Award Number: W81XWH-06-2-0012

TITLE: Research and Operational Support for the Study of Militarily Relevant Infectious Diseases of Interest to the United States Army and the Royal Thai Army

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REPORT DATE: January 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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**Title and Subtitle**
Research and Operational Support for the Study of Militarily Relevant Infectious Diseases of Interest to the United States Army and the Royal Thai Army

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**Abstract**
Cooperative agreement # DAMD17-01-2-0005 was implemented January 1, 2001 to provide funding support for Royal Thai Army at Armed Forces Research Institute of Medical Sciences (AFRIMS) engaged in research activities in collaboration with US Army. Administrative, logistical and scientific personnel required to support the ongoing U.S. Army AFRIMS research efforts, and utilities and maintenance required to support the U.S. Army AFRIMS research effort.
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I. INTRODUCTION

A. General

Collaborative studies into infectious diseases of military importance have been conducted at the Armed Forces Research Institute of Medical Sciences (AFRIMS) by both the U.S. Army Medical Component (USAMC) and the Royal Thai Army Medical Component (RTAMC) for 4 decades. Studies leading to develop drugs and vaccines to combat tropical diseases of military relevant importance.

B. Statement of Work

Administrative, logistical and scientific personnel required to support the ongoing U.S. Army AFRIMS research efforts, and utilities and maintenance required to support the U.S. Army AFRIMS research effort.

C. U.S. ARMY AFRIMS Research Efforts at Department of Entomology

Department of Entomology research efforts are the following:

1. Field Evaluation of Mosquito Control Strategies in Thailand
   - Evaluation of Passive Measures for the Control of Mosquitoes in a Military Setting
   - Evaluation of Anopheles Control methods in a Malaria Endemic Village in Thailand
   - Field Evaluation of Topical Arthropid Repellents in Thailand

2. Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines

3. Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

D. U.S. ARMY AFRIMS Research Efforts at Department of Immunology

Department of Immunology research efforts are the following: See page 29-30

E. U.S. ARMY AFRIMS Research Efforts at Department of Enteric Diseases

Department of Enteric Diseases research efforts are the following:

1. Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand
2. Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens

3. Characterization of Enteric Pathogens Isolated from Children and Adults in Maldives

4. Characterization of *Campylobacter jejuni* Isolates

5. Adaptation of the Established Rhesus Monkey Intragastric Challenge Model of Shigellosis to Study WRSD1 a Live Attenuated *Shigella dysenteriae*-1 Vaccine Candidates

6. The Production of Polyclonal Antibodies in ICR Mice (*Mus musculus*) and Wistar Rat (*Rattus norvegicus*) against F1 Capsular Protein Antigen of *Yersinia pestis*

7. The production of Antisera in Non-human Primates against Live *Shigella sonnei* 53G Strain


10. Establishment of a *Shigella sonnei* Challenge Model for Evaluation of Future Vaccine Candidates

11. Surveillance of Respiratory Pathogens in Patients Attending Royal Thai Army Hospitals

**F. U.S. ARMY AFRIMS Research Efforts at Department of Veterinary Medicine**

Department of Veterinary Medicine research efforts are the following:

1. Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta*)/*Plasmodium cynomolgi* Malaria Models

2. Care and Maintenance of Rhesus (*Macaca mulatta*) and Cynomolgus (*Macaca fascicularis*) monkeys and Management of Breeding Colonies

3. Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance/Quality Surveillance Program

4. A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs
5. Characterization and Validation of *Anopheles dirus* Sporozoite-Induced Mouse Malaria Models (ICR mouse/*Plasmodium berghei* and *P. yoelli*) for Screening Exoerythrocytic Antimalarial Drugs

6. Active and Passive Protection of Mice against Japanese Encephalitis Virus

7. Institutional Animal Care and Use Committee

G. U.S. ARMY AFRIMS Research Efforts at Department of Virology

Department of Virology research efforts are as follows:

1. Prospective Study of Dengue Virus Transmission and Disease in Primary Schools and Villages in Kamphaeng Phet, Thailand

2. The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue

3. A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naïve Infants


5. A Phase II, Prospective, Randomized, double Blind, Placebo controlled Field Efficacy Trial of a Candidate Hepatitis E Vaccine in Nepal, WRAIR #749, HSRRB Log #A-9117.1


7. Influenza Surveillance in Southeast Asia

8. Sentinel Surveillance for Emerging Diseases Causing Dengue-like or Acute Encephalitis Syndrome in the Philippines (SEDP)

9. Phase II, Randomized, Double-Blind, Single Center, Controlled Study of Two Doses of Different Formulations of the WRAIR Live Attenuated Tetravalent Dengue Vaccine Compared to a Placebo Control, Administered on a 0-6 Month Schedule, to Healthy Adults

10. Training and Workshops
H. U.S. ARMY AFRIMS Research Efforts at Department of Retrovirology

Department of Retrovirology research efforts are following:

1. A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp120 B/E (AIDSVAX® B/E) Boosting in HIV-uninfected Thai Adults (RV144, HSRBB Log No. A-11048, BB-IND 8795)

2. Extended Evaluation of The Virologic, Immunologic, and Clinical Course of Volunteers Who Become HIV-1 Infected During Participation in a Phase III Vaccine Trial of ALVAC-HIV and AIDSVAX® B/E (RV152, WRAIR #1184)

3. A Phase I Double-Blind Randomized Dose Escalating, Placebo-Controlled, Study of Safety and Immunogenicity of WRAIR/NIH Live Recombinant MVA-CMDR (HIV-1 CM235 env/ CM240 gag/pol) Administered by Intramuscular (IM) or Intradermal (ID) Route In HIV-Uninfected Adults (RV158, WRAIR #1143)

4. Protocol G, A Cross-Sectional Study to Screen for and Generate Broadly Neutralizing Monoclonal Antibodies from HIV Infected Individuals (RV212, WRAIR #1320)

5. The Molecular Epidemiology of HIV-1 among HIV Blood Testing Clients Attending the Thai Red Cross Anonymous Clinic in Bangkok, Thailand. (RV225, WRAIR #1383)

6. HIV Specific Immune Responses in Thai Individuals with HIV Dementia (RV238, WRAIR #1418)

7. Predictors of Neuro-Cognitive Decline and Survival in HIV-Infected Subjects (SEARCH 001, WRAIR #1161)

8. Preliminary study of early primary HIV infection in high risk cohort (SEARCH 004, HSRBB # A-14273.3)

I. Space and Utilities Required

Funding under the cooperative agreement is also directed by the Principal Investigator to the provision of site maintenance including space and utilities management for both the RTAMC and the USAMC in support of research activities.
II. BODY

A. Department of Entomology, AFRIMS FY07 Research Accomplishments


   - Evaluation of Passive Measures for the Control of Mosquitoes in a Military Setting
   - Evaluation of Anopheles Control Methods in a Malaria Endemic Village in Thailand
   - Field Evaluation of Topical Arthropod Repellents in Thailand

a. Investigators:

   - MAJ Jason Richardson, Ph.D
   - MAJ Brian Evans, Ph.D.
   - Alongkot Ponlawat, Ph.D.
   - MAJ Kendra Lawrence, Ph.D.

b. Objectives:

   The goals of this set of studies are to:

   1. Evaluate military tents pre-treated with insecticide while manufactured in the factory. By having tents already treated with insecticide, the tasks of Preventive Medicine troops would be reduced by not having to spray tents with insecticides after they are assembled at deployment sites.

   2. To redesign a novel integrated method of controlling malaria vector mosquitoes of the genus Anopheles in a malaria endemic village in Thailand which conventional methods proved to be less effective to ineffective.

   3. To evaluate the efficacy of six new compounds and formulations as mosquito repellents in the field conditions against malaria and dengue vectors. Their efficacy would be compared with DEET, the currently most effective insect repellent and long time used repellent by the military.

c. Methods:

   Evaluation of Passive Measures for the Control of Mosquitoes in a Military Setting

   Canvas tents which have deltamethrin-treated polyethylene threads interwoven through the fabric during manufacture (Vestergaard Frandsen A/S, Chemin de Messidor 5-7, CH-1006 Lausanne, Switzerland), will be evaluated in comparison with
untreated tents or control. All of the tents have two doors, one at each end, made up of outer canvas door-flaps and inner mosquito mesh door-flaps. In the treated tents, the mosquito mesh door-flaps are made from PermaNet™ polyester netting (Vestergaard Frandsen A/S, Chemin de Messidor 5-7, CH-1006 Lausanne, Switzerland), which is also pre-treated with deltamethrin.

A preliminary resistance assessment will be conducted to determine if and at what intensity the local mosquitoes are resistant to deltamethrin. We will use the bottle assay method of the Centers for Disease Control (CDC) for resistance assessment.

Fixed-time bioassays using the World Health Organization (WHO) plastic cone method will be conducted on the tent fabric using lab-reared or wild-caught mosquitoes to assess the insecticidal efficacy of the treated tents compared with the untreated. Knock-down (KD) and mortality rates will be determined. Mosquitoes will be exposed to the tent under the cones for ten minutes, after which they will be kept in containers and given access to sugar solution. Knock-down will be recorded after one hour and mortality after 24 hours. Reduction in biting rates will be evaluated by having human collectors expose their lower legs inside both the treated and untreated tents to attract host-seeking mosquitoes during the peak biting periods. Mosquitoes will not be allowed to bite but will be collected after they land on human skin, then counted and identified.

**Test Sites:**

Based on records of the local public health agency and preliminary adult mosquito surveillance conducted by AFRIMS, a heavy breeding area of vector mosquitoes in or around Fort Surasee of the Royal Thai Army in Kanchanaburi province, will be selected as the test site. This province is about 128 km. west of Bangkok, Thailand.

**Procedures:**

We will conduct a baseline surveillance of local mosquitoes to establish species diversity and population density prior to each monthly trial. Four untreated tents will be erected on a platform incorporating measures to discourage ants (water, glue traps or others). Tents will be located at least 30 meters apart. Four collectors will collect mosquitoes outside and away from the tents and four collectors inside each tent. The collection hours will be from 1800 hrs. to 0600 hrs. the next morning. The mosquitoes collected will be counted and identified.

Subsequently, four deltamethrin-impregnated tents will be erected with the existing untreated ones, buffer zones of at least thirty meters will also be designated between the treated and untreated tents to prevent any insecticidal interference between tents. WHO plastic cone bioassays will be performed on the treated and untreated (control) tents for knock-down and mortality effects of the insecticide against malaria vector mosquitoes i.e. anophelines. Knock-down after 1 hour and mortality after 24 hours will be evaluated. Bioassays for insecticidal activity of the treated tents will be conducted once a month to monitor any changes in residual activity within the test period due to weathering or other factors.
An evaluation of treatment effect on mosquito biting rates will then be conducted. Tents will be closed at one end and another end with an opened outer door flap and inner mosquito net flap hung loose, to allow moderate mosquito entry. Among the total of four treated and four untreated tents, one collector conducting human landing (HL) collections will be situated inside each treated or untreated tent, exposing their lower legs to collect host-seeking mosquitoes. Mosquitoes collected in each hour will be kept in separate vials and held for counting and species identification. The HL collectors will collect mosquitoes during the period from 1800 to 0600 the next morning, by collecting forty minutes and resting twenty minutes each hour. Four more workers will be designated for mosquito aspiration. By using a back-pack aspirator, each aspirator operator will aspirate mosquitoes resting on the tent inner walls and those on the tent floor. Each group of mosquitoes will be collected in separate paper containers, labeled for time and collection location (wall vs. floor), then held in a cardboard cups with sugar solution and observed after one hour for KD and 24 hrs. for mortality. Each back-pack aspirator will collect in either a pair of treated tents or a pair of untreated tents to prevent insecticide contamination in untreated collections. They will enter the tent each hour after the HL collector completes 40 minutes of collections. This will allow us to obtain both the treatment effect on mosquito biting rates and the knock-down or mortality rates of mosquitoes resting inside the tents. Human collectors for control and treatment tents will be rotated on a daily basis.

**Evaluation of Anopheles Control Methods in a Malaria Endemic Village in Thailand**

Kong Mong Tha, Thailand is one of the best characterized study sites available to military researchers as there has been continual evaluation of mosquito populations and malaria there since 1999. The area has been mapped using remote sensing/GIS. The focus will be an evaluation of vector control techniques to reduce populations of anthropophilic Anopheles. Control strategies to be evaluated include use of newer generation residual insecticides to treat both houses and vegetation surrounding the house. The village will be blocked into treatment and control areas. Adult mosquito populations will be monitored by human biting collections. Collections will be conducted dusk to dawn. Collections will be made inside and on house porches as well as outside away from houses. We will use GIS to evaluate the control. Key GIS components include remote sensing data (vegetation and water), environmental (temperature, humidity, rainfall), demographic (house, age, sex), and entomological (adult and larval distribution and habitats as well as entomological inoculation rates) indices.

**Field Evaluation of Topical Arthropod Repellents in Thailand**

These studies will be conducted in two areas of Thailand – Mae Sot and Lop Buri.

**Mae Sot:**

There will be a minimum of 5 days of participation: one day for screening and training, at least 3 days for testing, and one day for a post-study follow-up 72 hours
after study completion. A minimum number of days are provided because of the uncertainties associated with field studies. For example, inclement weather may prevent the challenge from occurring one night. Peak biting activity is normally for three hours per night but this can change depending on weather and time of year. On the first day, volunteers will be screened and assessed for study eligibility by the Principal Investigator (PI) or Associate Investigators (AI). After providing informed consent, all female volunteers will provide a urine specimen to a registered Thai nurse to determine pregnancy prior to any participation in the study. Pregnancy tests will be read by the nurse and female volunteers will know their results prior to study participation. Pregnant volunteers will not be allowed to continue to participate.

Also on the first day, volunteers will be randomly assigned to one of five treatments (repellent formulation) and will remain in that group for the remainder of the study. Each volunteer will be challenged at 6 post-application time points (2, 4, 6, 8, 10 and 12 hours). Peak biting activity of anopheline mosquitoes (Anopheles minimus and An. maculatus) occurs for approximately 3 hours per night (between 2100 and 2400 hours); therefore, a volunteer can only be challenged at two post-application time points each night (2 and 4 hours; 6 and 8 hours; or 10 and 12 hours) such that at least three days are required to capture all 6 post-application time points. Thus, a staggered application design will be employed so that all post-application challenge time points are measured during the peak biting time. However, peak biting activity can vary depending on time of year, weather conditions, and village location. Prior to the execution of each repellent trial, the peak biting activity will be verified using the most recent Human Landing Count (HLC) data and/or surveillance data collected several weeks prior to initiation of the trial. The goal will be to detect a difference in efficacy for duration periods of 2, 4, 6, 8, 10, and 12 hours between the treated legs and negative control legs. If the true efficacy is 95% for the treated legs we will have approximately 80% power to detect such a difference when using a minimum of 6 volunteers per repellent.

Lop Buri:

There will be a minimum of 8 days of participation: one day for screening and training, at least 6 days of testing, and one day for a post-study follow-up 72 hours after study completion. On the first day, volunteers will be screened and assessed for study eligibility by the PI or AI. After providing informed consent, all female volunteers will provide a urine specimen to a registered Thai nurse to determine pregnancy prior to any participation in the study. Pregnancy tests will be read by the nurse and female volunteers will know their results prior to study participation. Pregnant volunteers will not be allowed to continue to participate.

Also on the first day, volunteers will be randomly assigned to one of five treatments (repellent formulation) and will remain in that group for the remainder of the study. Each volunteer will be challenged at 6 post-application time points (2, 4, 6, 8, 10 and 12 hours). Peak biting activity of Aedes aegypti occurs twice daily (0800-0900 and 1600-1700 hours) and the afternoon peak biting period was selected for repellent evaluation due to the extended period of time required between repellent application and
evaluation. A volunteer can only be challenged at one post-application time point each day (2, 4, 6, 8, 10, or 12 hours) such that at least six days are required to capture all 6 post-application time points. Thus, a staggered application design will be employed so that all post-application challenge time points are measured during the peak biting time. Peak biting activity can vary depending on time of year, weather conditions, and village location. For each repellent trial, the peak biting activity will be verified prior to study execution using the most recent Human Landing Count (HLC) data and/or surveillance data collected several weeks prior to initiation of the trial. The goal will be to detect a difference in efficacy for duration periods of 2, 4, 6, 8, 10, and 12 hours between the treated legs and the negative control legs. If the true efficacy is 95% for the treated legs, we will have approximately 80% power to detect such a difference when using a minimum of 6 volunteers per repellent.

d. Results:

These studies were funded and scheduled to start in FY07. However, due to delays in protocol approval, the studies will now start in FY08. Site selection and baseline data were collected in FY07. In addition, companies which produce tents and which have the technology of impregnating tents with insecticides were identified. The field study site was selected and experimental design and plans were established.

e. Future Plans:

Protocol approval is expected in March of 2008 and execution will begin thereafter.

2. Title of research project: Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines

a. Investigators:

- MAJ Jason Richardson, Ph.D
- Dr. Kriangkrai Lerdthusenee, Ph.D

b. Objectives:

1. Conduct genetic characterization of O. tsutsugamushi infecting 12 colonies of Leptotrombidium chiggers spp. maintained at AFRIMS.

2. Evaluate the ability of each of the 12 chigger colonies to transmit O. tsutsugamushi to laboratory mice. Down-select 4-5 key chigger colonies for further studies. These chigger colonies should be infected with different strains of O. tsutsugamushi and should produce consistent, high infection rates when fed on mice.
3. Focus efforts on building up down-selected chigger colonies to the high levels required for potential vaccine studies.

4. Develop methods for assessing the efficacy of candidate vaccines using the chigger/mouse model. Criteria used to assess efficacy must include quantification of rickettsemia in the mice; however, additional methods (clinical or immunological responses) may also be assessed.

c. Methods:

1. Characterization of Strains/Isolates of Orientia tsutsugamushi: The goal is to characterize the 12 strains of O. tsutsugamushi infecting our 12 chigger colonies in order to determine phenotypic and genotypic relationship between different strains. The sequencing of O. tsutsugamushi strains currently being conducted in collaboration with Walter Reed Army Institute of Research.

2. Continue to evaluate the efficacy of chigger colonies to transmit O. tsutsugamushi to mice

3. Build-up key chigger colonies to levels sufficient to support vaccine challenge studies: The rearing and maintenance of Leptotrombidium chiggers is a long, slow process. The total life cycle (from egg to egg-laying adult) requires approximately 3 months (this is in contrast to 2-3 weeks for most mosquitoes). Each female chigger will only produce about 1000 eggs over her lifetime. Once a chigger colony is selected for use in vaccine trials, it requires approximately 6 months to build it up to a level required to support the trial.

4. Assess the efficacy of candidate vaccines using the chigger/mouse model. Initial efforts focus on determining the course of rickettsemia over time in the Lc-1 strain of O. tsutsugamushi and on the development and/or confirmation of diagnostic procedures (PCR, ELISA, etc.) to quantify rickettsemia in challenged mice. We will also continue to evaluate the effect of chigger infection with specific strains of O. tsutsugamushi on potential indicators of immunity, to include lymphocyte transformation, morbidity (as quantified by food consumption, weight gain/loss, activity, etc.), and mortality (time to death following chigger infection).

d. Results:

Summary of All Scrub Typhus Vaccine Trials (Chigger-Mouse-Model):

- Survival rate in single-dose group was 23.3% and the multiple doses were 24.0% for both double- and triple-Dose groups.

- About 18% of dead-mice died during the normal mortality period (17.4% in single-dose group & 18.4% in double-dose group). No mouse in the triple-dose group died during the normal mortality period.
• Of the vaccinated-mice, 82.6% and 81.6% of dead-mice in single- and double-dose groups exhibited a delayed onset of disease and a prolonged illness period prior to death. While in the vaccinated-mice of the triple-dose group, all mice (100%) showed a delayed onset of disease and a prolonged illness period prior to death.

• Among those vaccinated-mice with a delayed onset of disease and a prolonged illness period prior to death, more than half of them died during days 14-17 as demonstrated in single-, double- and triple-dose groups of 65.2%, 55.3% and 73.7% respectively.

• About a quarter total of vaccinated mice with a delayed onset of disease and a prolonged illness period prior to death died during days 18-23 as evidenced in the single-, double- and triple-dose groups with mortality percentages of 17.4%, 26.3% and 26.3%, respectively.

**Statistical Analysis on Survival time:**

Statistical Analysis on Survival time of the Vaccinated-mice versus the Reference-mice:

Using the Kaplan-Meier Test, the statistical analysis of our preliminary results, revealed that were significantly differences of “Median Survival Time (By determining the 50% probability of survival; @ 95% Confidence Interval)” of the single- and double-dose vaccination groups and the reference group and experiment-control groups (with the normal mortality period), indicated a significant delay in illness and death occurred in the vaccinated mice.

**Overall Preliminary Conclusion**

Our overall preliminary conclusion revealed that there was no difference in the survival rates between the vaccinated-mice of single-&multiple-dose groups. However, our data indicated that the vaccinated-mice of the multiple-dose groups had a longer delay in becoming ill. Therefore, they had the potential to be living longer when compared with mice of the reference group.

e. **Future Plans:**

• Continue to conduct mouse-challenge evaluation of candidate vaccines in 2008.

• The needle challenge in utilizing the “Mouse-model” (by which IP injection of the *Orientia tsutsugamushi* Lc-1 isolate) will be conducted in order to compare the results with the chigger challenge model.

• Determining the immune response developed by the vaccinated-mice from the serum samples by the ELISA technique.
3. Title of Research Project: Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

   a. Investigator:

      Dr. Jetsumon Prachumsri, Ph.D

   b. Objectives:

      1. Provide mosquitoes with consistent, reproducible salivary gland infections to support *Plasmodium vivax* sporozoite challenge.

      2. Provide live *P. vivax* sporozoite-infected mosquitoes and/or harvested, purified *P. vivax* sporozoites (on wet- or dry-ice) to WRAIR/NMRI investigators or collaborating institutions.

      3. Provide live, frozen and antigen slides of blood and exo-erythrocytic stages of *P. vivax* parasites for vaccine studies.

   c. Methods:

      1. Validation of basic system to provide sporozoite-infected mosquitoes in support of STEP/STO requirements. This system will be based on our current ability to feed mosquitoes directly on *P. vivax*-infected patients reporting to local malaria clinics. In brief, adult patients report to local malaria districts when they think they may have malaria. Ministry of Public Health (MOPH) personnel make thick blood smears and check the patients for malaria. As part of an approved Human Use Protocol, Department of Entomology personnel allow to draw 20 ml of patient blood to feed mosquitoes using membrane feeding technique. These studies are conducted weekly at Mae Kasa and Mae Sod malaria clinics. Aliquote of each blood sample will be spotted on filter paper and smeared on glass slide. Confirmation of parasite species will be accomplished by PCR of filtered blood and microscopic examination of blood smears. Infected mosquitoes are returned to AFRIMS and maintained in the AFRIMS insectary. Five to 10% of mosquitoes from each mosquito feed are checked for the presence or absence of oocysts approximately 7-10 days after infection. These mosquitoes are thereafter available for use in malaria sporozoite challenge studies.

      2. Refined Sporozoite Challenge System. In 2007 we will continue refining the system in order to reduce the variability in the mosquito infections (critical for ensuring consistent challenges) and to eliminate the risk of concomitant mosquito infections. The goal will be to develop a system that will i) consistently provide mosquito infection rates with >60% of blood-fed mosquitoes having +3/4 (>100 sporozoites) salivary gland infections, and ii) provide *P. vivax*-infected mosquitoes that do not harbor concomitant pathogens. Consistency in the challenge is a critical component of any vaccine trial. *Plasmodium vivax*-infected patients reporting to local malaria clinics will serve as the
starting point for development of the "refined system". Mosquitoes will be fed on venous blood provided to them in an artificial membrane feeding system. A series of carefully controlled experiments will be conducted using the membrane feeding system. We hypothesize that pooling blood from infected patients will reduce inherent variability in mosquito infections. In addition, pooling blood from several patients may offer the added benefit by increasing the genetic diversity of the sporozoite challenge, and thus may more truly evaluate the efficacy of any candidate vaccine. Replacement of Patient Sera with Commercial Sera: Blood will be collected from patients, and packed red blood cells separated from the sera and subsequently reconstituted with commercial sera. The reconstituted blood will be fed to mosquitoes in a membrane feeding system and mosquito infections quantified. This method has the advantage of removing anti-malaria antibody that may affect gametocyte infectivity (2) and replaces patient sera that is potentially infected with concomitant cellular pathogens with commercial sera that is known as pathogen-free.

3. Parasite Characterization: In the absence of an in vitro culture system, it will be necessary to feed mosquitoes on a \textit{P. vivax}-infected volunteer or the blood from a volunteer. Since it will be impossible to ensure that mosquitoes are infected with a single \textit{P. vivax} clone (as is currently done with \textit{P. falciparum}), it is critical that we develop a method of characterizing the parasites (i.e., genetic diversity of the parasites, resistance to antimalarial drugs, etc.). Once mosquitoes are infected, parasites from the infectious blood meal will be characterized by PCR using polymorphic gene targets, such as the nonapeptide repeat region of the circumsporozoite protein (PvCSP), and the region between interspecies conserved blocks 5 and 6 of the merozoite surface protein (PvMSP1).

4. We will continue our effort to establish a short term culture technique to produce gametocyte and study gametocyte infectivity. In this portion of the study we propose to evaluate the ability of produce infective gametocyte by in vitro culture of cryopreserved blood to infect mosquitoes. Effort will focus on establishment of cryopreservation techniques that will maximize parasite viability. Development of procedures to culture infective gametocytes would allow for production of sporozoites from \textit{P. vivax} specimens obtained from throughout the world. These sporozoites could then be used in a variety of experimental models (i.e., we could use sporozoites obtained from East Timor in our hepatoma cell model to evaluate resistance of Exo-erythrocytic stage parasites to primaquine and tafenoquine). We have used different cryopreservatives to freeze \textit{P. vivax} infected blood. The frozen samples will be thawed and cultured for short periods to evaluate the viability of the parasites and gametocyte infectivity to mosquitoes in 2007.

d. Results

1. New human use protocol to collect blood samples from patients has been completed and we have received an approval letter from the Thai Ministry of Public Health.

2. Updated standard operating procedure (SOP) has been prepared for the 2007 studies.
3. Sporozoites have been produced and used for preparation of IFA slide antigens and in vitro culture of liver stage parasites. In vitro culture of *P. vivax* parasites in human liver cells, HC04, have been started. Parasite infected liver cells were collected and prepared for study of parasite genes and proteins.

4. Analysis of genetic variation of vaccine gene candidates for *P. vivax* among Thai isolates were done. The data from this study will help us to estimate the case number of blood the will be required for *P. vivax* vaccine human challenge model which is planned for 2007.

e. Future Plans:

Different stages of parasites will be produced as listed below.

1. Blood stage parasites from different field sites will be cryopreserved and kept in liquid nitrogen to be used as starting parasites for in vitro culture.

2. Parasite pellets and antigen preparation as IFA slides will be prepared from *P. vivax* infected blood and kept frozen at -70 before being transported to AFRIMS and/or CONUS laboratories. Short term culture for blood stage *P. vivax* will be performed to obtain synchronous stage of the parasites. Separation of different blood stage will be done by using percoll gradient method.

3. Produce *P. vivax* sporozoites or sporozoite infected mosquitoes, blood from malaria clinics in Thailand will be fed to *Anopheles dirus* using membrane feeding method. Parasite development will be examined 7 and 14 days post feeding before harvesting of salivary gland sporozoites or transportation of live infected mosquitoes to CONUS laboratories. Shipment of *P. vivax* infected blood, sporozoites and/or infected mosquitoes will be coordinated by the PI (Departament of Entomology, AFRIMS) and CONUS investigators.

4. Exo-erythrocytic (EE) parasites will be produced by an in vitro culture technique using HC-04 hepatoma cells established at AFRIMS. Different developmental stages of liver parasites will be harvested and antigen slides will be prepared at different times for early EE parasites (day2), EE merozoites (day 7-14) and hypnozoite/no development forms (day 28) after sporozoite inoculation.

5. We plan to collect *P. vivax* parasites from 300 patients for this study in FY2007. Approximately 100 isolates of *P. vivax* will be used for mosquito feeding that will produce at least 30 batches of sporozoite infected mosquitoes. Sporozoites will be harvested from mosquitoes to prepare IFA slides, keep as sporozoite pellets and to be used for production of EE IFA slides. Blood stage parasites from 150 isolates will be cryopreserved, prepared for IFA slides and kept as frozen pellets for further genetic studies.

B. Department of Immunology AFRIMS FY07 Research Accomplishments
1. Title of Research Projects:

<table>
<thead>
<tr>
<th>Number</th>
<th>Projects</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human Malaria Vivax Challenge</td>
<td>Protocol in development. Undergoing ethical review.</td>
</tr>
<tr>
<td>2</td>
<td>Rhesus Pf AMA-1 Combinations Vaccine</td>
<td>Study complete; publication in progress</td>
</tr>
<tr>
<td>3</td>
<td>Immunologic studies of SIV/p27 adenovirus as a model system for future malaria antigen studies</td>
<td>Study completed; publication in progress</td>
</tr>
<tr>
<td>4</td>
<td>PvDBP Polymorphisms</td>
<td>Study completed; publication in progress</td>
</tr>
<tr>
<td>5</td>
<td>Tafenoquine Cure/Radical Cure Dose Ranging NIH</td>
<td>Manuscript preparation in progress</td>
</tr>
<tr>
<td>6</td>
<td>Bioassay/HPLC/LC-MS Validation - FDA</td>
<td>AS complete; LC-MS equipment validated; Method Validation protocol completed</td>
</tr>
<tr>
<td>7</td>
<td>Artesunate Phase II Protocol Development</td>
<td>Clinical Trial completed</td>
</tr>
<tr>
<td>8</td>
<td>Fever Surveillance in Sangklaburi</td>
<td>completed</td>
</tr>
<tr>
<td>9</td>
<td>Cambodia Malaria Prevalence Study</td>
<td>Final report completed</td>
</tr>
<tr>
<td>10</td>
<td>Leptospirosis in Sangkhlaburi</td>
<td>In life and data analysis completed; manuscript published</td>
</tr>
<tr>
<td>11</td>
<td>Molecular Assessment of Nepal Malaria Isolates</td>
<td>Pharmacodynamic/kinetic of lead antimalarial drugs in rhesus malaria model Ongoing</td>
</tr>
<tr>
<td>12</td>
<td>PF MSP-1 Genotyping – assay development</td>
<td>Evaluation and Testing of P. falciparum Parasite Antigens for Use in Active Immunizations Against Malaria Protocol approved, awaiting receipt of test article</td>
</tr>
<tr>
<td>13</td>
<td>Set up and Maintenance of Regulated LC-MS Laboratory</td>
<td>Data generated; publication in progress</td>
</tr>
<tr>
<td>14</td>
<td>Pharmacodynamic/kinetic of lead antimalarial drugs in rhesus malaria model</td>
<td>Data generation in progressIn life complete; publication submitted</td>
</tr>
<tr>
<td>15</td>
<td>Safety and Immunogenicity of Plasmodium vivax circumsporozoite Vaccine in Rhesus Monkeys</td>
<td>In life complete; publication submitted</td>
</tr>
<tr>
<td>16</td>
<td>Evaluation and Testing of P. falciparum Parasite Antigens for Use in Active Immunizations Against Malaria</td>
<td>Data generated; publication in progress</td>
</tr>
<tr>
<td>17</td>
<td>Evaluation of Avian Influenza hemagglutinin sequences in wild birds</td>
<td>Data generation in progressIn life complete; publication submitted</td>
</tr>
<tr>
<td>18</td>
<td>Kwai River Christian Hospital surveillance of influenza like illness</td>
<td>Protocol execution in progress</td>
</tr>
</tbody>
</table>

a. Investigators:

- Dr. Mark Fukuda, MD
- Dr. Bryan L. Smith, MD
b. Objectives:

1. To protect, project and sustain the military soldier against disease threats produced by the two major species of malaria, *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv). To support this mission through the evaluation of new or improved vaccines, prophylactic and therapeutic drugs, rapid diagnostic kits, and the maintenance of a center for excellence focused on the basic biology and epidemiology of malaria.

2. To assess emerging febrile diseases along high-risk regions in Thailand and throughout SE Asia.

c. Methods:

The Department of Immunology and Medicine has applied as many kinds of classical and state-of-the-art technologies as possible to the above multi-faceted research. Clinical research included mobile epidemiology team able to work in adverse conditions where malaria is present, including field sample collection and processing screening, reference microscopy, assessment of rapid diagnostics for various tropical infectious diseases, and a staff well-versed in conduct of clinical trails to GCP and ICH standards. The animal research teams are all trained in laboratory animal research and regulations, current AALAC requirements, and laboratory animal test and observation methods. State-of-the art methodologies are available for the study of vaccine and drugs to include advanced molecular biology methods such as sequencing, SNP analysis, and real-time PCR. Cellular immunology techniques are available which include flow cytometry and sorting technologies, ELISPOT, and molecular methods. Pharmacology assays include HPLC, LC-MS, a unique malaria bioassay to measure the *in vivo* antimalarial bioactivity of potential new antimalarial medications, sustained malaria cell culture and radioisotopic uptake, and antibody based methods for measuring *in vitro* drug sensitivity patterns of malaria strains against standard malaria drugs.

d. Results (accomplishments during the period of January-December 2007):

1. Malaria Drugs STEP Q, STO-AQ, STO-A4, STO-A5

Managed the implementation of departmental quality practices for the execution of studies in agreement with MRMC policies and US FDA standards in support of IV AS
drug development program. Work involved the generation and/or revision of nearly 60 SOPs; upkeep of personnel training and qualification records; space utilization for LCMS lab, sample repository, and field clinical lab; establishment of a controlled sample tracking and inventory system; qualification of equipment used for regulated studies; and continued interaction with Medical Maintenance and service contractors. Helped integrate Departmental QA/QC efforts with those of the subsequently established QA units at the AFRIMS, WRAIR and MRMC. Participated in the IPT teleconferences, providing metabolism and pharmacokinetics insight. Chaired the PK SC undertaking tasks to address related concerns. Completed the implementation of a metabolism-based drug interaction study for AS using markers of enzymatic activity and rhesus monkeys; presented the data to the ISSX and ASTMH meetings. Wrote protocol and built field site for a Phase II IV artesunate dose-ranging study that completed in Dec 2007.

The method of AS/DHA analysis in human plasma using LC/MS was validated according to a WRAIR approved protocol. Current efforts, are focused on measuring AS/DHA samples from ongoing Phase II Artesunate clinical trials. (AQ0047_06_AF: Maintenance of a cGLP Analytical Lab).

Published methods paper describing a simple, nonisotopic, semiautomated bioassay for the measurement of antimalarial drug levels in plasma or serum based on the quantization of histidine-rich protein II in malaria culture is presented. The assay requires only small sample volumes and was found to be highly sensitive and reproducible. The results closely paralleled those obtained with isotopic bioassays (R = 0.988, P < 0.001) and high-performance liquid chromatography-electrochemical detection (R = 0.978, P < 0.001).

Analyzed data from a clinical trial of tafenoquine monotherapy in adults for evaluation of radical curative ability and pharmacokinetics in P. vivax malaria. This study was fully successful in demonstrating that tafenoquine monotherapy can effectively eliminate both blood and liver stage parasites in a manner compatible with current therapies. Funded with NIH co-development grant with GSK, and partnered with Hospital of Tropical Diseases, Faculty of Tropical Medicine, Mahidol University.

Supported parasitology requirements for continuing efforts to develop a hepatocyte cell line to screen activity in the liver of antimalarial drugs and vaccine candidates. Collaboration with Department of Entomology. MIDRP funded.

2. Vaccinology and Immunology studies of in support of Malaria Vaccine Program and Highly Pathogenic Avian Influenza pathogenesis studies.

Several lines of evidence suggest that targeting pre-erythrocytic-stage parasites for malaria vaccine development can provide sterile immunity. The objectives of this study were (i) to evaluate preclinically the safety and immunogenicity of a new recombinant
pre-erythrocytic-stage antigen, liver-stage antigen 1 (LSA1), in nonhuman primates; and (ii) to investigate the potential for immune interference between LSA1 and the leading malaria vaccine candidate, RTS,S, by comparing the immune responses after single-antigen vaccination to responses after simultaneous administration of both antigens at separate sites. Using a rhesus monkey model, we found that LSA1 formulated with the GlaxoSmithKline proprietary adjuvant system AS01B (LSA1/AS01B) was safe and immunogenic, inducing high titers of antigen-specific antibody and CD4+ T-cell responses, as monitored by the production of interleukin-2 and gamma interferon, using intracellular cytokine staining. RTS,S/AS01B vaccination was well tolerated and demonstrated robust antibody and moderate CD4+ T-cell responses to circumsporozoite protein (CSP) and HBsAg. Positive CD8+ T-cell responses to HBsAg were detected, whereas the responses to CSP and LSA1 were negligible. For both LSA1/AS01B and RTS,S/AS01B, no statistically significant differences were observed between individual and concurrent administration in the magnitude or duration of antibody and T-cell responses. Our results revealed that both pre-erythrocytic-stage antigens were safe and immunogenic, administered either separately or simultaneously to rhesus monkeys, and that no significant immune cross interference occurred with concurrent separate-site administration. The comparison of the profiles of immune responses induced by separate-site and single-site vaccinations with LSA1 and RTS,S warrants further investigation.

Other Immunology studies have focused on the immunopathogenesis of Avian influenza using Thai viral isolates. We have found high susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-alpha and TLR ligands. There is worldwide concern that the avian influenza H5N1 virus, with a mortality rate of >50%, might cause the next influenza pandemic. Unlike most other influenza infections, H5N1 infection causes a systemic disease. The underlying mechanisms for this effect are still unclear. In this study, we investigate the interplay between avian influenza H5N1 and human dendritic cells (DC). We showed that H5N1 virus can infect and replicate in monocyte-derived and blood myeloid DC, leading to cell death. These results suggest that H5N1 escapes viral-specific immunity, and could disseminate via DC. In contrast, blood pDC were resistant to infection and produced high amounts of IFN-alpha. Addition of this cytokine to monocyte-derived DC or pretreatment with TLR ligands protected against infection and the cytopathic effects of H5N1 virus.

3. Diagnostics/Rapid Diagnosis of Malaria STEP-L/STO-L

Continued development of real-time PCR method to reliably diagnose Pv and Pf malaria from human blood. Developed new primer and probe sets to detect malaria generically (all Plasmodium species) and specifically for P. falciparum and P. vivax. This development used DNA alignment software to compare the sequence of the 18S rRNA gene of Plasmodium falciparum, vivax, malariae, and ovale and Homo sapiens sapiens. For generic primer and probe sets, common sequences for Plasmodium were chosen that had differences with human sequence. For specific Plasmodium primer and probe sets, sequences unique for only P. vivax or P. falciparum were chosen.
Chosen sequences were then checked via BLAST search for homology with any other sequence. Finally, primer and probe sets were designed using PrimerExpress™ (Applied Biosystems, Foster City California). The most optimal primer/probe set for Generic, *P. falciparum*, and *P. vivax* were determined. All PCR assays (Generic, *P. falciparum, P. vivax*) were optimized for MgCl2 concentration, probe concentration, and primer concentration. Also, limits of detection and quantitation were completed along with a small diagnostic specificity study.

In 2006 we developed and assessed primer sets as a diagnostic for leptospirosis. Developed 5 primer sets and tested annealing temperatures and cycle curves for each set on four different cultured serovars of leptospirosis. Using one of the primer sets, confirmed the presence of leptospirosis in four clinical samples that were positive by culture. Also, determined that whole blood or packed cells were the best samples for use in leptospirosis diagnostics using PCR. We also ran 16 samples in two different runs from Nepal suspected of being positive for leptospirosis. Along with conventional PCR, two primer/probe sets were developed to detect LipL32, an outer membrane protein found only in pathogenic leptospiro, using real time PCR. Both sets were optimized for MgCl2, probe, and primer concentrations. Primer sets were tested against laboratory strains of leptospirosis and also tested for limits of detection and quantitation. In Nov 2007, these primer sets were used to investigate an outbreak in Thailand.

Using ELISA, completed an assessment of two different proteins (*P. vivax* Circumsorozoite protein and Merozoite Surface Protein) to detect antibody responses that could be used as a surrogate marker of malaria exposure. We developed (antigen concentration and secondary antibody dilution) the ELISA with 40 different known negatives to clear background reactions. We then ran 587 different samples from a variety of anti-malarial drug clinical trials against both antigens to assess feasibility to detect increases in antibody response from malaria pre-exposure to post exposure. We found that PvCSP could detect antibody changes from baseline better in individuals who had several prior malaria exposures, but naive subjects had less of a response. PvMSP was a much better indicator. Also, antibody responses were most robust in subjects receiving no treatment or receiving mefloquine. The draft report and publication for this study is still pending.

We evaluated the level of malaria recrudescence and/or re-infection in 16 subjects from a clinical drug resistance study in Bangladesh. The alleles of MSP1, MSP2, and GLURP were tested at pre- and post-drug treatment in malaria parasite DNA collected from each individual. Publication still pending. We evaluated 57 samples collected from malaria subjects in Trat province, Thailand for MSP1, MSP2, GLURP for PCR correction (recrudescence/re-infection) and also ran restriction fragment length polymorphism assays for MSP1 and MSP2 on the same samples.

Multi-drug resistant *Plasmodium falciparum* (mdrpf) has become a worldwide public health threat. Presently, the most effective antimalarial treatment is artemisinin combination therapy (ACT), consisting of an artemisinin derivative and a longer-acting partner drug. In Southeast Asia mdrpf has increased in prevalence, and recent
alarming reports from the Thai-Cambodian border suggest reduced clinical cure rates following artesunate treatment. If artemisinin resistance exists, it is critically important to determine the molecular determinants. The SERCA-type PfATPase6, a sarcoendoplasmic reticulum calcium-dependent ATPase6, has been hypothesized to be a key target of artemisinin derivatives. Several point mutations found in Africa in the SERCA-type PfATPase6 have been associated with increased IC50s for artemisinin derivatives. However, point mutations in SERCA-type PfATPase6 have not been identified in SE Asia even though artemisinin derivatives have seen extensive use. We analyzed the sequence of the SERCA-type PfATPase6 alleles from field isolates collected from artesunate treatment trials conducted along the Thai-Cambodian border. We sequenced coding regions of the proposed artemisinin-derivative-binding pocket and the regions around residue position 769, a non-synonymous mutation conferring higher IC50 of artemether by \textit{in vitro} assay. Results from SE Asian parasites show that all field isolates share identical nucleotide sequence with the 3D7 allele, except for a synonymous change in the M5 helix, an area forming the artemisinin binding pocket.

This synonymous change was found in both treatment successes and failures. These results suggest that in SE Asian strains there are no correlations between clinical outcome and SERCA-type PfATPase6 sequences.

We have established a quality system for microscopy that involves the creation of a certified set of standard testing slides, a quality approved SOP for testing slide readers, and a system for evaluating the performance and training of tested microscopists. This set was recently used in support of the Artesunate Phase IIb DrT study.

\textbf{Emerging Infectious Diseases (GEIS)}

\textbf{Epidemiology of Falciparum Malaria Drug Resistance Patterns in Asia:}

Continued surveillance activities throughout Southeast Asia (Bangladesh, Myanmar, Thailand and Vietnam) for threat assessment of multi-drug resistant malaria. Assessed potential new field sites in Cambodia, eastern Bangladesh, Nepal, and northern Thailand (Chiang Dao). Parasite isolates continue to be analyzed longitudinally to assess for trends in antimalarial drug sensitivity patterns potentially signaling a diminution in the utility of the present armamentarium of malaria medications. GEIS funded, and coordinated with Public Health departments in the various countries.

Validated under field conditions a new non-isotopic method for \textit{in vitro} drug resistance assays, which is simpler, as robust, and avoids radioisotopes. The methods has been made available free of charge to the malaria research community as a public service (see http://malaria.farch.net). The test shows very reliable comparisons to the WHO microtest using a much simpler methodology. Funded by GEIS with support from Mahidol University and University of Vienna.
Continued a combined in vitro-in vivo antimalarial drug efficacy trial of standard artesunate/mefloquine therapy in Trat province in southeast Thailand. This study, intended to investigate previously anecdotal reports of high failures using this regimen is of paramount importance in informing malaria drug policy for the greater southeast Asia area. The study will be conducted employing directly observed therapy of all dosed antimalarial drugs and will draw inferences between IC50s measured using the above mentioned non-isotopic in vitro drug resistance assay. This study has been expanded to examine the same parameters in Tesan, Cambodia.

**Surveillance of Febrile Diseases along the Thai-Myanmar Border:**


**Abstract:** A hospital-based study was conducted along the Thai-Myanmar border to provide greater knowledge of the causes of febrile illness and to determine what zoonotic and vector-borne emerging infectious diseases might be present. A total of 613 adults were enrolled from June 1999 to March 2002. Cases were classified based on clinical findings and laboratory results. An etiologic diagnosis was made for 48% of subjects. Malaria was the most common diagnosis, accounting for 25% of subjects, with two-thirds *Plasmodium falciparum*. Serologic evidence for leptospirosis was found in 17% of subjects. Other etiologic diagnoses included rickettsial infections, dengue fever, and typhoid. The most frequent clinical diagnoses were nonspecific febrile illness, respiratory infections, and gastroenteritis. Clinical associations were generally not predictive of etiologic diagnosis. Apparent dual diagnoses were common, particularly for malaria and leptospirosis. Findings have been used to modify treatment of unspecified febrile illness in the area.

**Malaria Prevalence Study in Cambodia:**

Final analysis and publication of a completed 2005 investigation from the Department of Immunology and Medicine country-wide malaria prevalence survey in Cambodia. Results indicated that malaria prevalence is generally highest in clusters located in Rattanakiri, Stung Traeng, Preah Vihear and northern areas of Kampong Thom and Kratie. This is reflected in prevalence calculations by domain, which show that mean prevalence in domains 1, 2 and 3 were 6.9%, 2.8% and 0.2% respectively. Corresponding figures for prevalence by domain at mini-prevalence sites were 9.2%, 1.6% and 1.0%. The table below shows the prevalence of different species of malaria parasite by domain. Final molecular analysis of drug resistance markers is ongoing.
Parasite prevalence by domain from cross-sectional blood slide survey during household survey

<table>
<thead>
<tr>
<th>Domain</th>
<th>P. falciparum</th>
<th>P. vivax</th>
<th>Pf + Pv</th>
<th>Other*</th>
<th>Total Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.4</td>
<td>1.2</td>
<td>0.2</td>
<td>0.1</td>
<td>6.9</td>
<td>93.1</td>
</tr>
<tr>
<td></td>
<td>(128)</td>
<td>(31)</td>
<td>(4)</td>
<td>(4)</td>
<td>(167)</td>
<td>(2718)</td>
</tr>
<tr>
<td>2</td>
<td>1.3</td>
<td>1.4</td>
<td>0.04</td>
<td>0.03</td>
<td>2.8</td>
<td>97.2</td>
</tr>
<tr>
<td></td>
<td>(45)</td>
<td>(39)</td>
<td>(2)</td>
<td>(1)</td>
<td>(887)</td>
<td>(2723)</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>0.1</td>
<td>0</td>
<td>0.02</td>
<td>0.2</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(0)</td>
<td>(1)</td>
<td>(11)</td>
<td>(2729)</td>
</tr>
<tr>
<td>Total</td>
<td>1.8</td>
<td>0.8</td>
<td>0.1</td>
<td>0.04</td>
<td>2.7</td>
<td>97.3</td>
</tr>
<tr>
<td></td>
<td>(178)</td>
<td>(75)</td>
<td>(6)</td>
<td>(7)</td>
<td>(266)</td>
<td>(8159)</td>
</tr>
</tbody>
</table>

*Other species = 7 (P. malariae = 6, mixed Pm+Pv = 1)

e. Future Plans:

We plan to continue our multi-faceted emphasis on support for malaria product development in diagnostics, new drugs, and new vaccines. We anticipate heavy participation in a DoD wide effort on malaria microscopy QA procedures and have hired a dedicated expert teaching microscopist charged with the responsibility of developing a rigorous microscopy teaching and certification standard. Furthermore, we anticipate being the lead overseas lab for field-testing intravenous artesunate in phase I and II, as possibly phase III testing. We will continue efforts for tafenoquine development, especially towards an indication of radical cure for Plasmodium vivax. We will continue safety and immunogenicity testing of candidate malaria vaccines in rhesus, and progress towards vivax challenge studies for eventual human testing of vivax vaccines in Thailand. Emerging infection work in Sangkhlaburi will continue with emphasis on flaviviruses, leptospirosis and typhoidal illnesses, and this study will be expanded to another targeted site in Nepal. Lastly, we anticipate an expand role in regional malaria surveillance with a combination of in vivo, in vitro and genetic methods to define expanding malaria drug resistance.

C. Department of Enteric Diseases, AFRIMS FY06 Research Efforts

1. Title of Research Project: Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand

a. Investigators:

- Ladaporn Bodhidatta
- Umaporn Suksawad
- Ovath Thonglee
- Chittima Pitarangsi
- Boonchai Wongstitwilairoong
- Rungnapha Phasuk
b. **Objectives:**

Monitor diarrhea etiology and antimicrobial resistance of enteric pathogens at multiple sites within Thailand.

c. **Methods:**

Surveillance will be conducted in children under 5 years of age in hospitals and regional laboratories in several sites of Thailand to include Chiangrai, Phitsanulok, Nakornratchasima, Trang and Bangkok. Stool samples will be received from each participating site for on-site initial assessment followed by confirmatory tests and additional laboratory studies to include molecular studies and antimicrobial susceptibility testing at AFRIMS and Thai NIH, MOPH in Bangkok Thailand.

d. **Results:**

One protocol for a site in Bangkok (Pramongkutklao Military Hospital) has been approved and started for implementation. Over 100 stool samples have been collected and Campylobacter and rotavirus were identified as leading pathogens. The other protocol for regional surveillance in Thailand is under review by both the US and Thai authorities. Prior to study initiation, additional equipment and supplies will be provided to the microbiology laboratory at each site. Onsite training will also be conducted by AFRIMS nurses and technicians.

e. **Future Plans:** Continue study.

2. **Title of Research Project:** Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens

a. **Investigators:**

- Orntipa Sethabutr
- Rungnapha Phasuk
- Sasikorn Silapong
- Kaekwanya Nakjarung
- Tasawan Singhisilarak

b. **Objectives:**

Develop and standardize realtime PCR assays for the detection and characterization of enteric pathogens including Shigella, Enterotoxigenic *E. coli* (ETEC), Campylobacter, Cryptosporidia, Cyclospora, Norovirus, Sapovirus and Rotavirus
c. Methods:

Based on literature review and best available sequence data, multiple sets of primers and probes were designed for each pathogen of interest. The sets were initially evaluated against cultured material. Selected sets of primers and probes were then tested against frozen stool samples collected and archived from multiple Department of Enteric Diseases studies. Lower limits of detection for several sets of primers and probes were determined.

d. Results:

Probes and primer sets have been developed and evaluated for Shigella, ETEC, Campylobacter, Cryptosporidia, Cyclospora, Norovirus, Sapovirus and Rotavirus. Standardized and validated methods were applied to routine detection of pathogens in clinical specimens. During CY2007, over 2,500 frozen clinical stool specimens received from multiple study sites were processed and investigated for Norovirus, Sapovirus and Rotavirus infection. Rotavirus and Norovirus were the major pathogens detected by real time PCR.

e. Future Plans:

Transfer validated and evaluated assays of ETEC, Shigella and Cryptosporidium to JBAIDS platform. (b) Further characterize the genotype of Noro/Rotaviruses by PCR and nucleotide sequencing.

3. Title of Research Project: Characterization of Enteric Pathogens Isolated from Children and adults in the Maldives

a. Investigators:

- Ladaporn Bodhidatta
- Boonchai Wongsatitwilairoong
- Apichai Srijan
- Chittima Pitarangsi

b. Objectives:

Determine diarrhea etiology and antimicrobial resistance of enteric pathogens from children and adults with diarrhea in Male and 2 regional hospitals in the Maldives

c. Methods:

The study protocol was approved by both the US and local authorities. Additional equipment and supplies were provided to the microbiology laboratories at the Indira Gandhi Memorial Hospital in Male and 2 other regional hospitals. After obtaining informed consent, stool specimens were collected from diarrhea cases on presentation.
Initial stool examination, culture and identification of enteric pathogens were performed in Maldives. Confirmatory tests and additional studies to include molecular studies and antimicrobial susceptibility testing were conducted at AFRIMS in Bangkok.

d. Results:

A total of 57 adults and 73 children with diarrhea were enrolled in a one month cross sectional study during August - September 07. The common pathogens isolated and initially confirmed from adults with diarrhea were Rotavirus, *Aeromonas* and *V.parahemolyticus*. The common pathogens isolated and initially confirmed from children with diarrhea were Rotavirus, EPEC and *Campylobacter*.

e. Future Plans:

Additional testing for norovirus by a realtime PCR assay and for Giardia/Cryptosporidium and other viruses by EIA will be completed.

4. Title of Research Project: Characterization of *Campylobacter jejuni* isolates

a. Investigators:

- Oralak Serichantalergs
- Piyarat Poothong
- Panida Nopthai

b. Objectives:

Identify and characterize a safe human challenge strain of *Campylobacter jejuni* for future vaccine evaluation.

c. Methods:

Three-hundred and forty four *C. jejuni* isolated from adult travelers during Cobra Gold exercises in 1998-2003 and Bumrungrad hospital in 2001-2002 including 45 *C. jejuni* isolates from Multicenter studies, 2005 were selected and serotyped for Penner heat stable and Lior heat labile serotyping systems. Standardized PFGE was performed on each *C. jejuni* isolate with 2 restriction enzymes. Characterization of representatives of each major cluster was done by PCR for virulence, protein candidate antigens and lipooligosaccharide (LOS) genes. Isolates not containing genes associated with LOS or GBS related illness will be identified for further study.

d. Results:

Three hundred and forty-six *C. jejuni* isolates were screened by PFGE analysis. One hundred and six *C. jejuni* from the 105 PFGE major clusters were characterized by PCR for virulence genes and GBS related genes. Partial sequence of these genes has
been completed, analyzed and compared to \textit{C. jejuni} sequences in the GenBank. Potential candidate strains that lack of GBS related genes were identified and sent for further studies.

Finalized results of sequences analysis from 106 \textit{C. jejuni} isolates (virulence genes, protein antigen and lipooligosaccharide (LOS) in FY 07 were completed for 4 LOS genes \textit{[cgtA, cgtB, cstII (HS2), cstII (HS19)]}, 3 virulence genes \textit{[cdtA, cdtB, cdtC, flaA, flaC] and pVirB11}) and 3 candidate protein antigen genes \textit{[cmeC, peb1A, cadF, fspA and jlpA]}. \textit{FlaC, jlpA} and \textit{fspA} are among the newest possible vaccine candidate antigens. All sequences were aligned and blasted to partial amino acid sequences of the relevant genes from \textit{C. jejuni} NCTC 11168 or \textit{C. jejuni 81-176}. The results of alignments using Sequencher and Mega software were completed. The \textit{flaA} gene \textit{(1596 bp)} is the most variable gene amongst 106 isolates since there were both changes in deletion and addition in nucleotides compositions. Sequences of 106 \textit{flaA} gene were submitted for flagella typing in Campylobacter database web site.

Five isolates from 106 \textit{C. jejuni} were identified to lack LOS genes (GBS related) and these five Thai isolates were sent for further testing at NMRC for human challenge study.

Characterization of 45 Thai \textit{C. jejuni} isolates (Multicenter study, 2005) carried out in FY 07 for detection of virulence, protein antigens and LOS related genes was also completed. Development of Multilocus Sequence Typing (MLST) was initiated at Department of Enteric Diseases to use as a new typing technique by sequencing of 7 house-keeping genes for 148 \textit{C. jejuni} isolates in FY 06. In FY07, data from MLST results were completed for these 148 human \textit{C. jejuni} isolates. These data were submitted to the \textit{C. jejuni} online database (http://pubmlst.org/campylobacter/) to define the allelic profile or sequence type (ST) and examine the population structure as clonal complexes.

e. Future Plan:

Continue MLST study and analysis. Initiate sequencing of capsule antigen in selected \textit{C. jejuni} isolates and PCR of genes associated with common \textit{C. jejuni} of common Penner serotypes.

5. Title of Research Project: Adaptation of the established rhesus monkey intragastric challenge model of shigellosis to study WRSd1 a live attenuated \textit{Shigella dysenteriae}-1 vaccine candidate

a. Investigators:

- Dilara Islam, Ph.D
- Nattaya Ruamsap
- Ajchara Aksomboon
- Patchariya Khantapura
b. Objectives:

The primary objectives of these protocols are:

i) to evaluate safety, by monitoring the presence and severity of physical signs and symptoms in monkeys following immunization with multiple doses of WRSd1;

ii) to evaluate protective efficacy after immunization with multiple doses of WRSd1 by challenging immunized monkeys with S. dysenteriae-1 1617 strain (parent strain of WRSd1) compared to challenging control monkeys with S. dysenteriae-1 1617 strain;

iii) to evaluate the immune responses and inflammatory responses (by measuring cytokines) in blood, stool, colonic lavage and colonic biopsy and

iv) monitoring shedding of WRSd1 and 1617 strain by standard fecal culture procedure and by PCR assay.

The doses are:

i) Immunize group: Immunization on days 0, 3 and 6 with 2 x 10^10 cfu of WRSd1 & challenge on day 37 with 10^10 cfu of S. dysenteriae-1 1617 strain;

ii) Control group: no immunization and challenge on day 37 with 10^10 cfu of S. dysenteriae-1 1617 strain

C. Methods:

The study protocol was approved by the AFRIMS' IACUC. In order to establish the animal model of S. dysenteriae 1 infection, various specimens were taken for analysis, including, blood, stool, colonic lavage and colonic biopsies. Various clinical, immunologic, bacteriological, and histological tests were conducted on animal specimens from all monkeys. Serum IgA, IgG and IgM antibody titers, antibody secreting cells, fecal secretory-IgA and fecal cytokines were measured at different time points of the study.

All monkeys were monitored by direct observation for 30 minutes (after immunization and challenge) for occurrence of immediate adverse reactions. Also,
beginning on day (-3) and continued 7 days after last immunization and after challenge or until all animals are clinically normal, the monkeys were closely observed and scored twice daily using a clinical observation report (VM Form B20). Animals showing any signs of clinical disease were removed from the cage daily, physically examined by a veterinarian, and provided appropriate veterinary medical care.

d. Results:

Our result showed that WRSd1 vaccine is effective. Four of the five immunized animals were protected. No immunized animals developed dysentery but one animal developed severe vomiting with blood and died acutely; this might be the effect of Shiga toxin, as WRSd1 vaccine candidate can not protect against Shiga toxin, as the whole toxin gene is deleted in WRSd1. Five control monkeys developed diarrhea with blood and/or mucus, vomiting, dehydration, anorexia and depression within 1 to 3 days after challenge. One control monkey died acutely despite antibacterial treatment and supportive therapy.

Clinical result in both group of monkeys are shown in Table (below).

<table>
<thead>
<tr>
<th>Monkey Groups</th>
<th>Clinical observation: (After immunization)</th>
<th>Clinical observation: (After challenge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Immunization)</td>
<td>Anorexia (1/5)</td>
<td>Soft stool with mucous &amp; salivation, died with bloody vomiting content: (1/5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soft stool: (1/5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anorexia: (4/5)</td>
</tr>
<tr>
<td>Group 2 (Control)</td>
<td>No Immunization</td>
<td>Watery stool with mucous and blood: (1/5; died)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Loose stool with mucous and severe vomiting: (3/5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe vomiting with muco-bloody content: (1/5)</td>
</tr>
</tbody>
</table>

e. Future Plans:

The next following study will be a GLP study, where we will evaluate live, attenuated oral WRSd1 variant vaccine candidates, yielding information to further improve protective efficacy, reduced reactogenicity, and increased immunogenicity and compare with parent WRSd1 vaccine.

6. Title of Research Project: The Production of Polyclonal Antibodies in ICR Mice (*Mus Musculus*) and Wistar Rat (*Rattus Norvegicus*) Against F1 Capsular Protein Antigen Of *Yersinia Pestis*.

a. Investigators:

- Dilara Islam, Ph.D
- Nattaya Ruamsap
- Ajchara Aksomboon
• Patchariya Khantapura
• Chittima Pitarangsi
• Boonchai Wongstitwilairoong
• Anchalee Tungtaeng (Vet-Med, AFRIMS)

b. Objectives:

The objective of this protocol is to produce antisera in both rat and mice, by subcutaneous immunization with F1 capsular protein antigen of *Yersinia pestis*. These antisera will be used for ELISA procedure to measure the antibody titers in serum of infected feral rodents (*Suncus murinus*) (USAMC-AFRIMS protocol, PN 05-7). Positive reference sera is not available commercially, therefore we will obtain the reference sera by immunizing both rats and mice with F1 antigen. Three rat and three mice were immunized (subcutaneously). Normal rat and mice sera were collected to be used as negative reference sera. "ICR mice (mus musculus)" and rat “Rattus norvegicus” were used in this protocol.

c. Methods:

The study protocol was approved by the AFRIMS’ IACUC. After baseline blood collection, 3 mice and 3 rat were immunized subcutaneously (SC) with 0.1 ml of F1 antigen in Freund’s Complete Adjuvant. All animals were observed for complete recovery from anesthesia and they are returned to their cages. Initially there were 2 more subsequent immunizations on study days 21 and 35 at the same volume and route but the antigen is prepared in Freund’s Incomplete Adjuvant. On day 42, animals were anesthetized for bleeding to obtain 200 µl blood for checking antibody titer. They are observed for complete anesthetic recovery and they are returned to their cages. The IgG titer was ≥1,000 fold increased compared to the baseline level and then all the mice and rat were euthanized by CO₂ to obtained blood samples by cardiac puncture and exanguination. Serum separated and stored at -70°C freezer.

d. Results:

Positive reference sera, with a high titer is obtained by immunizing both rats and mice with F1 antigen. These sera will be used for Enzyme-linked immunosorbent assay to measure the antibody titers in serum of infected rodents of the Protocol “Surveillance for Plague in Feral Rodents in Vietnam”.

e. Future Plans: N/A.

7. Title of Research Project: The Production of Antisera in Nonhuman Primates Against Live *Shigella Sonnei* 53G Strain

a. Investigators:

• Dilara Islam, Ph.D
b. Objectives:

The objective of this protocol is to produce antisera against *S. sonnei* 53G by intragastric immunization of rhesus monkeys. These antisera will be used as reference sera in ELISA procedures. *S. sonnei* 53G antisera is not commercially available; therefore, we have to create the antisera to be used as reference sera in ELISA for WRAIR HURC Protocol# 1259.

c. Methods:

A total of 6 rhesus monkeys, (2 groups of 3 each) will be randomly assigned regardless of sex to Group 1 and Group 2. Initially Group 1 monkeys will be immunized by intragastric administration of a *S. sonnei* 53G strain with a dose of $2 \times 10^8$ cfu in 20 ml sterile PBS on study day 0 and boosted on day 21. If the goal antibody titer is not achieved then 2nd group of monkeys will be immunized similarly with *S. sonnei* 53G strain with a dose of $2 \times 10^9$ cfu in 20 ml sterile PBS on study day 0 and boosted on day 21.

### Experimental Groups and Immunizations Summary

<table>
<thead>
<tr>
<th>Monkey groups</th>
<th>Study Days</th>
<th>Immunizations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (3 monkeys)</td>
<td>0 and 21</td>
<td>Immunization with <em>S. sonnei</em> 53G strain at $2 \times 10^8$ cfu dose</td>
</tr>
<tr>
<td>Group 2 (3 monkeys)</td>
<td>0 and 21</td>
<td>Immunization with <em>S. sonnei</em> 53G strain at $2 \times 10^9$ cfu dose</td>
</tr>
</tbody>
</table>

Blood and stool samples will be collected.

d. Results:

The Protocol is submitted for IACUC approval.

e. Future Plans:

N/A.

8. **Title of Research Project:** “Exempt” Human Use Protocol: Establish ELISA Reference Sera to be used for Protocol “Establishment of a *Shigella sonnei* Challenge Model for evaluation of future vaccine candidates”
a. Investigators:

- Dilara Islam, Ph.D
- Nattaya Ruamsap
- Ajchara Aksomboon
- Patchariya Khantapura

b. Objectives:

The objective of this study is to generate reference plasma/sera from human or animal for use in Enzyme-linked immunosorbent assay (ELISA) to measure antibody responses against Shigella sonnei LPS and/or S. sonnei protein antigens (Ipa proteins).

c. Methods:

SOP: ETR-IM-000 is followed to select and screen samples to be used as positive and negative control sera. Serum samples from individuals with stool culture confirmed for S. sonnei were selected for positive reference sera and sera from Cobra Gold study with low titer against S. sonnei LPS were selected for negative reference sera. Titers (IgA/IgG/IgM) of individual serum sample was tested against. S. sonnei LPS by ELISA following SOP: ETR-IM-000. Purified S. sonnei LPS is purchased from Commonwealth Biotechnologies, Inc. USA. Positive reference sera: Depending on the obtained optical density (OD) value of individual tested serum sample; serum samples were selected to make the pool so the OD of the pool is not lower than 0.40. The expected OD value should be ≥ 0.40 for the pooled positive reference sera at dilution not lower than 1:100. Negative reference sera: A minimum of 5 samples with individual OD value between 0.1-0.2 (for all Ig classes) were pooled and will be used as negative reference sera.

d. Results:

Protocol is submitted for AFRIMS' Scientific Review.

e. Future Plans: N/A.


a. Investigators:

- Ladaporn Bodhidatta
- Apichai Srijan
- Paksathorn Puripanyakom
- Wilawan Oransatit
- Orapan Chivaratanond
b. **Objectives:**

Monitor diarrhea etiology and antimicrobial resistance of enteric pathogens at 3 sites in Kathmandu and Bharatpur, Nepal.

c. **Methods:**

Three human use protocols approved by both the U.S and Nepal authorities. Studies have been conducted in children in Kanti Children Hospital in Kathmandu and in Bharatpur Hospital, Bharatpur and in adults in Sukraraj Tropical Infectious Disease Hospital, Kathmandu. Several visits were made by the Principal Investigator, as well as nursing and laboratory staff prior to study initiation.

Microbiology laboratory, media preparation, ELISA and QA/QC capability have been established at WARUN. Laboratory procedures for specimens collected in Kathmandu are performed at WARUN. Stool samples received at Bharatpur Hospital are initially assessed on site and sent for confirmatory tests and additional laboratory studies at WARUN. New microbiology laboratory has been completed at Bharatpur Hospital by space renovation. Onsite trainings were conducted. AFRIMS will perform molecular studies and antimicrobial susceptibility testing of the specimens collected from all sites.

d. **Results:**

Approximately 1000 stool samples have been received. The leading pathogens identified so far in both children and adults were rotavirus, Aeromonas and ETEC. An outbreak of *Vibrio cholera* was detected during July to September 2007.

e. **Future Plans:** Continue study.

10. **Title of Research Project:** Establishment of a *Shigella sonnei* Challenge Model for Evaluation of Future Vaccine Candidates

a. **Investigators:**

- Ladaporn Bodhidatta
- Dilara Islam, Ph.D
- Umaporn Suksawad
- Apichai Srijan
- Nattaya Ruamsap
- Ajchara Aksomboon
- Patchariya Khantapura
- Boonchai Wongstitwilairoong
- Tasawan Singhsilarak
- Kaewkanya Nakjarung
b. Objectives:

To identify the optimal challenge dose of Shigella sonnei 53G that will elicit clinical diseases in at least 70% of the Healthy Thai volunteers.

c. Methods:

The study protocol was approved by 2 Thai IRBs (Ministry of Public Health and Faculty of Tropical Medicine) and the U.S IRB (HSRBB) with the main purpose to establish a Shigella sonnei challenge model in Thai adults. This will serve as the target attack rate for follow-on efficacy trials of future vaccine candidates against *Shigella sonnei*.

A sequential evaluation of three challenge doses of *S. sonnei* 53G at 100, 400 and 1600 cfu has been proposed. A group of 12 healthy Thai adult volunteers will be admitted to the Vaccine Trial Center (VTC) of the Faculty of Tropical Medicine, Mahidol University for a period of 8 to 11 days. Baseline stool, blood, serum, and urine samples will be taken. Volunteers will be orally challenged with *S. sonnei* 53G after a sodium bicarbonate solution to neutralize stomach acidity and will be closely monitored for symptoms and signs of shigellosis. Stools will be collected for clinical characterization and for culture and PCR, blood will be collected for immunology assays and clinical laboratory testings. After five days (120 hours) post challenge, volunteers will be treated with oral ciprofloxacin for 3 days unless specific early antibiotic treatment criteria are met. Volunteers will be hospitalized until they are symptom-free and 2 sequential stool cultures are negative for *S. sonnei* and 6 doses of oral ciprofloxacin therapy are completed. After discharge from the facility, volunteers will be asked to come for follow up visits on on Day 14 and Day 28. A telephone visit on Day 42 will also be made to monitor late complications.

d. Results:

The first cohort of 12 volunteers was admitted to the VTC on 11 September 2007. Volunteers were challenged with approximately 100 cfu of *S.sonnei* 53G suspended in sterile water after a sodium bicarbonate solution. Three out of twelve volunteers (25%) developed clinical diseases (fever and diarrhea/dysentery). No clinically significant adverse event was observed. After a safety review by medical monitors, the study can be proceeded to the second cohort of 400 cfu.

e. Future Plans:

The second cohort at the dose of 400 cfu is being conducted. If at least 70% attack rate of clinical disease has been achieved, the study will be considered successful. If not, the third cohort of 1600 cfu will be continued after a safety review.

11. Title of Research Project: Surveillance of Respiratory Pathogens in Patients Attending Royal Thai Army Hospitals
a. Investigators:

- Jariyanart Gaywee (RTA)
- Narongrid Sirisopana (RTA)
- Chirapa Eamsila (RTA)
- Pochaman Watcharapichat (RTA)
- Thippawan Chuenchitra (RTA)
- Khin Saw Aye Myint (Virology)
- Ladaporn Bodhidatta

b. Objectives:

To diagnose respiratory pathogens in patients attending Royal Thai Army hospitals and to provide influenza surveillance data to the WHO surveillance network.

c. Methods:

Eligible patients will be identified in the outlined study hospitals by study team staff based on the eligibility criteria. Eligible subjects will be presented the study information sheet and asked to participate in the study. If they agree, written informed consent will be obtained by study team staff medical history collected as outlined in the Demographic/Clinical Form by study team staff. Additional diagnostic tests and any treatment decisions will be solely made by the attending physician, in accordance with local standards and the available medical data.

d. Results:

Of the samples collected at the six sites from a total of 143 volunteers, 139 samples collected have been influenza negative by on site rapid testing; 4 were positive (2 influenza A & 2 influenza B). More comprehensive PCR testing at AFRIMS on an initial 38 samples found 34 negative and 4 positive (4 influenza A/H3). Further testing on these samples is pending.

e. Future Plans:

During the coming year, we plan to continue enrollment of volunteers at all sites.

D. Department of Veterinary Medicine AFRIMS FY07 Research Accomplishments

1. Title of Research Project: Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (Macaca mulatta)/Plasmodium cynomolgi Relapsing Malaria Model

   a. Investigator:

   - Dr. Montip Gettayacamin
b. Objectives:

1) Use the rhesus monkey/\textit{P. cynomolgi} model to determine the effectiveness of new causal prophylactic and radical curative compounds which are being synthesized and developed by the US Army antimalarial drug development program.

2) Use the rhesus monkey/\textit{P. cynomolgi} blood-stage malaria model to evaluate new antimalarial compounds for their blood schizonticidal activity.

c. Methods:

Malaria is one of the most important parasitic diseases worldwide. Traditional treatment for malaria includes drugs used to prevent disease (prophylaxis) and to cure the infection (therapeutic). Antimalarial drug screening in the rhesus monkey model is very effective for making comparisons between drugs. It is fairly rapid, relatively inexpensive, and makes reliable predictions of how drugs will act in man. Antimalarial drug screening in the rhesus monkey has played a key role in the development of every antimalarial drug licensed in the US for the past 30 years. This model provides a mechanism to identify effective new drugs for the enhanced prevention and treatment of malaria infections.

d. Results:

Three new experiments and two continuing experiments were conducted in this fiscal year using 54 monkeys. Imidazolidinedione (IZ) derivatives WR296580, WR299728, WR301772, WR283205, WR301855, WR299666, WR301795, WR294716, WR299849, WR299872 and WR299548) and a pyrimidine derivative (WR301798) were tested for causal prophylactic and radical curative activities in two combined test experiments. WR283205, six fluoroquinolones (Ciprofloxin, Ofloxacin, Grepafloxacin, Trovafloxacin, Sparfloxacin and norfloxacin), NPCB combination with chloroquine or mefloquine, FDA approved compounds (tinidazole, triamterene, mebendazole, chlorazanil), and azithromycin or doxycycline or clindamycin and primaquine combinations were screened for radical curative activity. WR296580, WR299728, WR283205, WR301855, WR301795 and WR299548 had tissue schizonticidal activity to delay parasite potency and relapse. NPCB/chloroquine combinations showed partial to complete radical curative activity. Triamterene and Tinidazole showed radical curative effect in one of the treated monkeys. Tinidazole/Primaquine/chloroquine, doxycycline/Primaquine/chloroquine combination had a radical curative activity. The other compounds had no activity.
e. **Future Plans:**

We anticipate conducting at least two experiments over the next calendar year.

2. **Title of Research Project:** Care and Maintenance of Rhesus (Macaca mulatta) and Cynomolgus (Macaca fascicularis) Monkeys and Management of Breeding Colonies

a. **Investigators:**

- Dr. Montip Gettayacamin
- Mr. Srawuth Komcharoen

b. **Objectives:**

Maximize the production of specific pathogen-free rhesus monkeys in the USAMC-AFRIMS production colony, using the best and most humane husbandry care, maintenance procedures, veterinary care, and disease surveillance and environmental enrichment procedures available.

c. **Methods:**

USAMC-AFRIMS maintains a breeding colony of rhesus macaques using a closed colony system. Approximately 150 rhesus monkeys are used in the breeding program. Two types of breeding are managed: compatible male and female pairs are housed in special paired-type caging, and multiple harem groups are established and maintained in large gang cages. Harems consist of one breeding sire and 5-15 adult females. Newborn monkeys are weaned at approximately 6 months of age, and then are reared to adulthood in gang cages with other weanlings. All colony primates are tested routinely for the presence of infectious diseases that pose a threat to either the health of the colony or to personnel working with the primates. Humane use of the animals is assured by the intense oversight of the Institutional Animal Care and Use Committee. Veterinary and technical care is extensive and continuous.

Whenever possible, animals are re-utilized in multiple protocols in order to optimize the use of this limited and essential resource.

d. **Results:**

Sixty-two (62) baby rhesus macaques were born in the colony in the last year.

e. **Future Plans:**

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free nonhuman primates to support USAMC-AFRIMS research needs. Maintain and expand the colony by obtaining 12 new breeding males, increasing the number of paired housing cages, and placing
breeding pairs in these new cages into additional animal rooms in the vivarium. Importing monkeys was delayed due to difficulties in getting CITES approval by the Thai Department of Park and Wildlife. The request was recently approved and the monkeys should arrive during the 2nd or 3rd quarter of CY08.

3. **Title of Research Project:** Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance/Quality Surveillance Program

   a. **Investigators:**
      
      • Dr. Montip Gettayacamin
      • Ms. Anchalee Tungtaeng

   b. **Objectives:**
      
      Maintain a breeding colony of specific pathogen-free laboratory rodents to meet the scientific research needs of the USAMC-AFRIMS, using state-of-the-art knowledge, equipment, and facilities.

   c. **Methods:**
      
      USAMC-AFRIMS maintains breeding colonies of laboratory rodents to meet the needs of AFRIMS research. Using state-of-the-art equipment, knowledge, and facilities, production is matched to the anticipated needs of individual research projects. Extensive and thorough recordkeeping ensures that outbred strains remain outbred, and that inbred strains remain truly inbred. An extensive quality assurance/quality surveillance program, which includes serologic assessments as well as necropsy/histopathologic analysis, ensures that the colony produces only high-quality disease-free animals. When necessary, new breeder stock is procured from a reliable vendor in the United States or Japan. Veterinary and technical care is extensive and continuous.

   d. **Results**
      
      Three thousand seven hundred twenty three (3,723) ICR mice (*Mus Musculus*) were produced for 9 active protocols. Quality assurance procedure monitors the health status of the animals produced in the colony and purchased from a local vendor.

   e. **Future Plans:**
      
      These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free rodents to support USAMC-AFRIMS research needs.
4. Title of Research Project: A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs

a. Investigators:
   - Dr. Montip Gettayacamin
   - Ms. Pranee Hansukjariya
   - Ms. Anchalee Tungtaeng

b. Objectives:

To evaluate potential antimalarial chemotherapeutic agents in the *P. berghei* ICR mouse - the modified Thompson Test model.

c. Methods:

The test system used for the determination of antimalarial activity of the compounds is a modification of the suppressive test known as the Thompson Test. Typically in this test, up to 22 groups of 5 mice are inoculated intraperitoneally (IP) with *P. berghei*-infected erythrocytes then treated with candidate drugs to determine the antimalarial activity. Infected erythrocytes are provided from donor mice. On experiment day 0, the donor mice are anesthetized then exsanguinated via cardiac puncture, the blood pooled and the level of parasitemia determined. The pooled blood is then diluted with normal mouse serum to a concentration of $1 \times 10^6$ *P. berghei*-infected erythrocytes per inoculum (0.1 ml). The groups of experimental and control mice are inoculated with this parasitized blood on day 0. On day 3, 4, and 5 mice are treated with either the candidate antimalarial drug or with vehicle alone, to serve as the negative control. The drug is administered orally (PO), subcutaneously (SC), intramuscularly (IM), and/or intraperitoneally (IP) up to three times a day, based on the individual and unique pharmacodynamics of the test compound. Each experimental group receives a different dose level, with up to 7 different dose groups per compound. A standard antimalarial drug may be tested along with the candidate drug for structure-activity determination and for quality assurance of the model. Blood films and body weights are taken on the third and sixth days post-infection, then at weekly intervals though day 31. Blood films are stained, examined by light microscopy, and the percent parasitemia determined. All mice are observed twice a day to assess their clinical signs. All mice with negative smears at 31 days are considered cured.

d. Results:

A total of 36 compounds were tested in 20 experiments.

e. Future Plans:

This mouse model for screening new candidate antimalarial compounds has been used for over 30 years and is very effective for making comparisons between drugs. It is
rapid, relatively inexpensive, and makes reliable predictions of how drugs will act in higher mammalian hosts, including humans. This is a core capability of the USAMC-AFRIMS Department of Veterinary Medicine and will be maintained so that many more compounds can be tested.

5. Title of Research Project: Characterization and Validation of Anopheles dirus Sporozoite-Induced Mouse Malaria Models (ICR mouse/Plasmodium berghei and P. yoelii) for Screening Exoerythrocytic Antimalarial Drugs

a. Investigators:

- Dr. Montip Gettayacamin
- Dr. Jetsumon Prachumsri
- Ms. Anchalee Tungtaeng
- Dr. Robert S. Miller
- Dr. Dennis Kyle

b. Objectives:

To evaluate potential causal prophylactic antimalarial agents in the P. yoelii mouse exoerythrocytic (EE) model at AFRIMS.

c. Methods:

A model involves infecting mice with sporozoites harvested from infected Anopheline dirus mosquitoes on day 0. The infected mice are dosed with test compound on day -1, prior to inoculation of sporozoites and then on day 1. There are 6-16 groups of 5 mice or up to 80 mice in each experiment. The routine test consists of 1 to 3 dosage levels of up to 5 compounds administered by one or two routes. One group will receive vehicle alone to serve as the control. Blood films, weight and clinical signs are followed to 30 days post-infection. Mice with negative smears at 30 days are considered to be protected.

d. Results:

This established model was validated and optimized. The model has been used for screening 41 novel compounds in 15 experiments.

e. Future Plans:

This new mouse model for screening new candidate antimalarial compounds is very effective and makes reliable predictions of new compounds and screens new antimalarial compounds against the exo-erythrocytic (liver stage parasites) before further testing in monkey malaria model. This core capability of the USAMC-AFRIMS Department of Veterinary Medicine will be maintained and many more compounds will be screened tested.
6. **Title of Research Project:** Active and Passive Protection of Mice against Japanese Encephalitis Virus

a. **Investigators:**

- Dr. Yvonne Van Gessel
- Dr. Rober Putnak (WRAIR)
- Dr. Shailesh Dewasthaly (Intercell Austria)
- Dr. Montip Gettayacamin
- Dr. Robert Gibbons

b. **Objectives:**

To establish a reliable mouse model of Japanese Encephalitis Virus (JEV) infection at AFRIMS. To evaluate a new second generation Japanese Encephalitis purified-inactivated vaccine (JE PIV) for active and passive cross-protection in this model when challenged with homologous and heterologous JEV strains.

c. **Methods:**

The study was divided into 4 distinct experiments. Experiments 1, “Determination of optimum challenge dose”, 2 “Passive protection model establishment” and and 3 “Determination of vaccine-induced (active) cross-protective potency against JEV challenge”, were completed in FY2006. In Experiment 4, “Determination of serum antibody induced (passive) cross protection against JEV” JEV immune serum produced from human volunteers vaccinated with the candidate JEV vaccine, IC51 (JE-PIV) during Phase 3 clinical trials was tested for passive protection in the ICR mouse model. Serum from volunteers vaccinated with IC51 (JE-PIV) was pooled into four batches based on antibody titer: high, medium, low and negative (non-responders). In addition, pooled serum from JE-VAX® vaccinees and JEV antibody negative serum was tested as positive and negative controls respectively. Groups of 6 to 7-week-old female ICR mice (N=10) received either 1:2 or 1:10 pre-diluted 0.5 ml of test serum via intraperitoneal (IP) injection and were challenged by the IP route 17 to 18 hours later with a lethal dose of either JEV SA14 or JEV KE-093 after disruption of the blood brain barrier by intracranial injection of sterile saline. Mice were observed for clinical signs for 21 days. Experiment 5: In addition to the experiments listed above, an in-vivo potency assay was run concurrently with all viral challenges in experiments 1 thru 4 to prove correct challenge dose.

d. **Results:**

Experiment 4: All mice in the negative control (non-immune) serum groups developed clinical disease or died. JEV strain SA14 challenged mice survival correlated inversely with titer of the serum from IC51 (JE-PIV) vaccine responders. The highest titered serum tested (107) rescued 9 of the 10 mice, with 60, 40, 30 and 0% survival observed in the next four treatment groups of effective input PRNT50 titers.
21.5, 21.4, 10.5 and 4.3 respectively. One of the ten mice (10%) in the lowest PRNT50 group (2.1) survived challenge. Serum from volunteers that had no detectable response to vaccination conveyed minimal protection with 10% survival observed in both the 1 in 2 and 1 in 10 dilution groups. JE-VAX® serum conveyed 100% and 60% protection at effective input titers of 27.5 and 5.5 respectively. For KE-093 challenged mice, the highest titered serum tested (107) conveyed 100% protection. Survival rates of 20%, 44%, 40%, 0%, and 30% were observed in serum PRNT50 titer groups of 21.4, 21.5, 4.3, 10.5, and 2.1 respectively. Serum from volunteers that had no detectable antibody response to vaccination conveyed a low level of protection at the 1:2 dilution with 30% survival and no protection (0% survival) at the 1:10 dilution. JE-VAX® serum conveyed 100% and 40% protection at effective input titers of 27.5 and 5.5 respectively. These results indicate that antibodies are able to protect against JE infection i.e. protection is antibody based, antibodies generated against IC51 (JE-PIV) vaccination protect mice equally against lethal challenge with JEV SA14 and KE-093 (genotype III and genotype I respectively) and that protection against JEV correlates with the (anti-JEV) input antibody titer as measured by PRNT_{50} assay.

The final report for all experiments under this protocol (1-5) was completed and submitted to the sponsor.

e. Future Plans:

The written reports to the sponsor will be included in the new product licensing packet submitted to the U.S. Food and Drug Administration by Intercell in 2008. The mouse model may be used to test other promising prophylactic or therapeutic treatments for JEV.

7. Institutional Animal Care and Use Committee

a. Personnel:

- Dr. Yvonne Van Gessel, IACUC Chair
- Dr. Sarah Hinds, Attending Veterinarian
- Dr. Kriangkrai Lerndthusnee, Scientist 1
- Dr. Wantanee Ratanasak, Alternate Scientist 1
- Dr. Warawadee Nirdnoy, Scientist 2
- Dr. Ladaporn Bodhidatta, Alternate Scientist 2
- Dr. Anon Srikiatkhachorn, Scientist 3
- Dr. Robert Paris, Alternate Scientist 3
- Dr. Kurt Schaecher, Scientist 4
- Dr. Sathit Pichyangkul, Alternate Scientist 4
- Dr. Julie Pavlin, Scientist 5
- Ms. Somporn Krasaesub, Statistician
- Ms. Pringsri Ingkaninun, Non-Affiliated/Non-Scientist
- Ms. Swalee Siriphol, Alternate Non-Affiliated/Non-Scientist
• SSG. Marc Bellaire, IACUC Administrator
• Ms. Angwara Arinhamapan, Alternate IACUC Administrator

b. Objectives:

1) To support the animal research of USAMC-AFRIMS by providing oversight for the USAMC-AFRIMS animal care and use program.

2) To review all proposed animal research protocols.

3) To assure IACUC members are trained in current SOP’s and issues related to managing a quality animal care and use program.

c. Methods:

The IACUC is the self-regulating body for animal research on behalf of the iAAnstitute. In accordance with regulations, the USAMC-AFRIMS IACUC meets a minimum of once every six months and typically meets once a month. All new protocols are reviewed by a full committee. The IACUC monitors the animal care and use program by conducting thorough reviews of the program and inspections of animal facilities semi-annually. Particular attention is paid to the justification for the use of animals, unnecessary duplication of studies, alternatives to animal use, early endpoints, pain and distress procedures, and euthanasia. Additionally the IACUC upholds that appropriate, documented training for principal investigators, technicians, and staff is in place prior to the initiation of any animal study, to include proper occupational health and safety requirements. Once a study is underway, the IACUC may perform post-approval compliance monitoring to verify quality animal care is intact.

d. Results:

The IACUC held six full committee meetings in 2007. The IACUC supported 24 active protocols during 2007, six of which were approved during the calendar year. Several amendments (20) were reviewed and approved. Two semi-annual facility inspections and program reviews were completed and reviewed. A training seminar was conducted in cooperation with Mahidol University and the Thai Association of Laboratory Animal Science (TALAS) in addition to training scheduled during normal meeting periods, totaling nine hours. Additionally, two IACUC members attended the 2007 American Association of Laboratory Animal Science (AALAS) convention.

e. Future Plans:

USAMC-AFRIMS will hold its third triennial Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALACi) site visit in March 2008. A major renovation of the Department of Veterinary Medicine animal facility is underway to
upgrade to current standards. Continued accreditation is expected, underscoring USAMC-AFRIMS’ commitment to quality animal care.

E. Department of Virology, AFRIMS FY07 Research Accomplishments

1. Title of Research Project: Prospective Study of Dengue Virus Transmission and Disease in Primary Schools and Villages in Kamphaeng Phet, Thailand

a. Investigators:

1. Principal Investigators:
   - In-Kyu Yoon, LTC, MD, MC (USAMC-AFRIMS)
   - Suwich Thampolo, MD, MPH
   - Dengue Office, Division of Vector-Borne Diseases
   - Ministry of Public Health (MOPH)
   - Chusak Pimgate, M.D., MC (USAMC-AFRIMS)

2. Associate Investigators (by institution):

   **Armed Forces Research Institute of Medical Science (AFRIMS):**

   **Department of Virology**
   - Robert V.Gibbons, M.D.,MPH, LTC, Chief
   - Richard G.Jarman, B.S., Ph.D, Chief, Laboratory Operations
   - Ananda Nisalak, M.D., Consultant in Arbovirology
   - Charity Ann M. Ypil-Butac, M.D., Consultant in data analysis
   - Butsaya Thaisomboonsuk, Ph.D., Head, Arbovirology Section
   - Chonticha Klunthong, Ph.D., Head, Molecular Research Section

   **Department of Entomology**
   - Jittawadee Murphy, Ph.D., MAJ, Chief
   - Thanyalak Fansiri, Entomology Study Coordinator, Mosquito Biology Section

   **Thai Ministry of Public Health (MOPH):**
   - Supamit Chunsuttiwat M.D., Senior Expert in Preventive Medicine, Disease Control Department, Ministry of Public Health

   **Institute of Urology and Nephrology, University College London, The Middlesex Hospital**
   - Henry A. F. Stephens, Ph.D., Clinical Scientist and Head of Tissue Typing
Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School (UMMS):

- Anon Srikiatkhachorn, M.D., Assistant Professor
- Daniel H. Libraty, M.D., Assistant Professor
- Alan L. Rothman, M.D., Associate Professor
- Sharone Green, M.D., Associate Professor
- Francis A. Ennis, M.D., Director

Department of Entomology, University of California, Davis:

- Thomas W. Scott, Ph.D., Professor of Entomology and Director, Davis Arbovirus Research Unit
- Amy C. Morrison, Ph.D., Assistant Research Entomologist

Department of Geography, San Diego State University:

- Arthur Getis, Ph.D., Stephen and Mary Birch Chair of Geographical Studies

a. Objectives:

The goal of the proposed study is to identify those factors that have the strongest influence on determining the early events in acute DV infections, and the eventual clinical manifestations of disease. An equally important goal is to characterize protective immune responses (e.g. CD4$^+$ and CD8$^+$ T-cell responses, neutralizing antibody responses) as we have found that low levels of pre-existing neutralizing antibodies to a subject’s own infecting virus isolate do not necessarily protect from symptomatic DV infection. We plan to prospectively identify host-specific factors (e.g., pre-existing memory T and B cell responses to DV, HLA genetic polymorphisms, viral burden and replication in the host), virus-specific factors (e.g. DV serotype, serotype infection sequence), and environmental factors (e.g. mosquito population patterns, mosquito viral burden) for asymptomatic and symptomatic secondary DV infections, particularly severe infections (DHF/DSS). Multi-year investigations are crucial to this study due to the year-to-year variations in the incidence and prevalence of circulating serotypes. An improved understanding of the correlations between the host, viral, and environmental factors and dengue disease severity will contribute to DV vaccine development and testing.

b. Study Specific Hypotheses:

1. Subjects with pre-existing neutralizing dengue antibodies above a definable threshold will be protected from DV infection or severe disease on subsequent exposure to virus.
2. The frequency of pre-existing CD4+ and CD8+ T-cells and their specific cytokine responses to stimulation with DV antigens will correlate with disease severity (protection or enhancement) and the plasma viral RNA levels measured in secondary DV infections.

3. Specific serotype sequence combinations of DV infections will elicit qualitatively and quantitatively distinct immune responses associated with illness of varying severity.

4. Higher viremia levels will be seen in secondary DEN-2 and DEN-4 virus infections in subjects with higher levels of in vitro antibody-dependent enhancing capability of pre-illness blood samples.

5. DV infection rates will cluster in households around a DV-infected index case and a correlation will exist between the number of susceptible contacts, and associated mosquito density, and mosquito infectivity (viral RNA levels).

6. DV disease severity will correlate with peak plasma viremia levels and associated mosquito density and mosquito infectivity (viral RNA levels).

7. Genes encoded within the human MHC, the NK killer inhibitory receptor (KIR) gene complex on chromosome 19, and the Fc gamma receptor gene complex on chromosome 1 influence the susceptibility, severity and resistance to primary and secondary DV infections.

d. **Methods:** In this study, we:

i) Continue the successful prospective, school-based, study platform to study dengue epidemiology in primary school children in KPP province, and

ii) Conduct a village-based, cluster surveillance study.

(a) This is a prospective school-based study of 2,000 children, which began in 2003 and will end in January 2008. Students in K2 to grade 6 are recruited and enrolled into the study. Baseline demographics are recorded and study numbers assigned. Each subsequent year, new K1-Grade 5 students are newly enrolled. Students are followed until they are either disenrolled, withdrawn by their parent/guardian, graduate from Grade 6 or when the study ends. Every year, plasma (PBMCs for Dengue Season 1 only) is collected from the entire cohort at the beginning of the surveillance period (June). Plasma and PBMCs are collected from the entire cohort at the end of the surveillance period (January). The hemagglutination inhibition (HAI) assay is performed on paired sera from the beginning and end of the surveillance period to assess for flavivirus seroconversion. Plasma and PBMCs obtained at the end of the surveillance period in January serve as pre-illness samples in subjects who have a DV infection that same calendar year.
During the active surveillance period extending between June and November, those children who are absent from school (or who report ill to the teacher), will be evaluated either by a village health worker or AFRIMS nurse using a questionnaire and oral temperature measurement. Any child who has a documented fever (temperature $\geq 38$°C) or reports illness with subjective fevers during the prior 7 days, is transported to the Public Health Office (PHO) where a public health nurse will do an evaluation. An acute blood specimen will be drawn. The child will be referred to the hospital at the discretion of the public health nurse. About 14 days later, an AFRIMS nurse visits the child to administer another questionnaire and to draw a convalescent blood specimen. The acute and convalescent specimens are evaluated by the AFRIMS dengue/JE IgM/IgG ELISA and HAI. The acute specimen will be evaluated further by dengue RT-PCR (and virus isolation techniques).

(b) Cases ‘triggering’ a cluster investigation are identified between Monday and Thursday of each week during the School-Based Component active surveillance period. Most specimens from acutely ill children arrive at the field station laboratory by 3pm each day. Upon arrival of the specimen, the database is reviewed to assess whether the child meets all index case inclusion and exclusion criteria. The field teams are notified of a possible case. The DV RT-PCR result (positive or negative) will normally be available by 11AM the following morning. No more than 30 positive and 30 negative clusters (as defined by the RT-PCR result of the index case) will be initiated in any given year. Once triggered, an Advance Team composed of a nurse and an entomological team supervisor visits the village and begins the consent form process. The exact location of all houses in each participating village has previously determined using a Global Positioning System (GPS) unit. Data points will be used to construct a digital map which will enable the team to precisely identify houses located within 100 meter radius of the index case and rapidly assess the likelihood of enrolling a minimum of 10 contacts. Once at least 10 contacts have been consented, the field teams will be dispatched to the village where the consent form process will continue. A clinical nurse will review the consent form, answer questions, address parental concerns, and obtain informed consent from the parents of susceptible contact children (ages 6 mo-15 yrs) residing within a 100 meter radius of the index household. Following the acquisition of parental consent, blood samples will be collected from 10-25 contacts. Those parents (and children) who are unavailable to be consented (and bled) are visited that same evening or the following morning. The clinical team will return to these homes approximately 5, 10 and 15 days after the initial visit to perform clinical assessments. The children bled on day 0 (initial specimen) are re-bled on approximately day 15 (follow-up specimen). DV RT-PCR will be performed on all acute specimens. If the day 15 blood is positive RT-PCR we will go out to draw blood on day 30 for doing ELISA. Dengue IgM/IgG ELISAs are performed on paired initial and follow-up specimens.

An entomological team collects mosquitoes, administers questionnaires, and performs insecticide spraying within the pre-determined meter radius of the index household. Another entomological team will collect mosquitoes but not perform insecticide spraying around the classroom and school bathroom areas of the index case.
e. **Results:**

1. School cohort study

   Year 2007 is the 5\textsuperscript{th} and final year of the study. At the beginning of the active surveillance period (1 June 2007), there were a total of 2,060 children enrolled in the school-based cohort. By the end of the surveillance period (1 December 2007), there were a total of 2,011 children who underwent blood draw for post dengue season serology testing.

   During the school-based active surveillance period in 2007, 1,782 school absences were evaluated. Of these, 757 were related to acute or recent (reported within 7 days prior to evaluation) febrile illness and 640 (85 %) of these had acute blood draws for dengue RT-PCR testing and EIA testing. Thirty-nine cases were positive for dengue by EIA (38 Acute Secondary Dengue Infection and 1 Recent Secondary Dengue Infection). Thirty-six children were positive for dengue by RT-PCR: 23 DEN-1, 8 DEN-2, 2 DEN-3 and 3 DEN-4. Six children with positive dengue RT-PCR were admitted to Kamphaeng Phet Provincial Public Hospital. All were discharged in good condition from the hospital.

   Based on RT-PCR results from acute specimens from ill children in the school-based surveillance, 30 cluster investigations were initiated from June until November 2007. 16 of these were based on dengue positive index cases and 14 were based on dengue negative index cases. The dengue positive index cases were identified as 12 DEN-1, 3 DEN-2, and 1 DEN-4.

   Five samples showed discordance results and were further investigated. At the beginning of the active surveillance period (1 June 2006), there were a total of 2,086 children enrolled in the school-based cohort. By the end of the surveillance period (1 December 2006), there were a total of 2,045.

   To make up for the expected eflux of school cohort subjects due to graduation from Grade 6 in March 2007, 387 new children were enrolled so that a total of 2432 children underwent scheduled blood draw in January 2007 from 11 participating schools. Of the new enrollees, 204 (53 %) were in Grade K1.

   In the school-based active surveillance period in 2006, 1837 school absences were evaluated. Of these, 871 were related to acute or recent (reportedly within prior 7 days of evaluation) febrile illness and 764 (88 %) of these had acute blood draws for RT-PCR testing and EIA testing. Eighty-four cases were positive for dengue by EIA. Seventy-two children were positive for dengue by RT-PCR: 43 were DEN-1 and 21 were DEN-4. Six JEV infection were diagnosed serologically but had no signs and symptoms of encephalitis. Twenty-two children with positive dengue RT-PCR were admitted to Kamphaeng Phet Provincial Public Hospital. All were discharged in good condition from the hospital.
Based on RT-PCR results from acute specimens from ill children in the school-based surveillance, 39 cluster investigations were initiated from June until November 2006. Twenty-two of these were based on dengue positive index cases and 17 were based on dengue negative index cases. The dengue positive index cases were identified as thirteen DEN-1, one DEN-2, eight DEN-4.

Overall during the 2006 active surveillance period, there were significantly more dengue cases than during the prior two surveillance years (2004 and 2005). Analysis of this season's obtained data is ongoing.

Number of Cases Related to Fever and Bled in 2007

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<th>School no.</th>
<th>T ≥ 38°C on evaluation during a given illness</th>
<th>If &lt; 38°C but the child reported to have subjective fevers during 7 days prior to evaluation</th>
<th>Children with reported or suspected fever (within past 7 days)</th>
<th>Bled (acute and 14 ± 4 days later)</th>
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Results of Serology Testing and Dengue RT-PCR Analysis Serology with Dengue RT-PCR results

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<td>Total</td>
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### School-Based Acute Illness RT-PCR Dengue Serotype with Confirmed EIA

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2. Village-based study

During the active surveillance period (1 June to 30 November 2007), a total of 30 cluster investigations were conducted: 16 positive clusters (involving 249 child contacts) based on dengue PCR positive index cases and 14 negative clusters (involving 222 child contacts) based on dengue PCR negative index cases.

Dengue RT-PCR was performed on specimens collected on days 0 and 15 from the child contacts (ages 6 months to 15 years) of index cases. Of these specimens, 26 samples in 16 positive clusters were positive for dengue by PCR; 2 samples in 14 negative clusters were positive for dengue by PCR.

No contacts were hospitalized in this season.

Out of 774 Aedes aegypti mosquitoes collected from cluster investigations, 8 were positive for dengue by RT-PCR (7 DEN-1 and 1 DEN-2). Seven of these positive mosquitoes were collected from positive clusters and the dengue serotypes of these mosquitoes were the same as that of their respective positive index cases. One positive mosquito (DEN-1) was from a negative cluster.

During the active surveillance period (1 June to 30 November 2006), a total of 39 cluster investigations were performed: 22 positive clusters (involving 339 child contacts) based on dengue PCR positive index cases and 17 negative clusters (involving 233 child contacts) based on dengue PCR negative index cases.
Dengue RT-PCR was performed on day 0 and day 15 specimens of the child contacts (ages 6 months to 15 years) of index cases. Of these specimens, 45 were positive for dengue by PCR in the 22 positive clusters; 6 were positive for dengue by PCR in the 17 negative clusters.

Four contacts were hospitalized for acute secondary dengue infection. All of them were discharge having recovered from their illness.

Out of 1442 Aedes aegypti mosquitoes collected from cluster investigations, 8 were positive for dengue by RT-PCR (4 DEN-1, 1 DEN-2, and 3 DEN-4). All of these positive mosquitoes were collected from positive clusters and the dengue serotype of these mosquitoes were the same as their respective positive index cases.

Cluster investigations:

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<th>Cluster No.</th>
<th>Subject No. of Index case</th>
<th>School No.</th>
<th>Type of cluster</th>
<th>Serotype of Index Case</th>
<th>Serology</th>
<th>Number of Enrollees</th>
<th>Number of Houses</th>
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<td>022300KDS51</td>
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<td>NEG</td>
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<td>Serologically on DAY 0</td>
<td>Serologically on DAY 15</td>
<td>Serotype on DAY 0</td>
<td>Serotype on DAY 15</td>
<td>Mosquito PCR (Female Ae. aegypti)</td>
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Positive Clusters:

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<th>Serologically on DAY 15</th>
<th>Serotype on DAY 0</th>
<th>Serotype on DAY 15</th>
<th>Mosquito PCR (Female Ae. aegypti)</th>
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<th>Serotype on DAY 0</th>
<th>Serotype on DAY 15</th>
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* Recent Secondary Dengue Infection

1. **Future Plans:**

   The post surveillance period blood draw of the school cohort is currently being undertaken. Data analysis of all results obtained during 2004-2007 will be performed in the next few months. Several manuscripts detailing interim study analyses are being prepared for publication. The final active surveillance period will occur from June 2007 to Nov 2007. Interaction with human subjects will end with the final post surveillance blood draw in January 2007. Data analysis and laboratory testing will continue beyond that point. Close out activities of the study are ongoing.

2. **Title of Research Project:** The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue

   a. **Investigators:**

      1. Principal Investigators:

67
• Siripen Kalayanarooj, MD (Queen Sirikit Institute of Child Health, Bangkok)
• Robert V. Gibbons, MD (USAMC-AFRIMS)

2. Associate Investigators:

• Ananda Nisalak, MD (USAMC-AFRIMS)
• Pra-orn Supradish, MD (QSNICH)
• Anchalee Krautrachue, MD (QSNICH)
• Lawan Wongtapradit, MD (QSNICH)
• Narong Nithipanya, MD (QSNICH)
• Warangkana Ratanaprakarn, MD (QSNICH)
• Anon Srikiatkhachorn, MD, Assistant Professor (UMMS)
• Daniel H. Libraty, MD, Assistant Professor (UMMS)
• Irene Bosch, PhD, Assistant Professor (UMMS)
• Alan L. Rothman, MD, Associate Professor, (UMMS)
• Sharone Green, MD, Associate Professor, (UMMS)
• Francis A. Ennis, MD, Director (UMMS)
• Henry A. F. Stephens, Ph.D., Clinical Scientist and Head of Tissue Typing, (University College London)

b. Objectives:

To identify the immunopathological mechanisms of dengue hemorrhagic fever (DHF), to analyze differences between DHF resulting from primary versus secondary infections, to identify a sensitive method for detection of plasma leakage, and to characterize the dengue specific T cell response. The project encompasses studies from 2003 to 2007.

c. Study Specific Objectives:

1. Characterize genetically and functionally the dengue virus-specific T lymphocyte response during, and after dengue virus infections (intracellular cytokine staining, HLA tetramers, T cell receptor gene usage).

2. Analyze interactions between dengue virus, virus-specific antibodies, and target cells in PBMC during acute dengue virus infections (quantify and characterize immune complexes, define the major cellular compartments in PBMC supporting dengue viral replication).

3. Determine if ultrasound or interstitial fluid albumin levels can predict early plasma leakage and shock. The ability to detect these shifts early in disease progression may help in prediction algorithms for DHF and permit early intervention with new therapies in the at-risk population.
4. Assess the utility of plasma sNS1 levels in predicting disease severity for subjects with primary or secondary infection due to any of the four dengue serotypes.

5. Analysis of the activation of innate immune responses in vivo during acute dengue virus infections (chemokine gene expression, inhibitory and activating NK receptor expression).

6. Identification of polymorphisms in immune response genes associated with disease manifestations and cellular immune responses during dengue virus infections (MHC class I and II, Fcγ receptor gene, KIR genes, NK receptors) and MHC class I chain-related (MIC) genes (ligands for lectin-like receptors),

7. Quantitative of viral burden in plasma and cell subsets of peripheral blood mononuclear cells (PBMC) for all four serotypes in primary and secondary dengue virus infections and determine if there is a correlation between viral load in these compartments and disease severity.

8. Measurement of neutralizing antibody elicited by primary infections, over an extended period of time. Few long-term studies of antibody titer following dengue infection have been performed previously. Neutralizing antibody will be measured on study day 1, 6 months, 1 year, and annually thereafter. Understanding wild type responses will help to set realistic standards for vaccines. Mature secondary responses determined by neutralization six months or more after infection will be correlated with class II HLA type.

9. Determination of memory T-cell responses following primary and secondary dengue infections, over an extended period of time. Understanding wild type responses and the durability of these responses over time will be crucial in setting standards for testing of candidate dengue vaccines.

10. Continue sequencing portions of the dengue genome from patients with mild dengue fever and those with severe DHF/DSS to test a hypothesis that severity of disease is strain related. In addition, compare the kinetics of plasma viral load and immune responses in primary and secondary infections with different DV serotypes.

11. Evaluate the accuracy of sequentially measured semi-quantitative d-dimer assay, as compared to standard clinical parameters, at predicting the clinical progression to severe clinical dengue.

d. Methods:

Children were enrolled if they were suspected of having an early DV infection (without evidence of DHF) or a fever without an identifiable source. Inclusion criteria included an oral temperature ≥ 38.5°C, fever onset not longer than 72 hours prior to the initial evaluation, weight > 6kg, flushed face, signed consent by parent or guardian. After informed consent is obtained, subjects are admitted to the hospital and a blood
specimen obtained. The result of the plasma test for DV RNA by RT-PCR is available the morning of study day 2. Children who are DV RT-PCR-negative are given the opportunity to leave the study, or to continue in the study for clinical observation. Those children remaining in the hospital undergo inpatient observation until one day following defervescence (fever day +1). Clinical information is collected and recorded daily. Radiographic studies are performed as outlined in the protocol. Serial blood samples are collected and analyzed for routine and dengue-specific blood and plasma tests were conducted to include, but not limited to:

- CBC, WBC differential, AST, Albumin
- Hemagglutination inhibition (HAI) assay for dengue
- Antibody-capture DV IgM/IgG enzyme immunoassay (EIA)
- RT-PCR for dengue, Plasma viremia titers
- Dengue virus isolation in Toxorhynchites splendens and typing
- IL-15, IL-18, MIP-1a, MIP-1b, and MCP-1, CD69, CD38, and Ki-67
- Labeled antibodies to identify T cell subsets, NK cells and B cells
- NS1 (soluble NS1 and anti-NS1 antibodies)
- Complement assays

**e. Results:**

There were 54 positive PCR cases in this report period (DEN 1 = 31; DEN 2 = 6; DEN 3 = 11, DEN 4 = 6; Negative PCR = 30. All subjects had ultrasound evaluation for plasma leakage. A subset (n=45) of dengue positive cases had interstitial fluid sampling done. Two cases were lost to follow-up (non-dengue diagnoses). No serious adverse events occurred.

**f. Future Plans:**

Long-term clinical follow-up is ongoing for prior years of enrollment. Analysis for markers that predict disease severity (NS1 protein/antibody levels, immune activation markers), that indicate plasma leakage is or will occur, and that indicate immunity will be done. Statistical analysis of DHF resulting from primary versus secondary DV infections with regard to the role viral serotype, viral burden and virus-antibody complexes plays on resulting disease severity is planned. Characterization of the dengue specific T cell response with regard to the magnitude of T cell expansion during infection and the functional characteristics of these cells is also planned.

3. **Title of Research Project:** A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Infants

**a. Background:**

The US Army seeks to acquire a licensed vaccine capable of protecting soldiers and their families from disease caused by infection with the dengue viruses. The Kingdom of Thailand shares this goal. For over 50 years the US Army has been active
in developing and testing various vaccine candidates. This study represents the first use of the most promising Army dengue vaccine candidate in an overseas (Thailand), infant population.

b. Principal Investigators:

- Robert V. Gibbons, LTC, MC (USAMC-AFRIMS)
- Veerachai Watanaveeradej, MD, Phramongkutklao Hospital (PMK), Thailand

Sub Investigators:

- Sriluck Simasathien, MD (PMK)
- Angkool Kerdpanich, MD (PMK)
- Ananda Nisalak, MD (AFRIMS)
- In-Kyu Yoon, MD (AFRIMS)
- Richard G. Jarman, PhD (AFRIMS)
- Supamit Chunsuttiwat, MD., MPH(MOPH)
- Bruce L. Innis, MD (GSK)
- Stephen J. Thomas, MAJ, MC (WRAIR)
- Kenneth H. Eckels, PhD (WRAIR)
- J. Robert Putnak, PhD (WRAIR)

c. Objectives:

The primary objective of this study is to assess the kinetics of dengue neutralizing antibodies to each dengue virus serotype one and four years following dose 2 of dengue/control vaccination in the setting of potential wild-type dengue virus exposure. And the secondary objective is to conduct passive surveillance for hospitalized dengue through annual follow-up visits and telephone contact between annual visits.

d. Methods:

1. Screen and enroll 51 healthy, flavivirus naïve, Thai infants between the ages of 12 and 15 months.

2. Provide 2 doses of the WRAIR tetravalent dengue vaccine as outlined in the study protocol.

3. A booster dose will be given at Year 3 post primary vaccination to all subjects previously vaccinated with dengue vaccine in protocol amendment 4.

4. Closely monitor the infants following each dose of vaccine for safety and tolerability.
5. Assess the immunogenicity of the dengue vaccine as outlined in the study protocol.

6. Follow-up for four year following dose 2 of dengue/control vaccination to assess for dengue-related hospitalizations and dengue antibody kinetics.

e. Results:

Forty-nine subjects have been enrolled in the long term follow up study (amendment 5) for assessment of dengue-related hospitalizations and dengue antibody kinetics after vaccination. Two subjects had withdrawn because of migration from the study area.

The clinical follow-up visit (year 2) and telephone contact (year 2 and a half) after primary vaccination have been completed. There were three serious adverse events (1 urinary tract infection, 1 dengue infection, 1 pneumonia), none of which were deemed related to study vaccine:

f. Future Plans:

The investigator desired to amend the protocol to administer a booster dose of post-transfection F17 dengue vaccine to all subjects enrolled in the dengue-001 amendment 4 who received dengue vaccine. The booster will be given at Year 3 following the primary (dose 1 and 2) vaccination series. In addition, the amendment 6 would allow for the collection of peripheral blood mononuclear cells (PBMCs) at the time of booster vaccination, and twice again at one month and two year following booster vaccination from all subjects. Long term follow up of the subjects would continue as outlined in the original protocol and previously approved amendments.

4. Title of Research Project: A Phase I/II, Open, Five-Year, Clinical Follow-Up Study of Thai Children Who Participated in Dengue-003 ("A Phase I/II Trial of A Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Children") With Evaluation of A Booster Dose Given One Year After Primary Dengue Vaccination Series

a. Investigators:

- Robert V. Gibbons, LTC, MC, USAMC-AFRIMS
- Sriluck Simasathien, MD, Phramongkutklao Hospital (PMK), Bangkok, Thailand

b. Objectives:

The primary objective of this study is to assess the immunogenicity of a booster dose of dengue vaccine administered approximately one year following the second dose.
c. **Methods:**

1. Enroll seven flavivirus antibody-naïve Thai children who participated in Study Dengue-003.

2. Provides booster dose of dengue vaccine given one year after the last dose of dengue vaccine in Dengue-003.

3. Assess the persistence of antibody one and two year following the booster dose.

4. Assess the safety and immunogenicity of a booster dose of dengue vaccine administered one year after primary dengue vaccination series.

5. To characterize cell mediated immune responses to each dengue virus serotype two years following the booster dose.

6. Four annual visits follow-up for passive surveillance for hospitalized dengue after 1 month post booster dose.

d. **Results:**

The study was approved to enroll 7 flavivirus antibody-naïve Thai children who participated in study Dengue-003 who received two doses of dengue vaccine. Seven subjects were enrolled. There were no subject withdrawals from the study. All subjects were administering a booster dose in February 2005 (Year-1). The protocol amendment 1 was approved and allow for acquire peripheral blood mononuclear cells (PBMCs) and sera to characterize cell-mediated immunity responses to vaccination and correlate these with N antibody titers at year 3 follow-up. The clinical follow-up visit 7 (Year - 3) has been completed.

e. **Future Plans:**

Next schedule follow up visit (Year-4) will be held on March 2008. The data analysis has been analyzed.

5. **Title of Research Project**: A Phase II, Prospective, Randomized, Double Blind, Placebo Controlled Field Efficacy Trial of a Candidate Hepatitis E Vaccine in Nepal WRAIR# 749, HSRRB Log# A-9117.1

a. **Investigators:**

**Principal Investigators:**

- M. P. Shrestha (WARUN)
- R. M. Scott (WARUN)
Associate Investigators:

- S. B. Bajracharya (SBH)
- M. P. Mammen (USAMC-AFRIMS)
- R. A. Kuschner (WRAIR)
- K. S. A. Myint (USAMC-AFRIMS)
- P. R. Pandey (SBH)
- K. J. B. Rana (SBH)
- K. N. Rayamajhi (SBH)
- J. Seriwatana (WRAIR)
- G. R. Shakya (SBH)
- G. B. Thapa (SBH)
- S.K. Shrestha (WARUN)
- N. Thapa (SBH)
- C. Jhang

b. Objectives:

To evaluate the protective efficacy for the prevention of definitive and probable hepatitis E disease provided by the candidate hepatitis E vaccine administered according to a 0, 1 and 6 month schedule.

c. Methods:

A candidate recombinant baculovirus expressed hepatitis E virus (HEV) vaccine was found to be safe and immunogenic in 88 American and 44 Nepalese volunteers. A 20μg formulation was selected for further evaluation in a randomized double blind placebo controlled efficacy trial in susceptible, active duty Nepal Army volunteers. The clinical phase started 30 April 2001 at the Nepalese Army Shree Birendra Hospital. Of 5,571 consenting volunteers screened, 3,692 were susceptible to HEV. Two thousand volunteers (8 females, 1,992 males) were enrolled, receiving either placebo or 20μg of active candidate vaccine. Volunteers were vaccinated at 0, 1, and 6 months with sera collected at months 0, 1, 3, 6, 7, 13, and 24. One tenth of the volunteers were followed on days 1, 3, 5, and 7 after each vaccination for local and general solicited adverse events (SoAE). Non-serious adverse events (NSAE) were recorded for 30 days after each vaccination and serious adverse events (SAE) were also collected throughout the study period. Sera and stool from cases meeting clinical and biochemical criteria compatible with viral hepatitis, were examined for HEV RNA by a reverse transcriptase-polymerase chain reaction, and serologically for HEV IgM and Ig, HAV IgM, HBsAg, HBcIgM and HCV IgG. Pregnancy was also recorded as SAE. Two subjects became pregnant during the study, about one year after third dose. The outcome was favorable in both cases. Seven deaths were documented and verified by Data Safety Monitoring Board (DSMB). They were 4 killed in action, 1 accident, 1 cholangiocarcinoma and 1 undetermined.
Of the 2000 enrolled subjects, 2000 received dose 1, 1890 received dose 2 and 1794 dose 3 (and 31 received dose 1 and 3). A total of 1566 subjects returned for concluding visit (Encounter# 8). Clinical study encounter with the subjects was concluded in January 2004.

d. Results:

The clinical phase of the trial has been successfully completed according to the protocol amendment 9. The DSMB unblinded 111 cases of suspected hepatitis following SOP provided by GSK on 23 June 2004. The study results were reported in the HEV Symposium of the American Society of Tropical Medicine and Hygiene (ASTMH) annual meeting in December 2005 as well as in the *New England Journal of Medicine* in March 2007.

The clinical portion of the study was completed in January 2004. A final clinical study report was submitted to the Food and Drug Administration on 21 August 2006.

e. Future Plans:

The WRAIR protocol # 749 reached five year term in October 2006. Memorandum requesting extension of this protocol for 18 months was submitted to Office of Research Management (ORM) on 12 January 2007. The study remains open in order to complete the plan to inform volunteers about the results of the study and the formulation (placebo or vaccine) they received. Measures are underway to provide a letter of appreciation to the volunteers who participated in the study.

The source document in all subject folders at WARUN and the Regulatory Documents at AFRIMS have been scanned and secured. The electronic files have also been secured off-site. Management of hard copy source documents and regulatory documents at WARUN has been discussed.

6. **Title of Research Project:** Japanese Encephalitis Surveillance in Nepal

a. **Investigators:**

- Robert V. Gibbons LTC, MC (USAMC-AFRIMS)
- Sanjaya Kr. Shrestha, MBBS, MD (WARUN, Kathmandu)

b. **Objectives:**

1. To determine JE diagnosis validation in Nepal

2. To determine the percentage of Japanese encephalitis and other causes of encephalitis among blinded samples provided to AFRIMS from Nepal.
c. **Methods:**

1. Blinded samples provided by National Public Health Laboratory (NPHL) to WARUN without personal identifier.
2. The specimens will then be processed, packaged, and shipped to the Department of Virology, AFRIMS, Bangkok.
3. Definitive quality control testing for JE diagnostics will be done at USAMC-AFRIMS.

d. **Results:**

In this report period, 440 samples including 83 cerebral spinal fluid (CSF) and 357 serum samples were sent from National Public Health Laboratory (NPHL) in Nepal to AFRIMS for JE IgM-capture ELISA assay. The results are shown in the table below.

**Result of blinded samples tested by JE IgM-capture ELISA assay.**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Total</th>
<th>JE (IgM ≥ 40 units)</th>
<th>Negative (IgM &lt; 40 units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>83</td>
<td>7 (8.43%)</td>
<td>76</td>
</tr>
<tr>
<td>Serum</td>
<td>357</td>
<td>72 (20.16%)</td>
<td>285</td>
</tr>
<tr>
<td>Total</td>
<td>440</td>
<td>79 (17.95%)</td>
<td>361</td>
</tr>
</tbody>
</table>

Seven of 83 CSF samples (8.43 %) showed positive result. 72 of 357 serum samples (20.16 %) showed positive result. For overall, we can detect JE IgM 17.95 % (79 samples) of all 440 samples from NPHL.

e. **Future Plans:**

Plans for CY2008 include: analysis of the data collected to date; discussion of the data with investigators at WARUN and NPHL; and manuscript writing. Remaining negative samples will be tested for other possible pathogens of encephalitis.

7. **Title of Research Project:** Influenza Surveillance in Southeast Asia

a. **Background:**

Influenza is an important cause of morbidity and mortality among populations at the extremes of age. Continuous viral surveillance and isolation of influenza viruses provides important information for the creation of annual vaccine formulations based on the identification of new and emerging strains of influenza. AFRIMS has been actively involved in influenza surveillance in Thailand and Nepal for several years. Expansion of AFRIMS influenza surveillance activities in the region will enhance DoD’s ability to detect and respond to an outbreak of pandemic influenza early in the course of the pandemic.
b. **Investigators:**

- Khin Saw Myint, M.D. (USAMC-AFRIMS)
- Robert V. Gibbons, M.D. MPH (USAMC-AFRIMS)
- Richard G. Jarman, Ph.D (USAMC-AFRIMS)
- Kamnuan Ungchusak, M.D., MPH (MOPH, Thailand)
- Chusak Pimgate, M.D. (USAMC-AFRIMS)
- John Mark Velasco, M.D. (USAMC-AFRIMS)
- Charity Ann Ypil-Butac, M.D. (USAMC-AFRIMS)
- Sanjaya K. Shrestha, M.D. (WARUN, Nepal)
- Rodney Coldren, M.D. (GEIS, Thailand)
- Linda C. Canas, B.Sc. (AFIOH, U.S.A.)

c. **Objectives:**

(a) To collect and characterize influenza viruses circulating within the human population in Asia including Thailand, Nepal, the Philippines and from the US Embassy/Consulate in the region.

(b) To provide influenza surveillance data to the US CDC and WHO surveillance network towards the annual re-formulation of the influenza vaccine.

(c) To report the circulating influenza strains and other respiratory pathogens to the Ministry of Health of host countries.

d. **Methods:**

Samples were collected from patients with clinically suspected influenza infection (case definition includes fever or history of fever $>38^\circ C$ within 72 hours with cough or sore throat). Participating physicians and staff identified patients who met the case definition during routine clinic visits. Emphasis was placed on quality samples that may provide genetic data for future influenza vaccines rather than a large number of samples to be tested for incidence and prevalence data. Clinical history forms, including basic demographic and clinical information, were completed by the OPD nurse or AFRIMS research nurses. Nasal specimens were collected and tested at field sites for rapid feedback to the physicians and patients. Nasal/throat swabs were collected and placed in viral media and stored at $-70^\circ C$. All specimens were shipped on dry ice to AFRIMS for typing and subtyping using molecular techniques. An aliquot is shipped to Armstrong Laboratory, San Antonio, Texas, for virus isolation and other definitive diagnosis.

e. **Results:**

AFRIMS continues to work in close collaboration with the US and Thai CDCs, the Thai Ministry of Public Health, and with NAMRU-2. The influenza surveillance is
divided into individual country projects each for Thailand, Nepal, U.S. Embassies in the region and the Philippines. Provision of staff, equipment, infrastructure development, and training is well underway. AFRIMS has recently set up training on molecular diagnostics in Kathmandu, Nepal and Kamphaeng Phet has plans to do similar trainings in the Philippines. The infrastructure of a dedicated respiratory pathogens laboratory is already completed. This will allow immediate processing of influenza samples, and ensure on-time reporting. Construction of a BSL-3 laboratory is well underway. The respiratory laboratory will be equipped with a real-time pcr machine, serology set up, viral isolation, computers for data entry, and capabilities for specimen storage and archiving.

1. Progress on influenza surveillance in U.S. citizens in U.S. Embassy/Consulate in Asia: the subject enrollment started since the protocol was approved by HURC on Feb 2006. There are now a total of 14 medical units/clinics from 13 countries in Asia participating in this study - Thailand (Bangkok), Burma (Rangoon), Bangladesh (Dhaka), India (New Dehli), Pakistan (Islamabad), Mongolia, Laos (Vientiane), Malaysia (Kuala Lumpur), Sri Lanka (Colombo), Vietnam (Hanoi and Ho Chi Minh City), Nepal (Kathmandu), China (Beijing), and Philippines (Manila). Total number of subjects enrolled since the study started till Dec 07 is 65; majority was from the US Embassy Medical Unit in Bangkok. The gender distribution was about equal.

2. Progress on influenza surveillance in Nepal: During the period of 12 January to 16 December 2007, 668 specimens were received from active sentinel surveillance sites in Nepal and from outbreak in Jhapa in July 2007. Real-time PCR done at AFRIMS reported 44.2% as FLU A/H1, 0.3% FLU A/H3, and 10% FLU B.

3. Progress on influenza surveillance in the Philippines: From 01 March to 30 November 2007, there were a total of 157 influenza-like illness samples collected from 5 sentinel sites in Cebu City, Cebu, Philippines. Male to female ratio was 1:1 and ages ranged from 6 months to 54 years old with a majority of the specimens collected from the 1-4 year old age group (54%) followed by the 5-9 year old age group (18%).

A total of 151 specimens underwent influenza real-time PCR subtyping at the Armed Forces Research Institute of Medical Sciences (AFRIMS). Twenty-six samples (17%) tested positive for influenza A/H3; 27 (18%) tested positive for influenza A/H1; 2 (1%) tested positive for Influenza B. No respiratory virus was detected in 96 (64%) of the specimens.

So far, 47 specimens have undergone further virus isolation and molecular characterization at the Air Force Research Institute for Operational Health (AFIOH). Coxsackie B was positive for 2 cases (4%); Parainfluenza 1 was positive for 2 cases (4%); Parainfluenza 3 was positive for 3 cases (6%). Molecular characterization was performed on all specimens positive for Influenza A. Of these, 10 Influenza A/H3N2 viruses contained a total of 13 mutations at key regions of the virus genome. Five of these 10 viruses contained novel mutations. Due to the biochemical nature in amino acid changes within key antigenic areas of these viruses, the potential efficacy of
current influenza vaccines may be reduced. Additional characterization of these novel mutations is currently being performed at the U.S. Centers for Disease Control.

4. Progress on influenza surveillance in Thailand: Total number of specimens enrolled for 2007 is 409. Real Time PCR result reported 7.1% FLU A/H1, 12.5% FLU A/H3, and 16.4% FLU B.

f. Future Plans:

1. To expand surveillance sites to include Manila (V Luna General Hospital) in the Philippines.

2. To expand surveillance to other countries in the region (Bhutan, Maldives and Bangladesh).

3. Set up BSL-3 facility at AFRIMS.

4. Set up Real-Time RT-PCR at KAVRU (Kamphaeng Phet) and PAVRU (Philippines).

8. Title of Research Project: Sentinel Surveillance for Emerging Diseases Causing Dengue-like or Acute Encephalitis Syndrome in the Philippines (SEDP)

a. Principal Investigators:

Armed Forces Research Institute of Medical Science (AFRIMS): Department of Virology

- In-Kyu Yoon, LTC, MC (USAMC-AFRIMS)
- Maria Theresa Alera, M.D (San Lazaro Hospital, Manila, Philippines)

Other Study Personnel:

Armed Forces Research Institute of Medical Science (AFRIMS): Department of Virology

- Charity Ann Ypil-Butac, M.D.
- John Mark Velasco, M.D.
- Robert V. Gibbons, M.D, MPH
- Richard G. Jarman, PhD
- Ananda Nisalak, M.D
- Butsaya Thaisomboonsuk, PhD
- Piyawan Chinnawirotipsan, PhD
- Thidarat Intararit, R.N.
Department of Entomology

- James Jones, Ph.D., LTC, MSC

San Lazaro Hospital

- Efren Dimaano, M.D

Philippine-Department of Health (DOH):

- Lyndon Leesuy, M.D.
- Vito G. Roque, Jr., M.D.
- Mario S. Baquilod, M.D., MPH

World Health Organization (WHO)

- Raman Velayudhan, M.D.

b. Objectives:

Primary objectives: Epidemiology

General:

To determine the spectrum of emerging diseases causing dengue-like syndrome (DLS) or acute encephalitis syndrome (AES) in the Philippines

Specific:

1. To determine the proportion of dengue-like syndrome that is caused by:
   - Dengue
   - Leptospirosis
   - Chikungunya
   - Scrub typhus
   - Murine typhus

2. To determine the proportion of acute encephalitis syndrome that is caused by:
   - Japanese encephalitis
   - Rabies
   - West Nile

Secondary objectives: Laboratory and Public Health Infrastructure

1. To enhance the diagnostic capabilities of the Philippines.
(2) To determine the mosquito species associated with the transmission of Japanese encephalitis (JE) and West Nile viruses.

c. Methods:

Study Design:

This was a cross-sectional, hospital-based passive surveillance study conducted among admitted patients in San Lazaro Hospital, a tertiary government hospital located in Manila, Philippines.

Study Population:

The study population consisted of male and female patients 2 years old and above who present with dengue-like syndrome or acute encephalitis syndrome and who signed an informed consent to participate in the study. Patients with DLS were enrolled in the study if they met the following inclusion criteria: any patient with history of fever (temperature of 38°C and above) within the past 2-7 days with either one of the following criteria: a positive tourniquet test, an eschar, migratory polyarthritis or calf pain OR two of the following, namely: headache, generalized rash, myalgias, arthralgias, retro-orbital pain or icteric sclerae. Patients with AES who present with acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) and/or new onset of seizures (excluding simple febrile seizures) were also enrolled. Criteria for exclusion were explainable causes of DLS or AES, AES preceded or associated with exanthem and known pregnancy.

Data Collection:

Patients who met the enrollment criteria were interviewed and examined. The patient’s personal, demographic, clinical and laboratory data were recorded. Acute blood specimens were drawn. In addition, CSF samples were obtained from AES patients. Initial laboratory testing focused on probable etiologies given the recognized endemic pathogens. Cases that remain undiagnosed after the initial stage of testing were further characterized using specialized testing to identify emerging pathogens. Patients were advised to come back 14 days after onset of illness where a convalescent serum was drawn.

Laboratory testing was conducted in Stages:

Stage 1: Laboratory tests routinely ordered by the San Lazaro Hospital clinical team as part of the management of the cases based on the prevailing standards of care of the various conditions in the Philippine setting.

Stage 2: AFRIMS in-house DEN/JE Enzyme immunoassay (EIA) was performed to help rule in/rule out either dengue or JE etiology; specimen with negative/inconclusive results were subjected to the next level of testing.
Stage 3: Laboratory tests performed for presumptive diagnosis

Stage 4: Confirmatory laboratory tests and other more advanced tests

Final diagnosis was based on study-specific case definitions. Cases which yielded negative results after Stage 4 testing were classified as those belonging to other etiologies.

The collaborating institutions were provided with laboratory test capabilities to perform AFRIMS in-house DEN/JE EIA. The investigators ensured that the materials required for the test performance were provided. On-site training of laboratory personnel and quality monitoring was conducted by AFRIMS staff. Proficiency evaluation was done.

d. Results:

Dengue-like Syndrome

As of 31 December 2006 a total of 260 patients were enrolled with dengue-like syndrome (DLS). One hundred four subjects were enrolled from 1 September 2005 to 23 January 2006 while 156 subjects were enrolled from 21 July 2006 to 14 December 2006. Majority of the patients came from the National Capital Region (Manila).

Two hundred thirty-two (89%) were positive for dengue by EIA or PCR. The following diagnoses were noted among the 19 patients who were considered as non-dengue cases: 2 cases of possible leptospirosis, 1 typhoid, 1 Plasmodium falciparum, 1 chikungunya, 1 pneumonia, 1 disseminated tuberculosis, and 1 possible rickettsia. Further characterization is pending for the remaining non-dengue cases.

All 4 dengue serotypes were documented to be present with the DEN-3 serotype predominant during both enrollment periods. Serotype distribution as follows: DEN-1 (2%), DEN-2 (16%), DEN-3 (74%), DEN-4 (8%). Preliminary sequencing on DENV-3 specimens collected before April 2006 inclusive, showed Asian genotype 1 strain.

Acute secondary dengue infection (76%) and DHF gr II (47%) clinical diagnosis were predominant. Male to female ratio was 1.2:1 and age range of the confirmed dengue cases ranged from 2 to 37 years old.

Acute Encephalitis Syndrome

A total of 15 patients presenting with acute encephalitis syndrome (AES) were enrolled from September 2005 to December 2006. Six (40%) patients were confirmed positive for JE with 3 patients enrolled in the month of July 2006. The ages of the confirmed JE cases ranged from 3 to 14 years old and male to female ratio was 1:2. The non-JE specimens were sent to the US Centers for Disease Control (CDC) for further testing and the specimens underwent a panel of IgM EIA tests including dengue,
Chikungunya (CHIK), Powassan virus/Tick-borne encephalitis virus (POW/TBE), Snowshoe Hare virus (SSH) and IgM MIA for West Nile (WN) and St. Louis encephalitis (SLE) virus with no positive findings

Three mortalities were reported to HURC with 2 of these among the DLS component and 1 from the AES component. The 3 mortalities that were recorded were not attributed as a consequence of participation in the study.

9. Title of Research Project: Phase II, Randomized, Double-Blind, Single Center, Controlled Study of Two Doses of Different Formulations of the WRAIR Live Attenuated Tetravalent Dengue Vaccine Compared to A Placebo Control, Administered on A 0-6-Month Schedule, to Healthy Adults

a. Principal Investigators:

- Robert V. Gibbons, LTC, MC (USAMC-AFRIMS)
- Veerachai Watanaveeradej, MD, Phramongkutklao Hospital (PMK), Thailand
- Sub Investigators:
  - Danaband Phiboonbanakit, MD (PMK)
  - Sriluck Simasathien, MD (PMK)
  - Angkool Kerdpanich, MD (PMK)
  - Adisorn Lumpaopong, MD (PMK)
  - Atik Sangahsapaviliyah, MD (PMK)
  - Nopaorn Phavichitr, MD (PMK)
  - Bruce L. Innis, MD (GSK)
  - Ananda Nisalak, MD (AFRIMS)
  - Lynch Julia A, MD (WRAIR)
  - Stephen J. Thomas, MAJ(P), MC (WRAIR)
  - Kenneth H. Eckels, PhD (WRAIR)
  - J. Robert Putnak, PhD (WRAIR)
  - In-Kyu Yoon, Md (AFRIMS)
  - Richrad G. Jarman, PhD (AFRIMS)

b. Objectives:

To evaluate the safety of T-DEN vaccine in terms of the occurrence of solicited adverse events (AE) within the 21-day solicited follow-up period following administration of study vaccine dose 1.

To explore the immunogenicity of T-DEN vaccine in terms of the GMT of neutralizing (N) antibody to each DEN serotype (DEN-1, -2, -3 and -4) determined 30 and 90 days after vaccine dose 2.
c. **Methods:**

1. Screen and enroll 120 Thai subjects, 20-25 year old healthy males and non-pregnant healthy females.
2. Randomized, double-blind, placebo-controlled study with 3 groups: T-DEN (post-transfection) F17, T-DEN (post-transfection) F19 and Placebo control.
3. Provide 2 doses of the tetravalent dengue vaccine or placebo at 0 - 6 month apart.
4. Evaluate the safety of vaccine in terms of AE, SAE, alert values, abnormal DEN physical examination finding and suspect or confirmed dengue.
5. Evaluate the immunogenicity of vaccine in terms of N antibody and measurable dengue viremia.
6. One screening visit and 10 study visits
7. The intended duration of the study will be 9 months (in addition to the screening period).

d. **Results:**

One hundred and forty six (146) potential subjects have been screened and 120 subjects have been enrolled. The visit 9 (1 month post dose 2) clinical follow-up have been completed. The laboratory testing is ongoing. There are 3 SAEs reported (1 abdominal hernia, 1 ectopic pregnancy, 1 appendicitis) none of which were demmed related to study vaccine. There are 2 pregnancy cases reported. The interim analysis of safety endpoints and partial immunogenicity (neutralizing antibody only) following dose 1 on blinded and unblinded, cleaned data is ongoing.

e. **Future Plans:**

Clinical follow-up would continue as outline in approved protocol. The specimens will be sent to test at location as specified in the protocol.

The demographics/base line characteristics, safety and immunogenicity data will be analyzed after complete clinical follow-up as outline in the protocol.

10. **Title of Research Project:** Training and Workshops

a. **Background:**

The Department of Virology, Armed Forces Research Institute of the Medical Sciences (AFRIMS), Bangkok, Thailand, seeks to expand its diagnostic capabilities in South and Southeast Asia by improving regional laboratory capabilities through the dissemination of diagnostic kits and the training of technical personnel.
b. Objectives:

1. To create and improve the laboratory infrastructure of South and Southeast Asian regional laboratories specializing in infectious disease surveillance.

2. To provide the training of laboratory personnel (technicians and supervisors) working in South and Southeast Asia and beyond in infectious disease diagnostic techniques.

c. Activities:

The department conducted numerous on-site and in-house diagnostic training activities.

1. Over 100 student scientists and medical technicians from Chulalongkorn University, Phramongkutklao Medical College, Faculty of Tropical Medicine-Mahidol University, Naval Medical Research Unit #2, Research Institute for Tropical Medicine, Chulalongkorn Hospital, Siriraj Hospital, Ramathibodi Hospital received training at AFRIMS in diagnostic laboratory modalities. In addition, we provided our laboratory visit to the staff from Ministry of Health-Myanmar, and WHO.

2. On-site training (Nepal and Philippines) in the proper performance of the AFRIMS DEN/JE IgM IgG EIA and basic instruction in QA and QC principles was provided to representatives of numerous Nepali health institutions including Nepal Public Health Laboratory (NPHL). The on-site training in serological assay was also provided to San Lazaro Hospital and Research Institute of Tropical Medicine in Philippines.

3. During the past year diagnostic kits or training was provided to the following laboratories:

- B.P. Koirala Institute of Health Sciences, Dharan, Nepal
- Nepal Public Health Laboratory, Kathmandu, Nepal
- Teku Hospital, Kathmandu, Nepal
- Bheri Zonal Hospital, Nepalgunj, Nepal
- Institute of Medicine, Kathmandu, Nepal
- ICDDR,B, Dhaka, Bangladesh
- Pasteur Institute, Ho Chi Minh City, Vietnam
- Research Institute of Tropical Medicine, Alabang, Philippines
- San Lazaro Hospital, Manila, Philippines

F. Department of Retrovirology, AFRIMS FY07 Research Accomplishments

1. Title of Research Project: A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp120 B/E (AIDSVAX® B/E) Boosting in HIV-uninfected Thai Adults (RV144, HSRRB Log No. A-11048, BB-IND 8795)
a. Investigators:

- Dr. Supachai Rerks-Ngarm
- Dr. Supamit Chunsuttiwatt
  Department of Disease Control,
  Ministry of Public Health
  Nonthaburi, Thailand

- COL Sorachai Nitayaphan
  RTA Component, AFRIMS

- Prof. Punnee Pitisuttithum
- Assoc. Prof. Jaranit Kaewkungwal
  Faculty of Tropical Medicine, Mahidol University
  Bangkok, Thailand

b. Objectives:

  Primary: To determine whether immunizations with an integrated combination of ALVAC-HIV (vCP1521) boosted by AIDSVAX® gp120 B/E prevent HIV infection in healthy Thai volunteers. Secondary: To determine whether immunization with this vaccine combination results in reduced HIV viral load “set point” among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To determine whether immunization with this vaccine combination results in an increased CD4 count measured at viral load “set point” among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To confirm the safety of this vaccine combination in Thai volunteers. To evaluate whether participation in this HIV vaccine trial is associated with behavior change that may increase the risk of HIV infection.

c. Methods:

  This will be a community-based, randomized, multicenter, double-blind, placebo-controlled clinical trial (vaccine:placebo = 1:1). Screening of potential volunteers will be carried out under a separate protocol entitled “Screening and evaluation of potential volunteers for a trial in Thailand of a candidate preventive HIV vaccine” (RV148). Eligible volunteers will be enrolled over approximately one year. The statistical assumptions of the study will require that 16,000 persons enroll into the study. Vaccinations for each individual will occur over a 24-week period (0, 4, 12, 24 weeks). Women will be tested for pregnancy and pregnant volunteers will not be vaccinated.
  The volunteers will be followed with HIV testing every 6 months for 3 years after immunization. Blood will be collected for plasma (for diagnostics and HIV-specific antibodies) at 0, 24 and 26 weeks, and every 6 months during the follow-up phase. The blood collection at 0 and 52 weeks will also be used for cryopreservation and archiving of PBMCs (for HIV-specific cellular immune responses). At week 24 and at each six-month follow-up visit, volunteers will have HIV testing, preceded by pretest counseling and followed (approximately 2-3 weeks later) by post-test counseling.
Assessment of HIV risk behavior will be performed at baseline and at each 6-month follow-up visit. Education on risk behavior reduction will be given at each vaccination visit and at each post-test counseling visit.

d. Results:

The first volunteer injected on 20 October 2003. Enrollment ceased 31 Dec 2005. 16,402 persons were enrolled and 16,396 received at least one vaccination. 13,974 persons received 4 vaccinations (85.2%). Of persons receiving at least one vaccination, overall follow-up is 82% at V11 (12 months post 4th vaccination). Interval follow-up rates have been > 98% (protocol estimate = 95%). As of 18 Nov 07 there were 39,245 person years of follow-up. 104,900 vials of vaccine were shipped and are 100% accounted for. There have been 227,586 volunteer visits (as of 1 Nov 07). Four Hundred Ninety Five Thousand Seven Hundred Eighteen (495,718) specimens have been archived. Eight Hundred Fifty Seven Thousand Six Hundred Eighty Eight (857,688) case report forms have been faxed.

The Data Monitoring and Safety Board met 18-19 July 2007 for the Interim Efficacy Analysis. Futility and safety were also reviewed. A Roadmap or “Contingency Plan” for various outcomes of the July 2007 DSMB meeting was created. A consensus communications dossier was made for each contingency. The DSMB recommended the study continue.

e. Future Plans:

The next DSMB meeting is scheduled for July 2008. A revision of the Roadmap is planned (version 2.0). Additional work with the Thai FDA to create licensure capacity is under way.

2. Title of Research Project: Extended Evaluation of The Virologic, Immunologic, and Clinical Course of Volunteers Who Become HIV-1 Infected During Participation in a Phase III Vaccine Trial of ALVAC-HIV and AIDSVAX® B/E (RV152, WRAIR #1184)

a. Investigators:

- Dr. Supachai Rerks-Ngarm
- Dr. Supamit Chunsutthiwat
  Department of Disease Control
  Ministry of Public Health
  Nonthaburi, Thailand

- COL Sorachai Nitayaphan
  RTA Component, AFRIMS
  Bangkok, Thailand
• LTC Robert Paris
• COL Jerome Kim
• Dr. Mark de Souza
  Department of Retrovirology,
  US Component, AFRIMS
  Bangkok, Thailand

• Assoc. Prof. Jaranit Kaewkungwal
  Faculty of Tropical Medicine, Mahidol University
  Bangkok, Thailand

b. Objectives:

This protocol seeks to establish whether a vaccine effect on HIV-1 viral load results in a reduction in the number of composite HIV-related clinical endpoints, which also includes a biomarker (CD4 count) component. This study also includes assays of both cellular (e.g., intracellular cytokine staining, CTL) and humoral (neutralizing antibody) responses to identify putative correlates of vaccine-associated immunity, as well as virologic characterization of infecting viruses by genotyping and selective sequencing to assess for selective vaccine efficacy.

c. Methods:

Volunteers attend study visits every 3 months to receive a clinical assessment, CD4 count, and viral load measurement, as well as collection of PBMC’s and plasma for research assays.

d. Results:

The protocol began enrollment in May 2006 and is on-going as HIV-infected volunteers from the phase III trial are identified. No analysis of data or abstracts has resulted from this study at this time.

e. Future Plans:

The protocol will continue to enroll HIV-infected participants from RV144 until the scheduled end of that protocol in August 2009. Volunteers will be followed for 5 years from enrollment or after they have been treated with antiretroviral therapy for 18 months.

3. Title of Research Project: A Phase I Double-Blind Randomized Dose Escalating, Placebo-Controlled, Study of Safety and Immunogenicity of WRAIR/NIH Live Recombinant MVA-CMDR (HIV-1 CM235 env/ CM240 gag/pol) Administered by Intramuscular (IM) or Intradermal (ID) Route In HIV-Uninfected Adults (RV158, WRAIR #1143)
a. Investigators:

- Dr. Mary A. Marovich  
  US Military HIV Research Program, Rockville, MD, USA
- Professor Prasert Thongcharoen  
  Dept of Microbiology, Faculty of Medicine, Siriraj Hospital  
  Mahidol University, Bangkok, Thailand
- Dr. Thira Woratanarat  
  Department of Retrovirology, US Component, AFRIMS  
  Bangkok, Thailand

b. Objectives:

**Primary:** To evaluate the safety and tolerability of MVA-CMDR (HIV-1 CM235 ENV/CM240 GAG/POL) administered by IM or ID injection to HIV-uninfected adult volunteers.

**Secondary:** To evaluate the ability of MVA-CMDR (HIV-1 CM235 ENV/CM240 GAG/POL) to induce HIV antigen specific cellular and humoral immune responses.

c. Methods:

This is a phase I double-blind, randomized, dose-escalating, placebo-controlled, study. Healthy HIV-uninfected adult volunteers (18 to 40 years old at the time of enrollment) will be enrolled up to 90 days prior to the first vaccination. Volunteers will be randomized to vaccine or placebo in a 5:1 ratio. Vaccinations will be on Days 0, 28, and 84 of each volunteer’s schedule; each vaccination will be followed with a phone call (within 24 to 48 hours) and a safety visit (within 14 days); and each subject will be followed up for approximately 40 weeks after the third vaccination. Total study duration will be 52 weeks for each volunteer. Volunteers attend study visits as scheduled to receive a clinical assessment, routine hematology, serum chemistry, liver function tests, urinalysis, ECG, and related laboratory tests for safety monitoring during the study conduct.

d. Results:

The protocol began enrollment on 27th November 2007 in Thailand. Twelve volunteers were enrolled at two sites (6 each): Siriraj Hospital and the AFRIMS Clinical Trial Center at Bumrungrad. All volunteers received their first vaccination in January 2008. No analysis of data or abstracts has resulted from this study at this time.

e. Future Plans:

The protocol will continue until its scheduled end in October 2008.
4. **Title of Research Project:** Protocol G, A Cross-Sectional Study to Screen for and Generate Broadly Neutralizing Monoclonal Antibodies from HIV Infected Individuals (RV212, WRAIR #1320)

**a. Investigators:**

- Professor Punnee Pitisuttithum  
  Vaccine Trial Centre  
  Faculty of Tropical Medicine  
  Mahidol University, Bangkok, Thailand

- Dr. Thira Woratanarat
- COL Jerome Kim
- LTC Robert Paris
- Dr. Mark de Souza
- Ms. Patricia Morgan  
  Department of Retrovirology,  
  US Component, AFRIMS, Bangkok, Thailand

**b. Objectives:**

This protocol seeks to generate broadly neutralizing monoclonal antibodies (mAbs) from volunteers who are HIV infected and have broadly cross-reactive serum neutralizing activity. It will also attempt to determine the clinical and laboratory characteristics of HIV infection that correlate with the presence of broadly cross-reactive neutralizing antibodies.

**c. Methods:**

Volunteers are seen at the AFRIMS Clinical Trials Center in Bangkok. They attend one study visit study every 3 months to receive a clinical assessment, CD4 count, and viral load measurement, as well as collection of PBMC’s and plasma for research assays.

This is a cross-sectional, multi-center study with one or two visits. At the first visit blood is drawn for serum to measure neutralizing activity. If there are clinically significant test results, a follow-up visit is scheduled to review all clinically significant test results with the volunteer and plan referrals for further evaluation, care and treatment. Volunteers who have broadly neutralizing antibodies are asked to return approximately 3 months after the screening visit. Blood is drawn for the preparation of PBMCs, clinical testing and to obtain additional serum to confirm that the volunteer continues to have broadly cross-reactive neutralizing antibodies.
d. Results:

The protocol began enrollment in August 2007. As of 31 Dec 2007, there were 42 volunteers enrolled at the AFRIMS site. No analysis of Thai data or abstracts has resulted from this study at this time.

e. Future Plans:

The protocol will continue to enroll HIV-infected participants until 100 volunteers have been enrolled.

5. Title of Research Project: The Molecular Epidemiology of HIV-1 among HIV Blood Testing Clients Attending the Thai Red Cross Anonymous Clinic in Bangkok, Thailand. (RV225, WRAIR #1383)

a. Investigators:

- CPT Miguel A. Arroyo
  Department of Retrovirology, US Component, AFRIMS, Bangkok, Thailand

- Dr. Sunee Sirivichayakul
  Division of Allergy and Clinical Immunology
  Department of Medicine, Chulalongkorn University,
  Bangkok, Thailand

- Professor Praphan Phanuphak

- Dr. Nittaya Phanuphak
  Thai Red Cross AIDS Research Center (TRCARC),
  Bangkok, Thailand

- Dr. Kiat Ruxrungtham
  Section of Allergy and Immunology, Department of Medicine
  Chulalongkorn University
  Bangkok, Thailand

- Dr. Jintanat Anaworanich
  Department of Retrovirology, US Component, AFRIMS
  and South East Asia Research Collaboration with Hawaii (SEARCH)
  Thai Red Cross AIDS Research Center
  Bangkok, Thailand

- Dr. Francine E. McCutchan and Dr. Paul T. Scott
  Department of Threat Assessment and Epidemiology
  U.S. Military HIV Research Program (USMHRP)
  Rockville, MD, USA
b. Objectives:

To describe the molecular epidemiology of HIV among clients undergoing voluntary counseling and testing Thai Red Cross Anonymous Clinic (TRCAC) for using in planning future vaccine trials in Bangkok, Thailand.

c. Methods:

This protocol aims to characterize the molecular epidemic of HIV-1 among clients attending the TRCAC. A total of approximately 3,000 samples and questionnaires were collected by TRCAC between July 1, 2006 and February 28, 2007 from subjects who requested HIV-1 testing and counseling. The HIV test results and questionnaire information will be linked to each sample by a unique number that does not identify the client. HIV-1 genotyping by MHAbce1 will be performed initially in all HIV-1 positive samples. Drug resistance and HIV-1 full-length genotyping may be performed on a subset of specimens of interest to the research team. Statistical analysis will be performed to determine associations between HIV-1 infection, genotype and, behavioral and demographic characteristics among the study population.

d. Results:

The protocol is expected to begin in February 2008. No analysis of data or abstracts has resulted from this study at this time.

e. Future Plans: Future Plans will depend on study results.

6. Title of Research Project: HIV Specific Immune Responses in Thai Individuals with HIV Dementia (RV238, WRAIR #1418)

a. Investigators:

- Dr. Victor Valcour
  Department of Internal Medicine, University of Hawaii
  Honolulu, HI, USA

- Dr. Samart Nidhinandana

- Dr. Suwicha Chitpatima
  Pramongkutklao Hospital
  Bangkok, Thailand
b. Objectives:

This is a proposal to obtain pilot data and to assess logistical feasibility for intended Hawaii-Thailand joint NIH Exploratory/Developmental Research proposal on the research topic of HIV-associated dementia (HAD). The proposal is written to include follow-up visits and evaluations to 24 months. There are two main scientific goals: 1) to assess the relationship between monocyte/macrophage activation and dementia in ARV naïve patients living in Thailand and 2) to assess the relationship among proviral DNA levels, blood HIV viral load, CSF HIV viral load, and cognitive status among ARV naïve patients with and without HIV dementia.

c. Methods:

Thirty volunteers were HIV-1 infected individuals (15 HAD and 15 non-HAD), and 30 matched HIV-negative controls. All individuals underwent neuropsychological testing. Blood draws were obtained and lymphocyte immunotyping, plasma HIV viral load and monocyte phenotyping was performed.

d. Results:

The current study has established that HIV associated Dementia (HAD) is present in patients infected with circulating recombinant form (CRF) 01_AE (subtype E). HAD HAART-naïve HIV-1-infected Thais with no active CNS opportunistic infection were screened for cognitive deficits. Fifteen individuals with HAD were identified and were then matched by age, education, and CD4 count with 15 HIV-1-infected non-demented (ND) individuals. All patients then completed the modified WHO international HIV NP battery. An independent review panel confirmed cognitive diagnoses using all available data. Neuropsychological z-scores were calculated using 30 age-, education-, and gender-matched HIV-negative Thais as controls.
Neuropsychological testing abnormalities were identified in most domains among HAD participants compared to HIV-negative controls. Abnormalities were most prominent in tests of verbal learning and recall, visuospatial construction, and selective attention. By contrast, only Color Trails 2 and EIWA Block Design distinguished ND individuals from controls.

The PBMCs from the HAD, non-HAD and HIV-negative controls groups were surface stained with M/M markers CD14/CD16/HLADR and CD14/CD69/HLADR. Non-parametric analysis (Mann-Whitney test) on these subsets of monocyte markers at baseline (V1) revealed significant differences associated with CD14/CD16/HLADR markers between controls and HIV groups (both HAD and non-HAD) (p< 0.001). We did not observe differences between HAD and non-HAD groups. We also compared the expression of these markers at V2 (6 months after initiation of ARV) to see if ARV induced could induce any change on expression of these markers of these monocytes. As expected, all individuals responded well to treatment with reduction in both plasma VL and augmentation of CD4+ counts. There was a significant reduction of expression for both percent and absolute number for CD14/CD16/HLADR M/M after ARV treatment in the HAD group and less impressively in the non-HAD group, meeting significance in only measurement of the absolute M/M number.

e. Future Plans:

Follow up for the 15 HAD and 15 non-HAD individuals with continuous collection of data. We are planning to submit a new protocol proposing an expansion of the immunological assessment in a similar cohort to better understand the underlying immunological mechanisms that may cause HAD.

7. Title of Research Project: Predictors of Neuro-Cognitive Decline and Survival in HIV-Infected Subjects (SEARCH 001, WRAIR #1161)

a. Investigators:

- Dr. Samart Nidhinandana (Principal Investigator)
  Department of Internal Medicine
  Section of Neurology, Pharmungkutklao Hospital
  Bangkok, Thailand

- Dr. Victor Valcour (co-Principal Investigator)
  Department of Internal Medicine
  University of Hawaii-Manoa,
  Honolulu, Hawaii, USA

- Dr. Jerome H. Kim
- Dr. Silvia Kim
- Dr. Mark deSouza
- Dr. Jintanat Ananworanich
Department of Retrovirology  
US Component, AFRIMS,  
Bangkok, Thailand

b. Objectives:

This protocol seeks to assess predictors of neuropsychological impairment in Thais and assess changes in neuropsychological testing parameters before and after antiretroviral therapy. A sub-study has also been performed to compare cellular responses, particularly activated monocytes, in volunteers with and without dementia.

c. Methods:

This 96-week study enrolled three groups of volunteers: 15 with HIV-associated dementia, 15 with no dementia and 30 HIV-negative controls. Volunteers attend study visits every 6 months to receive a clinical assessment, neuropsychological testing, CD4 count, and viral load measurement, as well as collection of peripheral blood mononuclear cells and plasma for research assays. Statistical analyses are planned at weeks 48 and 96.

d. Results:

All volunteers completed the study follow up in December 2007. Planned analysis at week 48 showed improvement of neuropsychological testing parameters after antiretroviral therapy. Volunteers with HIV-associated dementia performed worst at all time points compared to HIV-infected volunteers without dementia and HIV-negative volunteers. HIV DNA level in peripheral blood mononuclear cells predicted HIV-associated dementia. However, activated peripheral blood monocytes were maintained at high levels in HIV-negative controls and were non-discriminatory for HIV-associated dementia. The baseline data has been published in a peer-review journal and the 48-week data has been accepted as a poster presentation at the Conference on Retroviruses and Opportunistic Infections in Boston in February 2008.

e. Future Plans:

HIV-infected volunteers will be asked to enroll in a follow up protocol at the South East Asia Research Collaboration/The Thai Red Cross AIDS Research Centre. They will undergo neuropsychological testing, viral load measurement and archiving of peripheral blood mononuclear cells for HIV DNA level every 6 months for a period of 2 years. This roll over protocol called SEARCH 001.1 will commence in February 2008.

8. Title of Research Project: Preliminary study of early primary HIV infection in high risk cohort (SEARCH 004, HSRRB # A-14273.3)
a. Investigators:

- Dr. Jintanat Ananworanich (Principal Investigator)
  Department of Retrovirology, US Component, AFRIMS and SEARCH, Bangkok, Thailand

- Dr. Nittaya Phanuphak (co-Principal Investigator)
  The Thai Red Cross AIDS Research Centre, Bangkok, Thailand

- Dr. Jerome H. Kim, Dr. Robert Paris, Dr. Mark deSouza and Dr. Miguel Arroyo
  Department of Retrovirology, US Component, AFRIMS, Bangkok, Thailand

- Prof. Praphan Phanuphak and Dr. Sunee Sirivichayakul
  The Thai Red Cross AIDS Research Centre, Bangkok, Thailand

b. Objectives:

This protocol investigated the incidence, demographics, HIV subtype and genotypic resistance in acute HIV infection within a high-risk Thai cohort at the Thai Red Cross Anonymous Clinic (TRCAC), which has an HIV prevalence of about 17%.

c. Methods:

TRCAC uses 4th generation enzyme-linked immunoassay (AxSYM) for HIV diagnosis. This protocol utilized discarded samples from HIV testing that were routinely stored. AxSYM-negative samples were pooled and nucleic acid testing (NAT) was performed using Roche Amplicor v 1.5 ultrasensitive assay. Acute HIV infection samples were AxSYM-negative, NAT positive. In addition, AxSYM positive samples were tested with 1st generation US Food and Drugs Administration approved HIV-1 EIA (HIV-1 Microelisa System, Organon Teknika, Durham, NC). Acute HIV-infection samples were AxSYM-positive, 1st generation sensitive EIA negative and NAT positive. Demographic and risk behavior data from the TRCAC questionnaires were collected.

d. Results:

Plasma from 6426 clients was tested, and 11 subjects had acute HIV-1 infection. Seven of 5402 AxSYM-negative samples were NAT positive and 4 of 1024 AxSYM-positive samples were 1st generation EIA negative. The acute HIV infection prevalence was 20.3 per 10,000 persons at risk, and the estimated HIV incidence was 2.7 per 100 person-years. Median HIV RNA was 99,601 copies/ml. The majority had CRF01_AE strains and 1 had dual CRF_01AE and B recombinant. No samples showed resistance to antiretrovirals. The majority of the subjects were educated, employed and had income three times higher than the average Thai household monthly income. Few consistently used condoms. This study has completed. The results have been accepted for poster presentation at the HIV Pathogenesis meeting in Banff,
Canada in March 2008. The manuscript has been submitted to a peer-reviewed journal.

e. Future Plans:

A prospective study (SEARCH 010) has been funded by the U.S. Military HIV Research Program to diagnose clients of the TRCAC with acute HIV infection within the first 2-3 weeks of onset of infection. Volunteers with acute HIV infection will be asked to enroll in a cohort study that will follow their clinical, immunological and virological outcomes over a course of 2 years. Volunteers will be offered antiretroviral treatment and the effect of treatment will be assessed. Sub-studies evaluating immunologic and virologic parameters in the central nervous system and genital compartments will also be performed. The study will commence in mid 2008.
III. APPENDICES:

PERSONNEL ASSIGNED UNDER COOPERATIVE AGREEMENT

Department of Administration
1. Ms. Bung-on Kesdee
2. Mr. Weerasak Yeephu
3. Mr. Sompol Boonnak
4. Ms. Pattarapan Jullasing
5. Ms. Geerati Sornwattana
6. Ms. Yinglak Apisitsaowapa
7. Mrs. Somporn Krasaesub
8. Mr. Theerasak Ponepan
9. Mr. Prinya Yoophasook
10. Mrs. Khanitha Rojanasthien
11. Mrs. Bussara Sukpanichnant
12. Mrs. Lakhana Phaoharuhansa
13. Mr. Danuphol Junkaew
14. Ms. Usa Panichpathompong
15. Mrs. Sirin Limsurat
16. Ms. Nida Nopparatkailas
17. Ms. Panisara Puangsilpa
18. Ms. Tassanee Eamkamon
19. Ms. Amorn Upakaew
20. Ms. Lantharima Ngernsiri

Department of Logistics
21. Mr. Sawadi Boonnak
22. Mr. Charan Kajeechitr
23. Mr. Thongchai Duangkaew
24. Mr. Boonthum Jamjank
25. Mr. Komson Boonnak
26. Mr. Somporn Pinpo
27. Mr. Chatchai Saeng-ngern
28. Mr. Prasitchai Kruaysawat  
29. Mr. Yuthana Seemart  
30. Mr. Siriphong Amnuaisuksiri  
31. Mrs. Anchisa Maleenun  
32. Ms. Nongnoot Chaowaratana  
33. Mr. Tharanat Thanatepisansakun  
34. Ms. Saruta Anukul  
35. Mr. Jakkapong Maneerut  
36. Ms. Morakot Ubolraj  
37. Mr. Patrabhum Kwanyou  
38. Mr. Sanay Sangphaya  

**Department of Immunology**  
39. Mrs. Barnyen Permpanich  
40. Ms. Amporn Limsalakpetch  
41. Mr. Chaiyawat Mathavarat  
42. Ms. Srisombat Wannaying  
43. Ms. Apassorn Lim  
44. Ms. Sabaithip Sriwichai  
45. Ms. Duangkamon Siludjai  
46. Ms. Monticha Kongthaisong  
47. Mr. Sittidech Surasri  
48. Mr. Worachet Kuntawunginn  
49. Dr. Youry Se  
50. Ms. Kuntida Tangthongchaiwiriya  
51. Mr. Montri Arsanok  
52. Ms. Nillawan Buathong  

**Department of Virology**  
53. Khunying Dr. Ananda Nisalak  
54. Mrs. Chuanpis Ajariyakhajorn  
55. Mrs. Sumitda Narupiti  
56. Mrs. Naowayubol Nutkumhang
57. Mr. Somsak Imlarp
58. Mr. Wichien Sa-Nguansuk
59. Mr. Prachakkra Panthusiri
60. Ms. Wallika Kulthongkam
61. Mr. Wanchai Inpho
62. Mrs. Pannarat Chuakanobon
63. Mrs. Rungkarn Hangsuwan
64. Mr. Yongyuth Poolpanichupatam
65. Mr. Thanawat Boonnak
66. Ms. Rattiya Wannawong
67. Ms. Prinyada Rodpradit
68. Ms. Piyawan Chinnavirotpasan
69. Mrs. Wilaiwan Sridadeth
70. Ms. Wicha Panyalikhit
71. Mr. Winai Kaneechit
72. Ms. Chaleaw Saengchan
73. Ms. Nualanong Akaraputtiporn
74. Mrs. Choompun Manomuth
75. Mr. Supakit Wanasith
76. Dr. Charity Ann Ypil Butac
77. Ms. Taweewun Hunsawong
78. Dr. John Mark Sullesta Velasco
79. Ms. Frances Dominique Ybanez Villano
80. Ms. Joan Sabusay Reyes
81. Mr. Nazarino I. Adolfo
82. Mrs. Paichit Tothong
83. Ms. Sineewanlaya Wichit
84. Mrs. Vipa Thiravuth
85. Ms. Panor Srisongkram
86. Ms. Yuwadee Sae-Yong
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<th>No.</th>
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<td>87</td>
<td>Mr. Phongsak Maneerat</td>
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<td>Mr. Thonglor Detkokao</td>
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<td>Mr. Amnart Kayha</td>
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<td>Mr. Perapol Thanunok</td>
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<td>Mr. Chatree Kumhom</td>
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<td>Mr. Urai Klaraket</td>
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<td>Mr. Prasan Kankaew</td>
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<td>Mr. Nattapat Rochanarutaiprida</td>
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<td>Ms. Nongnuch Yimamnuaychok</td>
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<td>Mr. Yossasin Kertmanee</td>
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<td>Mr. Somsak Tiang-trong</td>
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<td>Ms. Kanchana Pantuwatana</td>
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<td>Ms. Bousaraporn Tippayachai</td>
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<td>Mr. Sommai Promsathaporn</td>
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<td>Ms. Sasathorn Rochanarutaiprida</td>
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