO is the gatekeeper for access to BSAT, ensuring that persons requesting access have met all the qualifying conditions. FSAP ensures that restricted persons do not
have access to BSAT through the SRA process (see section above); the Army’s BPRP further ensures that persons with access to BSAT are:

- Trustworthy;
- Mentally and emotionally stable;
- Physically competent;
- Free of unstable medical conditions;
- Able to exercise sound judgment;
- Willing to accept responsibility;
- Able to adapt to changing work environments;
- Free from drug and alcohol abuse;
- Willing to participate in random drug testing; and
- Willing to comply with all training requirements.

Enrollment in the Army’s BPRP involves:

- Initial interview;
- Personnel records review;
- Personnel security investigation;
- Medical evaluation;
- Drug testing; and
- CO’s final evaluation and briefing.

The order of steps in the process is discretionary; nevertheless, each step must take place and be fully documented.

20.2.12.1 Initial Interview

The CO is required to conduct a personal interview of a potential enrollee in the BPRP to assess suitability and reliability. The CO must inform the candidate of the Privacy Act of 1974 [47] and the Health Insurance Portability and Accountability Act [48] to obtain consent to proceed with the screening process. Although not required by the regulations, the initial interview may also include a written questionnaire. The candidate is asked questions that will allow the CO to determine whether he or she has engaged in any activities that would be either mandatory or potentially disqualifying factors. Mandatory disqualifying factors are those that are beyond the discretion of the CO for deciding reliability and suitability. If exceptional extenuating circumstances exist, the reviewing official may request an exception for the individual’s enrollment through command channels. The following are mandatory disqualifying factors:

- Current substance or alcohol dependence.
- Drug or substance abuse within 5 years prior to the initial interview.
- Trafficking, cultivating, processing, or manufacturing illegal or controlled drugs within the past 15 years.
- Drug or substance abuse while enrolled in BPRP.
- Inability to meet safety requirements.
- Meeting the criteria of a restricted person as defined by 18 USC § 175b [49].

Other potentially disqualifying factors include:
- Alcohol-related incidents or alcohol abuse.
- Drug or substance abuse greater than 5 years prior to initial interview.
- Mental or physical medical condition, medication usage, or medical treatment that may result in:
  - Altered state of consciousness;
  - Impaired judgment or concentration;
  - Increased risk of impairment if exposed to BSAT;
  - Impaired ability to wear PPE; and
  - Inability to meet physical requirements;
- Inappropriate attitude, conduct, or behavior.

The CO must inform the candidate that he or she will be subject to random, unannounced drug testing as part of continuous monitoring; an initial negative test is required prior to certification in the BPRP. The CO must also explain to the candidate about:
  a) Continuous monitoring;
  b) The requirement for self-reporting; and
  c) Use of prescription drugs.

The initial interview is a good opportunity for the CO to get to know the candidate and to begin a relationship based on mutual trust and respect.

20.2.12.2 Personnel Records Screening

Once the CO has completed the initial interview and found the candidate to be suitable for enrollment, the applicant’s personnel records are screened by a supporting personnel officer. The screening official will determine the individual’s citizenship and identify it to the CO. Any Potentially Disqualifying Information (PDI) discovered during the screening process is immediately communicated to the CO. Individuals with extended federal government service may have information in their personnel records from the inception of their employment. In contrast, information in contract employees’ personnel records may be limited to the length of their employment with that company. Anything that may indicate unsatisfactory employment history or dereliction of duty, such as job applications, enlistment contracts, and any other available pertinent record should be reviewed for PDI. The CO acts on any PDI discovered during the personnel records screening; however, the CO does not retain any records of this information.

20.2.12.3 Personnel Security Records Screening

The current minimum Personnel Security Investigation (PSI) requirement for unescorted access to BSAT within DoD is a favorably adjudicated single-scope background investigation [50]. This level of PSI is conducted to confer top secret clearance. However, a security clearance is not required for BPRP enrollment. The personnel
security manager will request a copy of the PSI from the Office of Personnel Management on behalf of the CO. The personnel security officer will expeditiously provide any adverse information to the CO, ensuring Privacy Act requirements are not violated. Personnel scheduled for initial assignment to BPRP positions must have the appropriate and favorably adjudicated PSI completed within the 5 years preceding certification to the BPRP. PSI files contain sensitive information and should only be retained for the time necessary to determine suitability and reliability. The CO will review the results of the personnel security investigation to determine if the individual meets the suitability and reliability requirements of the BPRP [51]. The FSAP is not prescriptive, with respect to PSI, above what is required to obtain an SRA for suitability assessment of individuals with access to Tier 1 BSAT.

20.2.12.4 Medical Evaluation

The Competent Medical Authority (CMA) medically evaluates the candidate to ensure that the individual seeking enrollment in the BPRP is physically, mentally, and emotionally stable; alert; competent; dependable; and free of unstable medical conditions that may impact BPRP duties [18]. The CMA meets with the candidate and reviews the individual’s medical records to identify any PDI. Medical PDI includes any medical condition, medication use, or medical treatment that may result in an altered level of consciousness, impaired judgment or concentration, impaired ability to safely wear required PPE, or impaired ability to perform the physical requirements of the BPRP position, as substantiated by the medical authority to the CO. The candidate may also provide the CMA copies of medical records from a personal healthcare provider. If medical records are incomplete or inadequate, the CMA will conduct the appropriate medical evaluation. This may include a mental health evaluation if the CMA determines such an evaluation is prudent or upon request by the CO [18]. Medical PDI is reported to the CO with recommendations regarding the person’s fitness for assignment to these duties or limitations in duties or reasonable accommodations that might allow the individual to perform his or her duties without compromising worker safety.

20.2.12.5 Drug Testing

All candidates for BPRP must complete drug testing within 6 months prior to initial certification. All drug test results will be provided to the CO before the individual is certified in the BPRP. Positive drug test results indicating illegal drug use will result in disqualification.

20.2.12.6 Certifying Official’s Final Evaluation and Briefing

After the candidate has completed all phases of the screening, the CO conducts a final evaluation of all the information received during the screening process and conducts a final interview. During the final interview, the candidate will have an opportunity to review and discuss any BPRP-relevant issues, including PDI discovered during the screening process and the circumstances surrounding such an event, and before the CO’s decision on the candidate’s suitability and reliability for the program. During this time the CO:

- Reviews the duties and responsibilities of the individual’s BPRP position, including required PPE use;
- Discusses the expectations for continuous monitoring;
- Reviews disqualifying factors, including any incidents or medical issues that may have occurred since the initial interview;
- Reminds the individual that prescription drug use must be under the supervision of a healthcare provider; and
- Reviews the self-reporting requirements of the BPRP.
At the end of the interview, the CO should inform the candidate whether he or she is suitable for the program, and the individual signs DA Form 3180 indicating his or her understanding of the program and willingness to comply with the requirements. If the candidate is determined to be eligible, the CO ensures that the candidate has completed all the core safety, security, and emergency training. The CO will notify the RO immediately after the individual is certified in the BPRP [18].

Individuals certified in the BPRP are subject to continuous monitoring. Continuous evaluation includes, but not limited to:

- Self-reporting;
- Peer and supervisor observation and reporting;
- Periodic unannounced drug testing;
- Periodic personnel security investigations;
- Periodic medical evaluations by the CMA; and
- CO observation and evaluation.

The FSAP recommends the RO’s involvement in the development, implementation, and administration of the Tier 1 BSAT suitability assessment program. The RO must ensure that access to Tier 1 BSAT is limited to individuals in the suitability program with the entity’s ongoing suitability monitoring, and have current FSAP approval to access SAT. Ongoing efforts to harmonize the DoD regulations governing BSAT with the FSAP are expected to clarify the role of the RO in the BPRP or suitability assessment program.

### 20.2.13 Biosecurity

Safeguarding BSAT is a high priority for the DoD [43], [44] and the FSAP [52]. According to the Office of Science and Technology Policy, the term “biosecurity” refers to the protection, control of, and accountability for high-consequence biological agents and toxins and critically relevant biological materials and information within laboratories to prevent unauthorized possession, loss, theft, misuse, diversion, or intentional release [53]. AR 190-17, *Biological Select Agents and Toxins Security Program* [54] prescribes the policy, responsibilities, procedures, and minimum standards for safeguarding BSAT. Biosecurity plans are based on risk assessments, are entity-specific, and constitute sensitive information. A site-specific security plan based on risk assessments must be developed by all CDC-registered entities with BSAT. An effective site-specific security plan will have initial and continuous input from and interactions with:

- Security personnel;
- Commanders or directors;
- Subject-matter experts;
- Local law enforcement officers;
- ROs;
- Biosafety officers;
- Occupational health CMAs;
- Facilities management personnel; and
- Information security management personnel.
An effective biosecurity plan is based on operational processes, accounts for all BSAT from creation or acquisition to destruction, does not violate any laws, weighs both primary and secondary affects, and is reviewed and updated at least annually.

The biosecurity program for CDC-registered entities with BSAT can be broadly divided into at least five major components:

1) BSAT security;
2) Physical security;
3) Personnel security;
4) Operational security; and
5) Information security.

### 20.2.13.1 Biological Select Agents and Toxins Security

There are a number of factors that contribute to the challenge of effective BSAT inventory and accountability within containment laboratories. Temperature-sensitive microbes, confined spaces, sharing of limited freezer space by multiple investigators, co-existence of both LT and WS BSAT, multiple users, and illegible specimen labels can all contribute to ineffective BSAT inventory and accountability. Uniform labels with human-readable information and barcodes, inventory verification and wrapping of all LT BSAT with tamper-evident materials, centralized storage of wrapped LT BSAT within the containment laboratory, and controlled access to LT BSAT materials can preserve the integrity of the stored specimens and provide an accurate real-time inventory of these materials. These LT BSAT management strategies can be instituted without affecting ongoing research. Entities must establish standard operating procedures for incoming, outgoing, and intra-entity BSAT transfer. All transfers must be conducted with chain-of-custody documentation, which is retained and verified with BSAT inventory databases. BSAT destruction documents should be confirmed with the BSAT databases. BSAT inventory audit should include review of laboratory notes and verification of BSAT WS materials. All BSAT materials must be maintained in CDC-registered laboratory spaces with restricted access to prevent theft, loss, or release of these materials. All personnel with access to BSAT must be trained in FSAP regulations, including reporting requirements. Entities must also conduct complete inventory audit of a PI:

a) When the PI with BSAT holdings leaves the entity;
b) In the event of a theft or loss of BSAT; and
c) Upon physical relocation of a collection of BSAT materials.

These practices will also prepare the entity for any unannounced inspections. Effective BSAT inventory and accountability practices will preserve the integrity of the specimens and increase research efficiency within the containment laboratories.

### 20.2.13.2 Physical Security

A physical security plan developed using site-specific risk assessment can detect, deter, or delay threat and provide sufficient time to respond to the threat. Security barriers such as perimeter fences, armed guards, walls, locked doors, secured laboratories, and locked freezers can deter intrusion and deny access to BSAT. FSAP regulations require:

- Controls limiting access to CDC-registered spaces to approved individuals with access to BSAT;
• Provisions to safeguard animals and plants infected with select agents;
• Review and update of access logs to CDC-registered spaces;
• Prevention of access credentials sharing;
• Procedures for reporting loss of access credentials;
• Procedures for personnel changes;
• Three barriers (physical structures that are designed to prevent access to unauthorized individuals) to access Tier 1 BSAT;
• Intrusion detection systems where Tier 1 BSAT is manipulated or stored;
• Response time not exceeding 15 minutes for a force capable of interrupting a threat to Tier 1 BSAT manipulation and storage spaces; and
• Procedures for access control power failures [19], [52].

20.2.13.3 Personnel Security
The FSAP and DoD consider personnel security integral to detecting insider threat. The personnel security office at the entity level works with the RO to facilitate SRA documentation and fingerprinting for individuals requesting access to CDC-registered spaces. Personnel security also includes: verification of background information, security investigations, personnel dossier reviews, identifying violators of security and safety procedures, and identifying individuals who threaten or support those who threaten to do harm to others. The biosecurity plan should include personnel security measures based on a site-specific risk assessment. A robust “insider threat awareness” training program developed and continuously updated based on site-specific risk assessments is administered to individuals with access to Tier 1 BSAT. Insider threat awareness training is an annual requirement [19].

20.2.13.4 Operational Security
Effective operational security posture builds on existing operational procedures and mitigates threats based on site-specific risk assessments [52]. Operational security measures for an entity with BSAT should include:

• Training personnel on securing BSAT;
• Monitoring individual access to areas containing SAT;
• Monitoring BSAT activities inside containment suites through security closed-circuit television or by using an escort;
• Control of after-hour and weekend access to containment laboratories with BSAT;
• Screening visitors, packages, and delivery trucks at the entry point;
• Procedures in place for immediate notification to the RO, Commander or director, security forces, and law enforcement if theft or loss of SAT is suspected;
• Training personnel to identify and report suspicious activities;
• Prominently displayed identification badges on individuals within the entity;
• Constant building security surveillance;
• Intrusion detection systems;
• Surveillance of backup power generators; and
• Peer reporting procedures for any sudden changes in behavior among approved individuals with access to SAT [52].

20.2.13.5 Information Security
FSAP regulations require registered entities to develop and implement procedures for information control and information security [55]. Information security procedures and protocols must:
• Ensure all external connections to systems that manage security for the registered space are isolated or have controls that permit and monitor authorized and authenticated users;
• Ensure authorized and authenticated users only access information necessary to fulfill their roles and responsibilities;
• Prevent malicious code from compromising the confidentiality, integrity, or availability of information systems that control safety and emergency equipment, engineering controls for the containment laboratories, and access to registered space;
• Include regular patching and updates to operating systems as well as to individual applications;
• Protect network operating systems with security firewalls;
• Protect hardware assets;
• Include data encryption;
• Ensure remote access capability;
• Establish robust information backup systems in the event of primary system failure;
• Establish procedures for purging electronic storage media prior to disposal; and
• Establish procedures for shredding paper documents and computer disks [55].

20.2.14 Incident Response and Emergency Management
A robust incident response plan and a knowledgeable and competent emergency management team are critical to an entity involved in developing medical countermeasures against dangerous pathogens and toxins. An incident is an occurrence, natural or human-made, that requires a response to prevent the theft, loss, or release of an SAT or to protect human life and animal and plant health [56]. FSAP, DA, and DoD regulations require entities with SAT to develop, exercise, and routinely update a comprehensive, site-specific incident response plan to ensure the security and safeguarding of BSAT in the event of human-made threats and natural disasters. A site-specific incident response plan protects human life before property, is focused on laboratories and not just the entire facility, is developed as a result of collaboration between research staff and leadership, includes responder participation and training, and addresses primary and secondary effects and the impact on workers at the facility [56]. Developing an incident response plan at the entity level should be a team effort involving (but not limited to) the RO, AROs, biosafety officer, facility engineers, PI or researcher, security manager, occupational health physician or CMA, and entity leadership, with input from local first responders (fire department, emergency medical and law enforcement).
Laboratory leadership, supervisors, biosafety specialists and subject-matter experts within a registered entity with SAT should develop incident response information specific to the agents, toxins, and procedures conducted in that laboratory. Individuals working in the laboratory must be trained on how to respond to an incident with the materials they handle in the laboratory, emergency exit procedures, and the use of communication devices within the laboratory. Laboratory incident response information must also include decontamination protocols, first-aid, and reporting requirements [19]. Laboratory and facility incident response plans should be practiced via exercises with entity staff and external first responders (fire department, emergency medical and law enforcement); this practice is critical and will save lives and property in the event of a real incident.

The incident response plan should consider and mitigate vulnerability assessments specific to the laboratory and the facility. The incident response plan must include provisions for theft, loss, or release of SAT, inventory discrepancies, and security breaches [19].

20.2.14.1 Theft, Loss or Release

Response to suspected theft or loss of SAT should include immediate notification to the entity RO and Commander or director for an immediate investigation and verification of pertinent SAT inventory. An investigation should include physical inventory and reconciliation of all LT SAT with database records, review of laboratory usage records, transfer records, destruction records, and WS records. Once theft or loss has occurred, the investigation and recovery of SAT is a law enforcement function. Law enforcement, state, and federal agencies, including FSAP, must be notified of theft or loss of SAT; in terms of FSAP, initial notification is followed by a completed APHIS/CDC Form 3 within 7 days. The entity should be prepared to support law enforcement with all its recovery efforts.

Release of SAT from primary containment could occur during movement (breakage of specimen tubes), due to loss of engineering controls (e.g. equipment malfunction, power outage), or as a result of an unforeseen event inside the containment laboratory. SAT release can pose a significant additional risk of exposure to workers if they are not adequately protected with PPE and if the release is not captured and neutralized. Workers potentially exposed to SAT should be immediately evaluated by occupational medicine staff, and appropriate follow-up care must be provided to the affected workers. Local and state public health agencies and FSAP must be notified of SAT release, including potential exposures to workers. Theft, loss, or release of SAT is also reported up the chain of command in the DA and DoD laboratories.

20.2.14.2 Inventory Discrepancies

SAT inventory discrepancies (overage or shortage) should be immediately reported to the entity RO and AROs. The PI and research staff must conduct an investigation to resolve or confirm the inventory discrepancy. The memorandum of inventory discrepancy investigation should include:

- Identity of the SAT;
- Amount of discrepancy;
- Date of last inventory and by whom;
- Current or last known storage location;
- Names of individuals who discovered the discrepancy;
- Names of individuals who are notified of the discrepancy; and
- Explanation or resolution, if available.
Theft and loss of BSAT must be reported to FSAP [19].

20.2.14.3 Security Breaches

A security breach can occur due to a disruption in an established security network or failure to follow established security procedures and policies, or during active and deliberate intrusion from unauthorized sources (e.g., intruders, enemy forces). The RO and the Commander or director must be notified of all security breaches to restricted areas containing SAT. Security breaches may include:

- Access to SAT by individuals not approved by the FSAP;
- Individuals “piggy backing” into restricted areas;
- Tampering of access controls, locks, and seals securing SAT;
- Unauthorized access to SAT inventory databases;
- Tampering of security badges, passcodes, or other entry credentials to restricted areas containing SAT;
- Unauthorized removal of SAT from restricted areas;
- Sharing of access credentials by workers;
- Damage to building infrastructure resulting in easy access to SAT; and
- Compromises due to hacking or deliberate manipulations in computer programming controlling containment access.

Lessons learned should be incorporated to enhance security systems and decrease security breaches [56].

The FSAP requires the RO to ensure that individuals with access to SAT are trained annually on entity incident response plans.

20.3 SUMMARY

The intent of the FSAP and the DoD’s BSP is the same: to allow peaceful research to continue while restricting BSAT access to individuals and parties who intend to misuse them and do harm. Overall, current regulatory requirements promote laboratory safety and security of BSAT by requiring laboratory registration; pre-screening of individuals requesting access to BSAT; personnel reliability or suitability assessments for individuals seeking access to Tier 1 BSAT; BSAT inventory management; pre-approval and monitoring of BSAT transfers; reporting requirements for theft, loss, release, or identification of BSAT; pre-approval for certain genetic alterations to BSAT (restricted experiments); and periodic onsite inspections by regulatory agencies. Regulatory burden on entities with BSAT can be significant; however, it is critical for the public to have confidence that work involving BSAT is conducted in a manner that prioritizes laboratory and public safety and protection of the environment.

Government agencies, academia, and private companies have all imposed additional standards well beyond what is mandated by the current federal regulations governing BSAT. Biological surety and security requirements to access BSAT in DoD laboratories currently meet or exceed that of the FSAP. DoD also imposes additional biological surety and security measures, beyond those required by the FSAP, on contractors using DoD-owned BSAT. Having different eligibility standards to access and work with BSAT can have significant impact on collaborative research; harmonization of administrative policies and practices of facilities registered with FSAP.
is expected to promote increased collaboration among scientists. Currently, DoD is synchronizing its biological
surety regulations with the FSAP regulations in accordance with Executive Order 13546 [22].

Scientific advances in synthetic biology are likely to challenge the current regulations governing BSAT;
however, current U.S. regulations governing BSAT are consistent with the broad international framework of
agreements intended to prevent development and proliferation of chemical and biological weapons.

20.4 REFERENCES

KGaA: Weinheim, Germany; 2008.


[4] Noah DL, Huebner KD, Darling RG and Waeckerle JF. The history and threat of biological warfare and

[5] Poupard JA, Miller LA and Granshaw L. The use of smallpox as a biological weapon in the French and


18 October 1907.* http://www.icrc.org/ihl.nsf/52d68d14de6160e0c12563da005f6b1b/4360cf9a132f97d7

Weapons: Research, Development, and Use From the Middle Ages to 1945.* Oxford, UK: Oxford

[9] Rutecki GW. A revised time-line for biological agents: revisiting the early years of the germ theory of

8(8):450-454.


Research, Development and Use from the middle ages to 1945.* Edited by Geissler, E and Moon, JEvC,


**Disclaimer:** The opinions, interpretations, conclusions, and recommendations contained herein are those of the authors and do not necessarily reflect the official policy or position of the Department of the Army, the Department of Defense, or the U.S. Government.
### State-of-the-Art in Research on Medical Countermeasures Against Biological Agents

The Final Report of Task Group 186 represents a first-of-its-kind International compendium on the state-of-the-art in Medical Biological Defence.

**Author(s)/Editor(s)**

Multiple

**Date**

August 2016

**Pages**

550

**Distribution Statement**

There are no restrictions on the distribution of this document. Information about the availability of this and other STO unclassified publications is given on the back cover.

**Keywords/Descriptors**

- Biological warfare agents
- Infectious Disease
- Medical countermeasures

NATO RTG/HFM-186 began as an Exploratory Team 91 (ET 91) which met in Paris at the RTA Headquarters on September 3-4, 2008. Representatives from the Netherlands, Canada and the United States composed the Technical Activity Plan and Terms of Reference for “State-of-the-art in research on medical countermeasures against biological threat agents”. RTG/HFM-186 was approved by the Spring 2009 RTB. Nations participating in the Task Group included Canada, the Czech Republic, France, Netherlands, Germany, Norway, and the United States. The Task Group convened 4 times during 2009 and 2011. The deliverable for HFM-186 was a Technical Report covering the current state of medical preparedness against 15 of the most dangerous and deadly viruses, bacteria and toxins considered to be biological threat agents of military importance. The Task Group delivered an extensive report in 20 Chapters (> 500 pages) on the subject matter. It is important to note, however, that this area is dynamic, not only in the advancement of new technologies to develop vaccines, therapies and diagnostic tests, but also in the fact that there are emerging, re-emerging, and genetically-engineered biological agents that will arise to threaten us. We must not treat this HFM-186 Technical Report as a final report to sit on a shelf, but as a beginning report continually updating the “State-of-the-art in research on medical countermeasures against biological threat agents”.

---

**REPORT DOCUMENTATION PAGE**

|--------------------------|----------------------------|---------------------|-------------------------------------|

| 5. Originator | Science and Technology Organization  
North Atlantic Treaty Organization  
BP 25, F-92201 Neuilly-sur-Seine Cedex, France |

| 6. Title | State-of-the-Art in Research on Medical Countermeasures Against Biological Agents |

<table>
<thead>
<tr>
<th>7. Presented at/Sponsored by</th>
</tr>
</thead>
</table>

The Final Report of Task Group 186 represents a first-of-its-kind International compendium on the state-of-the-art in Medical Biological Defence.

<table>
<thead>
<tr>
<th>8. Author(s)/Editor(s)</th>
<th>9. Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple</td>
<td>August 2016</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>10. Author’s/Editor’s Address</th>
<th>11. Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple</td>
<td>550</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>12. Distribution Statement</th>
</tr>
</thead>
</table>

There are no restrictions on the distribution of this document. Information about the availability of this and other STO unclassified publications is given on the back cover.

<table>
<thead>
<tr>
<th>13. Keywords/Descriptors</th>
</tr>
</thead>
</table>

- Biological warfare agents
- Infectious Disease
- Medical countermeasures

<table>
<thead>
<tr>
<th>14. Abstract</th>
</tr>
</thead>
</table>

NATO RTG/HFM-186 began as an Exploratory Team 91 (ET 91) which met in Paris at the RTA Headquarters on September 3-4, 2008. Representatives from the Netherlands, Canada and the United States composed the Technical Activity Plan and Terms of Reference for “State-of-the-art in research on medical countermeasures against biological threat agents”. RTG/HFM-186 was approved by the Spring 2009 RTB. Nations participating in the Task Group included Canada, the Czech Republic, France, Netherlands, Germany, Norway, and the United States. The Task Group convened 4 times during 2009 and 2011. The deliverable for HFM-186 was a Technical Report covering the current state of medical preparedness against 15 of the most dangerous and deadly viruses, bacteria and toxins considered to be biological threat agents of military importance. The Task Group delivered an extensive report in 20 Chapters (> 500 pages) on the subject matter. It is important to note, however, that this area is dynamic, not only in the advancement of new technologies to develop vaccines, therapies and diagnostic tests, but also in the fact that there are emerging, re-emerging, and genetically-engineered biological agents that will arise to threaten us. We must not treat this HFM-186 Technical Report as a final report to sit on a shelf, but as a beginning report continually updating the “State-of-the-art in research on medical countermeasures against biological threat agents”.

---

STO-TR-HFM-186
Les publications de l’AGARD, de la RTO et de la STO peuvent parfois être obtenues auprès des centres nationaux de distribution indiqués ci-dessous. Si vous souhaitez recevoir toutes les publications de la STO, ou simplement celles qui concernent certains Panels, vous pouvez demander d’être inclus à ce service.

CENTRES DE DIFFUSION NATIONAUX

**ALLEMAGNE**
Stréitkräfteamt / Abteilung III
Fachinformationszentrum der Bundeswehr (FIZBw)
Gorch-Fock-Straße 7, D-53229 Bonn

**BELGIQUE**
Royal High Institute for Defence – KHID/IRSD/RHID
Management of Scientific & Technological Research for Defence, National STO Coordinator
Royal Military Academy – Campus Renaissance
Renaissancelaan 30, 1000 Bruxelles

**BULGARIE**
Ministry of Defence
Defence Institute “Prof. Tsvetan Lazarov”
“Tsvetan Lazarov” bul no.2
1592 Sofia

**CANADA**
DGSIIT
Recherche et développement pour la défense Canada
101 Colonel By Drive, 6 CBS
Ottawa, Ontario K1A 0K2

**DANEMARK**
Danish Acquisition and Logistics Organization (DALO)
Lautrupbjerg 1-5
2750 Ballerup

**ESPAGNE**
Área de Cooperación Internacional en I+D
SDGIPLATIN (DGAM)
C/ Arturo Soria 289
28033 Madrid

**ESTONIE**
Estonian National Defence College
Centre for Applied Research
Riiu str 12
Tartu 51013

**ETATS-UNIS**
Defense Technical Information Center
8725 John J. Kingman Road
Fort Belvoir, VA 22060-6218

**FRANCE**
O.N.E.R.A. (ISP)
29, Avenue de la Division Leclerc
BP 72
92322 Châlignon Cedex

**GRECE (Correspondant)**
Defence Industry & Research General Directorate, Research Directorate
Fakinos Base Camp, S.T.G. 1020
Hologars, Athens

**HONGRIE**
Hungarian Ministry of Defence
Development and Logistics Agency
P.O.B. 25
H-1885 Budapest

**ITALIE**
Centro Gestione Conoscenza
Secretariat General of Defence
National Armaments Directorate
Via XX Settembre 123/A
00187 Roma

**LUXEMBOURG**
Your Belgique

**NORVEGE**
Norwegian Defence Research Establishment
Attn: Biblioteket
P.O. Box 25
NO-2007 Kjeller

**PAYS-BAS**
Royal Netherlands Military Academy Library
P.O. Box 90.002
4800 PA Breda

**POLogne**
Centralna Biblioteka Wojskowa
ul. Ostrobramska 109
04-041 Warszawa

**PORTUGAL**
Estado Maior da Força Aérea
SDFA – Centro de Documentação
Alfragide
P-2720 Amadora

**REPUBLIQUE TCHÈQUE**
Vojenský technický ústav s.p.
CZ Distribution Information Centre
Mladoboleslavská 944
PO Box 18
197 06 Praha 9

**ROUMANIE**
Romanian National Distribution Centre
Armaments Department
9-11, Drumul Taberei Street
Sector 6
061353 Bucharest

**ROYAUME-UNI**
Dstl Records Centre
Rm G02, ISAT F, Building 5
Dstl Porton Down
Salisbury SP4 0JQ

**SLOVAQUIE**
Akadémia ozbrojených sil gen.
M.R. Štefánika, Distribučné a informačné stredisko STO
Demínová 393
031 06 Liptovský Mikuláš 6

**SLOVENIE**
Ministry of Defence
Central Registry for EU & NATO
Vojkova 55
1000 Ljubljana

**TURQUIE**
Milli Savunma Bakanlığı (MSB)
ARGE ve Teknoloji Dairesi
Başkanlığı
06650 Bakanliklar

**AGENCES DE VENTE**

**The British Library Document Supply Centre**
Boston Spa, Wetherby
West Yorkshire LS23 7BQ

**Canada Institute for Scientific and Technical Information (CISTI)**
National Research Council Acquisitions
Montreal Road, Building M-55
Ottawa, Ontario K1A 0S2

AGARD, RTO & STO publications are sometimes available from the National Distribution Centres listed below. If you wish to receive all STO reports, or just those relating to one or more specific STO Panels, they may be willing to include you (or your Organisation) in their distribution. STO, RTO and AGARD reports may also be purchased from the Sales Agencies listed below.

Requests for STO, RTO or AGARD documents should include the word ‘STO’, ‘RTO’ or ‘AGARD’, as appropriate, followed by the serial number. Collateral information such as title and publication date is desirable. Full bibliographical references and abstracts of STO, RTO and AGARD publications are given in “NTIS Publications Database” (http://www.ntis.gov).

Requests for electronic notification of STO reports as they are published, please visit our website (http://www.sto.nato.int/)

If you wish to receive STO reports, or just those relating to one or more specific STO Panels, they may be willing to include you (or your Organisation) in their distribution.

If you wish to receive electronic notification of STO reports as they are published, please visit our website (http://www.sto.nato.int/) from where you can register for this service.

### NATIONAL DISTRIBUTION CENTRES

<table>
<thead>
<tr>
<th>Country</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>BELGIUM</td>
<td>Royal High Institute for Defence – KHID/IRSD/ RHID</td>
</tr>
<tr>
<td></td>
<td>Management of Scientific &amp; Technological Research for Defence, National STO Coordinator</td>
</tr>
<tr>
<td></td>
<td>Royal Military Academy – Campus Renaissance</td>
</tr>
<tr>
<td></td>
<td>Renaissancelaan 30</td>
</tr>
<tr>
<td></td>
<td>1000 Brussels</td>
</tr>
<tr>
<td>BULGARIA</td>
<td>Ministry of Defence</td>
</tr>
<tr>
<td></td>
<td>Defence Institute “Prof. Tsvetan Lazarov” “Tsvetan Lazarov” bul no.2</td>
</tr>
<tr>
<td></td>
<td>1592 Sofia</td>
</tr>
<tr>
<td>CANADA</td>
<td>DSTKIM</td>
</tr>
<tr>
<td></td>
<td>Defence Research and Development Canada</td>
</tr>
<tr>
<td></td>
<td>101 Colonel By Drive, 6 CBS</td>
</tr>
<tr>
<td></td>
<td>Ottawa, Ontario K1A 0K2</td>
</tr>
<tr>
<td>CZECH REPUBLIC</td>
<td>Vojenský technický ústav s.p.</td>
</tr>
<tr>
<td></td>
<td>CZ Distribution Information Centre</td>
</tr>
<tr>
<td></td>
<td>Mladoboleslavská 944</td>
</tr>
<tr>
<td></td>
<td>PO Box 18</td>
</tr>
<tr>
<td></td>
<td>197 06 Praha 9</td>
</tr>
<tr>
<td>DENMARK</td>
<td>Danish Acquisition and Logistics Organization (DALO)</td>
</tr>
<tr>
<td></td>
<td>Lautrupbjerg 1-5</td>
</tr>
<tr>
<td></td>
<td>2750 Ballerup</td>
</tr>
<tr>
<td>ESTONIA</td>
<td>Estonian National Defence College</td>
</tr>
<tr>
<td></td>
<td>Centre for Applied Research</td>
</tr>
<tr>
<td></td>
<td>Riiia str 12</td>
</tr>
<tr>
<td></td>
<td>Tartu 5103</td>
</tr>
<tr>
<td>FRANCE</td>
<td>O.N.E.R.A. (ISP)</td>
</tr>
<tr>
<td></td>
<td>29, Avenue de la Division Leclerc – BP 72</td>
</tr>
<tr>
<td></td>
<td>92322 Châtillon Cedex</td>
</tr>
</tbody>
</table>

### GERMANY

- Streitkräfteamt / Abteilung III
- Fachinformationszentrum der Bundeswehr (FIZBw)
- Gorch-Fock-Straße 7
- D-53229 Bonn

### GREECE (Point of Contact)

- Defence Industry & Research General Directorate, Research Directorate
- Fakinos Base Camp, S.T.G. 1020
- Holargos, Athens

### HUNGARY

- Hungarian Ministry of Defence Development and Logistics Agency
- P.O.B. 25
- H-1885 Budapest

### ITALY

- Centro Gestione Conoscenza
- Secretariat General of Defence National Armaments Directorate
- Via XX Settembre 123/A
- 00187 Roma

### LUXEMBOURG

- See Belgium

### NETHERLANDS

- Royal Netherlands Military Academy Library
- P.O. Box 90.002
- 4800 PA Breda

### NORWAY

- Norwegian Defence Research Establishment, Attn: Biblioteket
- P.O. Box 25
- NO-2007 Kjeller

### POLAND

- Centralna Biblioteka Wojskowa
- ul. Ostrobramska 109
- 04-041 Warszawa

### PORTUGAL

- Estado Maior da Força Aérea
- SDF – Centro de Documentação
- Alfragide
- P-2720 Amadora

### ROMANIA

- Romanian National Distribution Centre
- Armaments Department
- 9-11, Drumul Taberei Street
- Sector 6
- 061353 Bucharest

### SLOVAKIA

- Akadémia ozbrojených sil gen
- M.R. Štefánika, Dluženské
- informačné stredisko STO
- Demínová 393
- 031 06 Liptovský Mikuláš 6

### SLOVENIA

- Ministry of Defence
- Central Registry for EU & NATO
- Vojkova 55
- 1000 Ljubljana

### SPAIN

- Área de Cooperación Internacional en I+D
- SDG PLATIN (DGAM)
- C/ Arturo Soria 289
- 28033 Madrid

### TURKEY

- Milli Savunma Bakanlığı (MSB)
- ARGE ve Teknoloji Dairesi Başkanlığı
- 06650 Bakankilar – Ankara

### UNITED KINGDOM

- Dstl Records Centre
- Rm G02, ISAT F, Building 5
- Dstl Porton Down, Salisbury SP4 0JQ

### UNITED STATES

- Defense Technical Information Center
- 8725 John J. Kingman Road
- Fort Belvoir, VA 22060-6218

### THE BRITISH LIBRARY DOCUMENT SUPPLY CENTRE

- Boston Spa, Wetherby
- West Yorkshire LS23 7BQ
- UNITED KINGDOM

### CANADA INSTITUTE FOR SCIENTIFIC AND TECHNICAL INFORMATION (CISTI)

- National Research Council Acquisitions
- Montreal Road, Building M-55
- Ottawa, Ontario K1A 0S2
- CANADA