Genomic Characterization of Adenovirus 21 Strains Associated With Outbreaks of Febrile Respiratory Illness in United States Military Recruit Training Centers Between 1996 & 2005

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2 **Febrile Respiratory Illness in United States Military Recruit Training Centers Between**

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5 Running title: Ad21 infections among US military recruits

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1   **ABSTRACT**

2

3   Adenovirus type 21 (Ad21) is a well-known causative agent of acute respiratory disease

4   among both military recruits and children. In an effort to characterize the molecular

5   epidemiology of Ad21 infections in the military environment, genome-typing work was

6   carried out on a collection of 75 Ad21 strains isolated from the pharyngeal swabs of military

7   recruits presenting with symptoms of febrile respiratory illness between 1996 and 2005 at

8   eight US training centers. One further strain from an ill serviceperson deployed at sea was

9   also characterized. Restriction enzyme analysis with *Bam*HI discriminated two distinct DNA

10   variants, Ad21a and Ad21b. Further analysis with *Bgl*I, *Bgl*II, *Bst*EII, *Hind*III, *Kpn*I, and *Sma*I

11   discriminated two new subtypes, Ad21a1 and Ad21b1. Ad21a was the most prevalent

12   genome type, accounting for 69 of the 76 strains examined. Genome type Ad21a1 was

13   identified only among the strains isolated at the Marine Corps Recruit Depot, San Diego,

14   CA. Genome types Ad21b and Ad21b1 were identified among strains isolated in 2005 and

15   seem to have emerged after a 4-year (1999–2002) disappearance of all Ad21 genome

16   types. After the reintroduction of the Ad4/Ad7 vaccine in 2008, Ad21 is expected to become

17   a predominant adenovirus serotype in US recruit training centers once again. Knowledge of

18   circulating genome types and their epidemic behavior will be of significant value to ongoing

19   surveillance efforts in these highly susceptible and impacted populations.

1   **INTRODUCTION**

2

3   Adenoviruses (Ads) of species B, C, and E are major causative agents of respiratory

4   disease in infants, young adults, and immunocompromised patients (12, 21). Ads were first

5   isolated in 1954 in the United States from military recruits with febrile respiratory illness

6   (FRI) (11), and since then have been recognized as a unique threat to military training

7   centers around the world. In the military setting, Ad serotypes 4 (species E), 3, 7 and 21

8   (species B) can cause essentially continuous outbreaks of FRI that can affect the majority

9   of the recruit population and result in hospitalization of large numbers of individuals. Aside

10 from the unacceptable impact on recruit health and welfare, these outbreaks disrupt

11 training, challenge medical readiness, and saddle training commands with significant

12 financial burdens (8, 14, 30). Outbreaks of Ad-associated FRI were well documented

13 among American military trainees and deployed troops (23) before the implementation of

14 live Ad4/Ad7 vaccination starting in 1971 (8, 27, 28). The vaccine was highly effective,

15 limiting the impact of Ads infection to sporadic and relatively minor outbreaks associated

16 with infection by the otherwise less prevalent Ad21 and, occasionally, other serotypes not

17 directly targeted by the vaccine. The vaccination protocol was abandoned in 1996 when the

18 sole manufacturer of the licensed vaccine permanently discontinued production. Vaccine

19 availability was thereafter rationed with a focus on outbreak events and peak Ad seasons

20 until the last stocks were exhausted in 1999. Unchallenged by vaccines, Ads (especially

21 Ad4) once again became the primary cause of FRI among military recruits in US training

22 centers. For perspective, it should be noted that FRI is the primary cause of hospitalization

23 and incapacitation of recruits (3, 9, 20).

1　　　　Ad21, a species B serotype, was first isolated in association with trachoma and

2　conjunctivitis from a 1-year-old child in Saudi Arabia in 1956 (5) and was first identified in

3　association with FRI among US military recruits, during the testing of the recruit-targeted

4　Ad4 vaccine (30). Ad21 was a dominant source of adenoviral illness at training centers

5　during the years when Ad4 and Ad7 were being effectively controlled by vaccination,

6　although it never caused a return to epidemic levels of FRI (6, 9). Ad21 vaccines were

7　designed and tested, but concerns of possible interference between the Ad21 vaccine and

8　the Ad4/Ad7 vaccine were raised, and the effort to apply the Ad21 vaccine was abandoned

9　(9, 26).

10　　　　Outside the United States, the association of Ad21 with acute respiratory disease

11　among both military recruits and children is well documented (15, 29-31). However,

12　detailed molecular characterization of Ad21 isolates and genome typing by restriction

13　enzyme analysis of the viral genome is limited to a few published studies (2, 19, 29). An

14　epidemic outbreak of Ad21 infection causing severe respiratory disease in children was

15　documented in Auckland, New Zealand, in 1965 (4, 15). In The Netherlands and Great

16　Britain in the early 1960s a high prevalence of Ad21 was observed in both pediatric and

17　military populations (29, 31). This serotype was detected only sporadically in Europe during

18　the following 20 years, but an increase in incidence of Ad21 infections was again observed

19　in 1985. While the early outbreaks of the 1960s were associated with an extremely high

20　rate of pneumonia (40%) among sampled Ad-positive patients, the reemergence of Ad21 in

21　1985 in Europe was associated with milder clinical manifestations of respiratory disease

22　and the appearance of a new genomic variant. This suggests that both the virulence and

23　the rate of transmission may be highly variable among Ad21 genome types, and that

1 appropriate evaluation and response to Ad21-associated outbreaks of FRI may require

2 genome type characterization for this particular viral agent.

3   In preparation for the restoration of Ad4/Ad7 vaccine, estimated for 2008 (22), we

4 have initiated studies aimed at characterizing the molecular epidemiology of Ad infections

5 in the military environment. In this paper we report the genomic characterization of 75 Ad21

6 strains isolated from military recruits with FRI during the 10-year period (1996–2005)

7 spanning the initial curtailment and eventual discontinuation of the Ad4/Ad7 vaccination

8 protocol. We also report the characterization of a single strain isolated from an ill

9 serviceperson deployed at sea.

10

11 **MATERIALS AND METHODS**

12

13 **Surveillance and sample collection.** Since 1996, the Naval Health Research Center

14 (NHRC) has conducted population-based surveillance of FRI-associated Ad infections as

15 the Navy node for the Department of Defense Global Emerging Infections Surveillance and

16 Response System. Surveillance currently includes eight military training camps throughout

17 the United States (Fig. 1). Dedicated NHRC staff at seven sites and collaborators at the

18 eighth site (Cape May) monitor their trainee populations for symptoms of FRI (fever [oral

19 temperature ≥ 38°C/100.5°F] plus a respiratory symptom, such as cough or sore throat).

20 Clinical specimens are routinely obtained from a subset of qualifying recruits who seek

21 medical care, frozen at -80°C, then sent on dry ice to NHRC to be tested for Ad, influenza A

22 and B, parainfluenza 1, 2, and 3 and respiratory syncytial virus. Surveillance is also

23 conducted onboard deployed ships with ultra-low temperature storage capability.

1

2   **Origin of Ad21 virus strains**. Genome-typing work was carried out on one Ad21 strain

3   isolated from a nontrainee military person aboard the USS Peleliu (LHA-5) while deployed

4   at sea and 75 Ad21 strains isolated from the pharyngeal swabs of military trainees

5   assigned to the eight US training centers (Fig. 1): Fort Jackson, SC (FJ); Fort Benning, GA

6   (FB); Fort Leonard Wood, MO (FLW); Naval Recruit Training Center, Great Lakes, IL

7   (NRTC); Lackland Air Force Base, San Antonio, TX (LAFB); Coast Guard Training Center,

8   Cape May, NJ (CGTC); Marine Corps Recruit Depot, Parris Island, SC (PI); and Marine

9   Corps Recruit Depot, San Diego, CA (SD). All samples were collected between 1996 and

10   2005 from individuals reporting ill with symptoms of FRI. All viral isolations were initially

11   carried out in A549 cell monolayers and confirmed to be Ads by immunofluorescence in the

12   College of American Pathologists-certified NHRC diagnostic microbiology laboratory. The

13   Ad isolates analyzed in this study were identified as serotype 21 by either a

14   microneutralization assay (17) or PCR (18), or both. Serotyped isolates were then stored at

15   -80°C for further analysis.

16

17   **Viral DNA purification and restriction enzyme analysis.** Ad isolates were shipped frozen

18   on dry ice to the Lovelace Institute for further characterization. All isolates were passed

19   once onto monolayers of A549 cells in 25 cm^2 flasks and subsequently amplified in

20   monolayers of A549 cells in 75 cm^2 flasks for viral DNA extraction. When extensive

21   cytopathic effect was evident, intracellular viral DNA was isolated from infected cells by the

22   method developed by Shinagawa and colleagues (24), with modifications as previously

23   described (13). For genome-type identification, 1 μg of viral DNA was initially digested with

1 10 U of restriction endonuclease *Bam*HI following manufacturer's recommendations

2 (Promega, Madison, WI). Viral DNA was further characterized by digestion with *BgI*I, *BgI*II,

3 *BstE*II, *Hind*III, *Kpn*I, and *Sma*I. DNA fragments were separated by horizontal gel

4 electrophoresis on 1% agarose gels run in 1X TBE buffer (0.09 M Tris-borate; 0.002 M

5 EDTA pH 8). Restriction profiles were visualized by UV transillumination at 303 nm after

6 staining with ethidium bromide, and photographed in a Gel Doc imager (Bio-Rad, Hercules,

7 CA).

8

9 **Genome type denomination.** Genome types were denominated following the system

10 developed by Li and Wadell (16) through comparison of the generated restriction patterns

11 with published profiles. Genome types or DNA variants were discriminated based on their

12 distinct profile of *Bam*HI restriction fragments and designated p for the prototype strain, and

13 a, b, c, etc., for subsequently identified variants. Additional restriction enzymes

14 discriminated subtypes a1, a2, etc. Restriction profiles were also denominated using the

15 system developed by Adrian (1) to facilitate comparisons with the limited available

16 literature.

17

18 **PCR amplification and sequence analysis of the hypervariable region of the hexon**

19 **gene and the fiber gene knob**. Fragments of 1524 bp spanning the seven hypervariable

20 regions of the hexon gene of Ad21 were generated by PCR using a high-fidelity Taq

21 Polymerase (iProof, Bio-Rad, Temecula) and the following primer pairs: forward Hex 1: 5'-

22 GGCTGAGTTGCTTTC-3' and reverse Hex 8: 5'-CCATTCATGTAGTCATAAGAG-3', or

23 forward Hex 3: 5'-CCCAATACATCTCAGTG-3' and reverse Hex 6: 5'-

1   CACATCAGGATCATAGC-3'. The amplification mix contained 1.5 mM MgCl2, 1 U of iProof

2   Taq polymerase, 200 μM dNTP, 1X iProof HF buffer provided by the manufacturer, and 1

3   μM of each primer in a total volume of 50 μl. The cycling conditions consisted of an initial

4   step of denaturation for 30 s at 94 °C followed by 50 cycles of 30 sec at 94°C, 45 sec at

5   49°C and 45 sec at 72°C. A final step of extension for 5 min at 72°C was added. PCR

6   fragments were purified with Montage PCR columns and Micropure-EZ columns (Millipore,

7   Billerica, MA). Sequencing from both stands was achieved with the primer sets listed in

8   Table 1 (Hex 1–8).

9   The fiber gene was sequenced using a similar approach with the following primer

10 pairs: forward, Ad21 fiber up: 5'-CTACCCCTATGAAGATGAAAGCAC-3' and reverse, Ad21

11 fiber knob down 1: 5'-AAGGGATAAGCTGTAGTACTTGGC-3', or forward, Ad21 fiber up

12 and reverse, Ad21 fiber knob down 2: 5'-AAATGGGAAGGGGGAGG-3'. Cycling conditions

13 for amplification of the fiber gene were: 5 min at 94°C followed by 49 cycles of denaturing

14 at 94°C for 30 s, annealing at 60.5°C for 30 s and extension at 72°C for 30 s. A final step of

15 extension for 5 min at 72°C was added. PCR fragments were purified with Montage PCR

16 columns and Micropure-EZ columns (Millipore). Sequencing services were contracted from

17 the DNA Research Services of the University of New Mexico Health Sciences Center. All

18 primers used for the amplification of hexon and fiber-coding regions, and the sizes of the

19 corresponding amplification products, are listed in Table 1. Sequence data were deposited

20 in GenBank under accession numbers DQ349207-DQ349216 for hexon gene sequences,

21 and DQ349217, DQ349218, DQ369951–DQ369958 for fiber gene sequences.

1   Sequence data analysis was carried out using Seqman software for contig assembly

2   and Megalign software for sequence alignments (both programs are components of the

3   Lasergene suite, DNASTAR, Inc., Madison, WI).

4

5   **RESULTS**

6

7   **Adenovirus type 21-associated FRI**

8   According to data from continuous surveillance of Ad-associated FRI by the NHRC

9   laboratory, Ad21 was identified to be present in 58% of all serotyped Ad-positive clinical

10   samples in 1996 (n=24), 5% in 1997 (n=1618), 14% in 1998 (n=260), 3.6% in 1999

11   (n=248), 0% in 2000 (n=261), 0.4% in 2001 (n=271), 0% in 2002 (n=220), 0% in 2003

12   (n=212), 1% in 2004 (n=475), and 2.6% for January through September 2005 (n=307). The

13   circulation of Ad21 strains was documented in six of the eight sites under surveillance

14   during the 10-year period: NRTC, FLW, FJ, FB, SD, and PI. One strain was recovered from

15   the pharyngeal swab of an active-duty, nontrainee military person aboard the USS Peleliu

16   (LHA-5) while deployed at sea.

17   The following Ad21 strains were characterized by restriction enzyme analysis: Five

18   random representatives of the 14 strains isolated in 1996 from GL; 22 random

19   representatives of the 82 strains isolated in 1997 from GL, FLW, and FJ; 29 random

20   representatives of the 36 strains isolated in 1998 from SD, FLW, FB, and FJ; all 9 strains

1   isolated in 1999 from SD; the only strain isolated in 2001 in FJ; all 4 strains isolated in 2004

2   from PI; and all 5 strains isolated in 2005 from FLW, PI, FJ, and the USS Peleliu.

3

4   **Genome type analysis of Ad21 strains circulating between 1996 and 2005**

5   Two major genome types and a total of four different DNA variants were identified

6   among the 76 isolates subject to whole-genome restriction enzyme analysis with seven

7   endonucleases. The genomes of all 75 isolates were initially characterized by digestion

8   with *Bam*HI, *Bgl*II, *Hind*III, and *Sma*I. Restriction profiles are shown in Figure 2 in

9   comparison to those of the prototype strain AV-1645 [Saudi Arabia, 1956; (5)]. Following

10   the denomination system proposed by Li and Wadell (16), analysis with *Bam*HI

11   discriminated two distinct DNA variants, 21a and 21b. Further analysis with *Bgl*II

12   discriminated two new subtypes, 21a1 and 21b1. All genome types exhibited prototype-like

13   *Hind*III restriction profiles (profile "p" according to the denomination system proposed by Li

14   and Wadell (16), and profile "1" according to the system developed by Adrian et al. (1) and

15   identical *Sma*I restriction profiles that were found to correspond to the *Sma*I pattern 2

16   described by van der Avoort et al. following the system of Adrian and colleagues (1)(29)). In

17   order to conduct a more thorough comparison of the NHRC strains with Ad21 strains from

18   other studies, further analyses with the endonucleases *Bgl*I, *BstE*II and *Kpn*I were carried

19   out. Restriction profiles obtained with these enzymes are shown in Fig. 3. All isolates

1   exhibited identical *Bgl*I and *BstE*II profiles matching pattern 2 described for these

2   endonucleases by van der Avoort et al. (29). Two *Kpn*I patterns deviating from the

3   prototype were identified and denominated "a" and "b". Pattern "a" appeared identical to the

4   *Kpn*I pattern 4 described by van der Avoort et al. (29)

5   The results of genome typing by restriction enzyme analysis with the complete set of

6   7 enzymes are shown in Table 2. The temporal and geographic distribution of the 75

7   genome-typed isolates associated with outbreaks of FRI in recruit training camps is shown

8   in Fig. 4. The genome type and month of detection for the strain recovered aboard the USS

9   Peleliu is also shown in this figure, but it should be noted that shipboard surveillance was

10   only recently initiated and is temporally irregular.

11

12   **Sequence analysis of hexon and fiber genes**

13   The sequence of the 5' end of the hexon gene, spanning the first 1450 nucleotides

14   and comprising the start codon and the seven hypervariable regions (HVR1–HVR7), was

15   determined for 10 Ad21 strains representing the 4 genomic variants and different sites and

16   years of isolation. The complete sequence of the fiber gene was determined for the same

17   10 strains. Accession numbers are shown in Table 2.

18   All strains examined showed identical hexon and fiber gene sequences. The

19   consensus sequence for the hexon and fiber of NHRC Ad21 strains were compared with

1   those of the prototype strain of Ad21 [AV-1645, Saudi Arabia, 1956, (5)] and the closely

2   related prototype strain of Ad50 [Wan (7), GenBank accession number AY737798]. The

3   corresponding sequences of Ad34p (Compton, GenBank accession number AY737797)

4   and Ad35p (Holden, GenBank accession number AY128640) were also included in the

5   analysis because of the previous record of circulation in the United States of intermediate

6   strains (identified as Ad21/H21+35) representing apparent recombinants of Ad21 and

7   Ad35, a closely related serotype of subspecies B2 (10). Additional GenBank sequences

8   corresponding to field strains of Ad21 were included when available.

9   Sequence alignments of the amino-terminal 460 amino acids of the hexon

10   polypeptide clearly identified the seven HVRs of the NHRC strains as 21-like (Figs. 5 and

11   6). Differences in the amino acid sequences of the hexon gene between the NHRC Ad21

12   strains and the prototype strain AV-1645 were detected in HVR-1, HVR-3, HVR-4, and

13   HVR-5. No major differences were detected between the hydrophobicity plots of the

14   corresponding polypeptides (data not shown).

15   Differences in the sequences of the fiber genes of the NHRC Ad21 strains and the

16   prototype strain AV-1645 were detected in the antigenic shaft and knob domains as shown

17   in Fig. 7. The fiber protein of the NHRC strains is closely related to that of the prototype AV-

18   1645 (97.2% amino acid sequence identity) and also to the fiber polypeptide of Ad50 strain

19   Wan (98.8% amino acid sequence identity). A six-amino acid motif AATSSK present in

1   positions 226-231 in the sequence of the NHRC Ad21 fiber, but not in the fiber of the

2   prototype strain AV-1645, was identical to that present in the same position in the fiber of

3   Ad50p and also in the fibers of the recently described Malaysian strains of Ad21 SIBU97,

4   MY7/1, and MY8/1 (19). A similar motif TVASSK is also present in the fibers of Ad34p and

5   Ad35p.

6

7   **DISCUSSION**

8

9   The relative contribution of Ad21 to the etiology of Ad-associated FRI among military

10 recruits appears to be determined by the levels of circulation of Ad4 and Ad7. During the

11 years when infection by Ad4 and Ad7 was being effectively controlled by vaccination, Ad21

12 acquired (or possibly retained) a niche in military recruit camps as revealed by the fact that

13 this serotype was present in 58% of all serotyped Ad-positive clinical samples in 1996 (the

14 last full year of vaccination), and was also the serotype that appeared to take over after

15 introduction of the vaccine (26). Surveillance data in succeeding years showed 5% Ad21 in

16 1997, 14% in 1998, 3.6% in 1999, 0% in 2000, 0.4% in 2001, 0% for 2002-2003, 1% in

17 2004, and 2.6% in 2005. It is interesting to note that, as the percentage of Ad21 detection

18 dropped off between 1996 and 1999, it was being progressively replaced by the serotypes

19 previously controlled by the use of the vaccine; surveillance showed a mix of primarily Ad7

1 and Ad4 in 1997, then almost entirely Ad4 in later years (6). The gradual nature of this

2 transition may reflect the gradual loss of the vaccine, which continued to be used

3 sporadically during outbreaks and peak seasons between 1997 and 1999. Ad4 continues to

4 dominate through the current year (NHRC, unpublished data) in the absence of the

5 vaccine.

6 In contrast to their moderate level of recognized impact among US recruits, Ad21

7 genome types have caused massive documented outbreaks of respiratory disease in other

8 countries, including New Zealand, The Netherlands, and Germany (4, 15, 29), and

9 essentially replaced Ad4 and Ad7 as the dominant serotype in European recruit camps for

10 periods of several years (29, 30).

11 Although the effects of genetic variability on Ad virulence and fitness have not been

12 thoroughly examined, several studies have suggested that newly emerging genome types

13 of species B1 serotypes cause outbreaks of disease characterized by more severe clinical

14 presentations (25, 32).

15 Four different genome types were identified among the 765 Ad21 strains

16 characterized in this study. Using a panel of seven endonucleases, the four DNA variants

17 were defined and named Ad21a, Ad21a1, Ad21b, and Ad21b1 following the denomination

18 system proposed by Li and Wadell (14). Ad21a was the most prevalent genome type,

19 accounting for 69 of the 76 strains examined. Ad21a was the only Ad21 genome type

1   detected at FLW, NRTC, FB, FJ, and PI during outbreaks of FRI between 1996 and 1999.

2   A unique *Bgl*II variant of genome type Ad21a — Ad21a1 — was identified only at SD in

3   1998, and was the only Ad21 genomic variant seen at SD during the study. Ad21a

4   reappeared in 2004 and 2005 at PI after a 4-year (2000–2003) apparent absence of Ad21

5   from all the surveyed recruit training facilities. Two genome types not previously detected

6   among US recruits, Ad21b and Ad21b1, were identified by restriction enzyme analysis with

7   *Bam*HI and *Bgl*II. Ad21b was found in 2005 at PI and aboard the USS Peleliu. Ad21b1 was

8   found in 2005 at FLW. These variants appear to have emerged after the aforementioned 4-

9   year disappearance of Ad21.

10   The comparison of the four newly identified NHRC Ad21 genome types with those of

11   strains circulating in the United States, Europe, and Asia in previous years is limited by the

12   very few published studies examining the molecular epidemiology of Ad21 infections. The

13   only previous records of genomic characterization of Ad21 strains isolated in the United

14   States (2, 29) describe four isolates corresponding to DNA variants D2 and D5 (as denoted

15   by Adrian) circulating between 1976 and 1981. Genomic analysis data were also available

16   for Dutch and German Ad21 strains (2, 29) and also for Malaysian strains isolated during

17   an outbreak of enterovirus 71-associated hand, foot, and mouth disease (19). Based on

18   their *Bam*HI restriction profiles and following the denomination system of Li and Wadell

19   (14), the D2 strains prevalent in Europe before 1980 (29) and the Malaysian Ad21 DNA

1   variants could be described as Ad21a-like genome types. The NHRC strains of genome

2   type 21a show an intriguing resemblance to the Malaysian Ad21 strains, but correspond to

3   different genome types from those identified among strains isolated the United States

4   between 1976 and 1981 (10). Some of these strains, genome typed as variant D2 (29),

5   were shown to be intermediate variants Ad21/H21+35 by cross-neutralization assays with

6   reference antisera by Hierholzer and colleagues (10). Although no comprehensive

7   serological analysis of antigenic reactivity was conducted in our study, the sequence

8   analysis of the hexon and fiber genes of the NHRC Ad21 strains clearly identified both

9   regions encoding major neutralizing epitopes as Ad21-like.

10   Ad21 grows much more slowly than Ad4 and Ad7 in tissue culture (30), and has

11   been shown to have low rates of transmission under certain circumstances (14). This may

12   explain why it does not cause as widespread or continuous outbreaks as Ad7 and Ad4 do

13   in the absence of a specific vaccine. In US recruit training centers, the contribution of Ad21

14   to the etiology of FRI was not historically affected by application of the Ad4/Ad7 vaccine.

15   However, in the presence of the vaccine, the proportion of Ad21-associated FRI increases

16   dramatically due to the control of Ad4- and Ad7-associated FRI (9), suggesting that the

17   vaccine has no effect on Ad21 and also that Ad21 is not directly competing with Ad4 and

18   Ad7. In any case, in the presence of the Ad4/Ad7 vaccine, Ad21 does not appear to greatly

1   increase in impact, but rather becomes the dominant Ad by default. The impact of Ad21 in

2   this context is still significant, however, accounting for 3–4% of FRI (9).

3   After the reintroduction of the Ad4/Ad7 vaccine anticipated to occur in 2008 (22),

4   Ad21 is expected to become a predominant Ad serotype in US recruit training centers once

5   again. Knowledge of circulating genome types and their epidemic behavior will be of

6   significant value to ongoing surveillance efforts in these highly susceptible and impacted

7   populations.

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11

1   **FIGURE LEGENDS**

2

3   **FIG. 1.** Sites participating in the nationwide surveillance of FRI.

4

5   **FIG. 2.** Restriction enzyme analysis of NHRC Ad21 strains with *Bam*HI, *Bgl*II, *Hind*III and

6   *Sma*I. Lane **P**: prototype strain of Ad21, AV-1645; lane **M**: molecular weight markers (1kb +

7   100 bp ladders, Bio-Rad, Hercules, CA). Letter **p** denotes a prototype-like restriction profile;

8   letters **a**, **b**, etc. denominate distinct restriction profiles; numbers **1** and **2** refer to the

9   corresponding pattern identified by van der Avoort et al. (29).

10

11   **FIG. 3.** Restriction enzyme analysis of NHRC Ad21 strains with *Bgl*I, *BstE*II and *Kpn*I. Lane

12   **P**: prototype strain of Ad21, AV-1645; lane **M**: molecular weight markers (1 kb + 100 bp

13   ladders, Bio-Rad, Hercules, CA). Letters **a**, **b**, etc. denominate distinct restriction profiles;

14   numbers **1** and **2** refer to the corresponding pattern identified by van der Avoort et al. (29).

15

16   **FIG. 4.** Geographic and temporal distribution of Ad21-associated cases of FRI in military

17   training camps and corresponding genome types.

18

19   **FIG. 5.** Alignment of the deduced amino acid sequence of the seven hypervariable regions

20   (HVRs) of the hexon gene of NHRC Ad21 strains with those of the prototype strains of

21   Ad21 (AV-1645, AY008279), Ad50 (Wan, AY737798), Ad34 (Compton, AY737797), and

22   Ad35 (Holden, AY128640). Sequences alignments were carried out with Clustal W and

23   Megalign (Lasergene suite, DNASTAR, Inc., Madison, WI)

1

2   **FIG. 6.** Analysis of the relative sequence identity between the predicted NHRC Ad21 hexon

3   polypeptide and the hexon polypeptides of Ad21p, Ad50p, Ad34p, and Ad35p. Sequences

4   alignments were carried out with Clustal W and Megalign (Lasergene suite, DNASTAR,

5   Inc., Madison, WI).

6

7   **FIG. 7.** Alignment of the deduced amino acid sequence of the fiber protein of NHRC Ad21

8   strains with those of the prototype strains of Ad21 (AV-1645, U06107), Ad50 (Wan,

9   AY737798), Ad34 (Compton, AY737797), and Ad35 (Holden, AY128640). Sequences

10 alignments were carried out with Clustal W and Megalign (Lasergene suite, DNASTAR,

11 Inc., Madison, WI). ⸱⸱⸱⸱ fiber tail domain; ⸺ fiber shaft domain; **----** fiber knob domain.

1 **TABLE 1**: Primer sets used in the amplification and sequencing of the hexon and fiber

2 genes of NHRC Ad21 strains.

REGION	PCR and sequencing primers	Amplicon[a]
Hexon gene partial cds[b].	Forward Hex1: 5'-GGCTGAGTTGCTTTC-3' Reverse Hex8: 5'-CCATTCATGTAGTCATAAGAG-3'	1524 bp
HVR1-HVR7[c]	Forward Hex3: 5'-CCCAATACATCTCAGTG-3' Reverse Hex6: 5'-CACATCAGGATCATAGC-3'	702 bp
	Reverse Hex2: 5'-GCAATCCACTGAGATG-3' Reverse Hex4: 5'-GCAAAAGACCCATAGC-3' Forward Hex5: 5'-GCTATGGGTCTTTTGC-3' Forward Hex7: 5'-GTGACAGAACCAGATCTTC-3'	Seq[d] Seq Seq Seq
Fiber gene partial cds.	Forward Fiber tail: 5'-CTACCCCTATGAAGATGAAAGCAC-3' Reverse Fiber knob 1: 5'-AAGGGATAAGCTGTAGTACTTGGC- Reverse Fiber knob 2: 5'-AAATGGGAAGGGGGAGG-3'	684 bp 1004 bp

3

4 [a] Length of obtained PCR product. [b] cds – coding sequence. [c] HVR – Hypervariable Region. [d] Seq - primer

5 used for sequencing only.

TABLE 2. Origin of NHRC Ad21 isolates, identified genome types and accession
numbers for hexon and fiber gene sequences

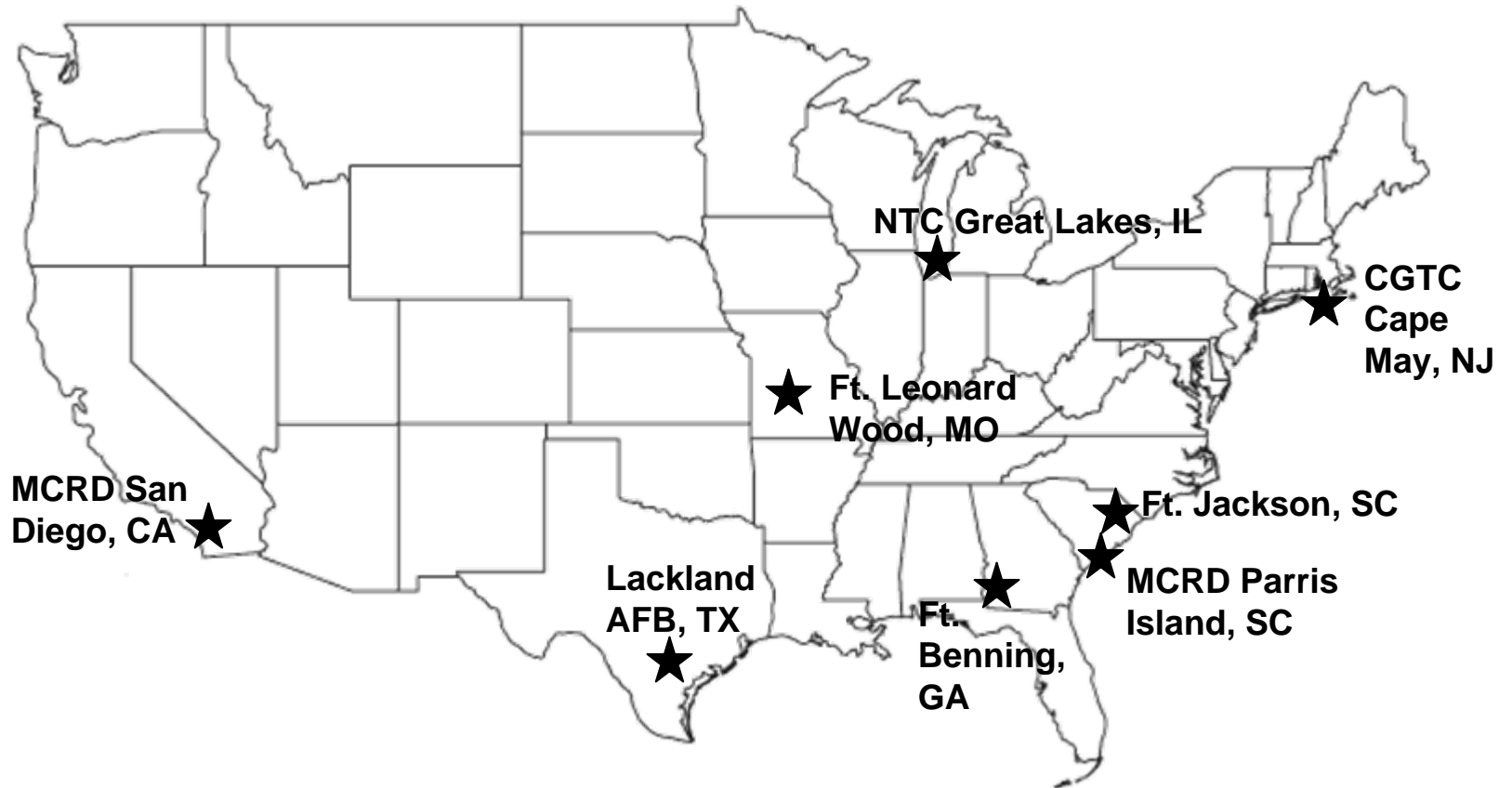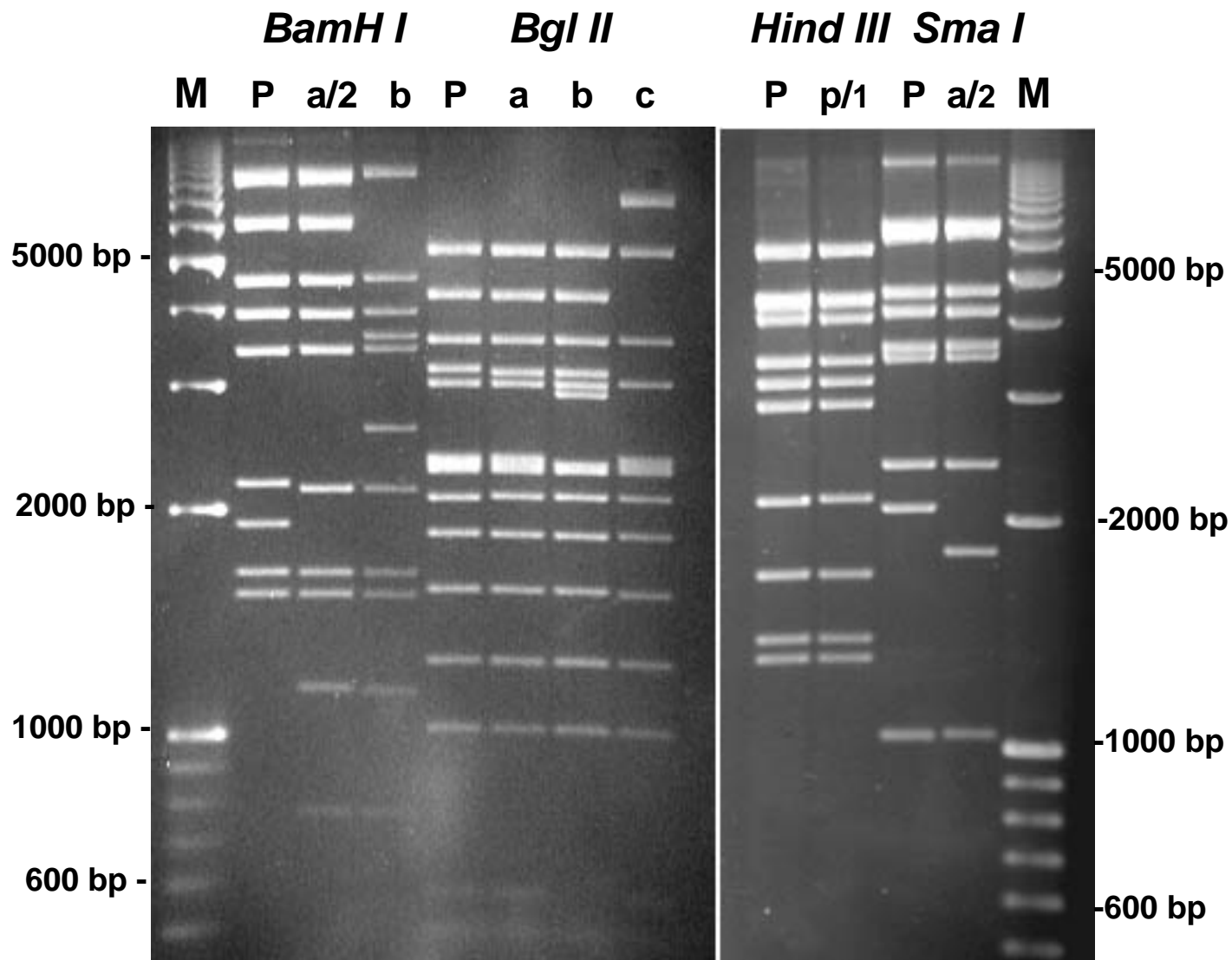| Genome type	Digestion profile (from Figs. 2A and 2B)							Location (number of isolates)	Hexon HVR1–7 sequence accession number *
	*Bam*HI	*Bgl*I	*Bg* II	*Bst*EII	*Hind*III	*Kpn*I	*Sma*I		
Ad21p AV-1645	p/1	p/1	p/1	p/1	p/1	p/1	p/1	Saudi Arabia, 1956	AY008279
Ad21a	a/2	a/2	a	a	p/1	a/4	a/2	PI, (n=16)	DQ349209 DQ349207
								NRTC (n=12)	DQ349211
								FLW (n=18)	DQ349212
								FB (n=13)	D349216
								FJ (n=9)	DQ349215
Ad21a1	a/2	a/2	b	a/2	p/1	a/4	a/2	SD (n=5)	DQ349214
Ad21b	b	a/2	a	a/2	p/1	b	a/2	PI (n=1)	DQ349208
								USS Peleliu (LHA-5) (n=1)	DQ349210
Ad21b1	b	a/2	c	a/2	p/1	b	a/2	FLW (n=1)	DQ349213

4

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

```
Ad21p_AV-1645.pro  MATPSMLPQWAYMHIAGQDASEYLSPGLVQFARATDTYFNLGNKFRNPTVAPTHDVTTDRSQRLMLRFVPVDREDNTYAYKVRYTLAVGDNRVLDMASTF  100
Ad21_NHRC.pro      .................................................................................................... 100
Ad50p_Wan.pro      .................................................................................................... 100
Ad34p_Compton.pro  .................................................................................S.................... 100
Ad35p_Holden.pro   .................................................................................S.................... 100

Ad21p_AV-1645.pro  FDIRGVLDRGPSFKPYSGTAYNSLAPKGAPNTSQWIAEGVKKE----DGGSDEEEEKNLTTYTFGNAPVKAEGGDITKDKGLPIGSEITDG-EAKPIYAD 195
Ad21_NHRC.pro      ..........................................----N.E.............................................-...... 195
Ad50p_Wan.pro      .................................LNK----------.DEEDG.DDQQA............A-E....-E.....L.VPSEGGP...... 188
Ad34p_Compton.pro  .........................A...LDK..TSTGLVD..NT.DG..AKKA............A-E...-D...V.L.VSTEG-P..... 197
Ad35p_Holden.pro   .........................A.....K..PTAAAAGN.EEEH.T.EKTA....A.......A-Q...-E.....L..SAEN.S..... 198

Ad21p_AV-1645.pro  KLYQPEPQVGDETWTDTDGTTEKYGGRALKPETKMKPCYGSFAKPTNVKGGQAKQKTTEQPQNQQVEYDIDMNFFDEASQKANFSPKIVMYAENVDLETP 295
Ad21_NHRC.pro      ......................A.............................................L.............Q................. 295
Ad50p_Wan.pro      ...........E.S......D...........................V.-K.EEGK--..........LR..MTGLK............... 285
Ad34p_Compton.pro  ...........L..K..E...V...................I.....V.PK.DDGTNNI.........LR..RSELK..........C. 297
Ad35p_Holden.pro   ...........L..K..E......T.N.......Y....L......P.NS.-.SSEKI......E...NS..RT............G.... 297

Ad21p_AV-1645.pro  DTHVVYKPGTSEESSHANLGQQSMPNRPNYIGFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDP 395
Ad21_NHRC.pro      .................................................................................................... 395
Ad50p_Wan.pro      ........A.DA......................................................................................... 385
Ad34p_Compton.pro  ........V.DA..ET.................................................................................... 397
Ad35p_Holden.pro   .........EDT..E..................................................................................... 397

Ad21p_AV-1645.pro  DVRIIENHGVEDELPNYCFPLDGVGVPISSYKIIEPNGQ-GADWKEPDINGTSEIGQGNLFAMEINLQANLWRSFLYSNVALYLPD            480
Ad21_NHRC.pro      .......................TT............E-........................S.....................            480
Ad50p_Wan.pro      ...V.................PR.D...G..T..DETTT..DLEPK.I...AK..P..............................SYKYTPANV 480
Ad34p_Compton.pro  ...V.................PRTD...E.K...DQS-T.TNV.PT.S..LAK..P.............................            480
Ad35p_Holden.pro   ...V.................I...TT...S.V...EDNNN....EV......................................            480
```

```
Ad21p AV-1645.pro  MATPSMLPQWAYMHIAGQDASEYLSPGLVQFARATDTYFNLGNKFRNPTVAPTHDVTTDRSQRLMLRFVPVDREDNTYAYKVRYTLAVGDNRVLDMASTF  100
Ad21 NHRC.pro      ....................................................................................................  100
Ad50p Wan.pro      ....................................................................................................  100
Ad34p Compton.pro  .............................................................................S......................  100
Ad35p Holden.pro   .............................................................................S......................  100


Ad21p AV-1645.pro  FDIRGVLDRGPSFKPYSGTAYNSLAPKGAPNTSQWIAEGVKKE----DGGSDEEEEKNLTTYTFGNAPVKAEGGDITKDKGLPIGSEITDG-EAKPIYAD  195
Ad21 NHRC.pro      ..........................................----N.E...........................................-.......  195
Ad50p Wan.pro      ....................................LNK----------.DEEDG.DDQQA...........A-E...-E.....L.VPSEGGP......  188
Ad34p Compton.pro  ..........................A...LDK.TSTGLVD..NT.DG..AKKA.............A-E...-D...V.L.VSTEG-P......  197
Ad35p Holden.pro   ..........................A.....K..PTAAAAGN.EEEH.T.EKTA....A.......A-Q...-E.....L..SAEN.S......  198


Ad21p AV-1645.pro  KLYQPEPQVGDETWTDTDGTTEKYGGRALKPETKMKPCYGSFAKPTNVKGGQAKQKTTEQPQNQQVEYDIDMNFFDEASQKANFSPKIVMYAENVDLETP  295
Ad21 NHRC.pro      ...........................A...............................L................Q.......................  295
Ad50p Wan.pro      ..........E.S......D.........................V.-K.EEGK--.......LR..MTGLK.......................  285
Ad34p Compton.pro  ...............L..K..E....V..............I.....V.PK.DDGTNNI........LR..RSELK..............C.  297
Ad35p Holden.pro   ...............L..K..E.......T.N.....Y....L......P.NS.-.SSEKI......E...NS..RT...........G....  297


Ad21p AV-1645.pro  DTHVVYKPGTSEESSHANLGQQSMPNRPNYIGFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDP  395
Ad21 NHRC.pro      ....................................................................................................  395
Ad50p Wan.pro      ...........A.DA.....................................................................................  385
Ad34p Compton.pro  .........V.DA..ET..................................................................................  397
Ad35p Holden.pro   ..........EDT..E...................................................................................  397


Ad21p AV-1645.pro  DVRIIENHGVEDELPNYCFPLDGVGVPISSYKIIEPNGQ-GADWKEPDINGTSEIGQGNLFAMEINLQANLWRSFLYSNVALYLPD         480
Ad21 NHRC.pro      .......................TT.........E-.................S...............................         480
Ad50p Wan.pro      ...V.................PR.D...G..T..DETTT..DLEPK.I...AK..P.........................SYKYTPANV  480
Ad34p Compton.pro  ...V.................PRTD...E.K...DQS-T.TNV.PT.S..LAK..P........................            480
Ad35p Holden.pro   ...V...............I...TT...S.V...EDNNN....EV..................................            480
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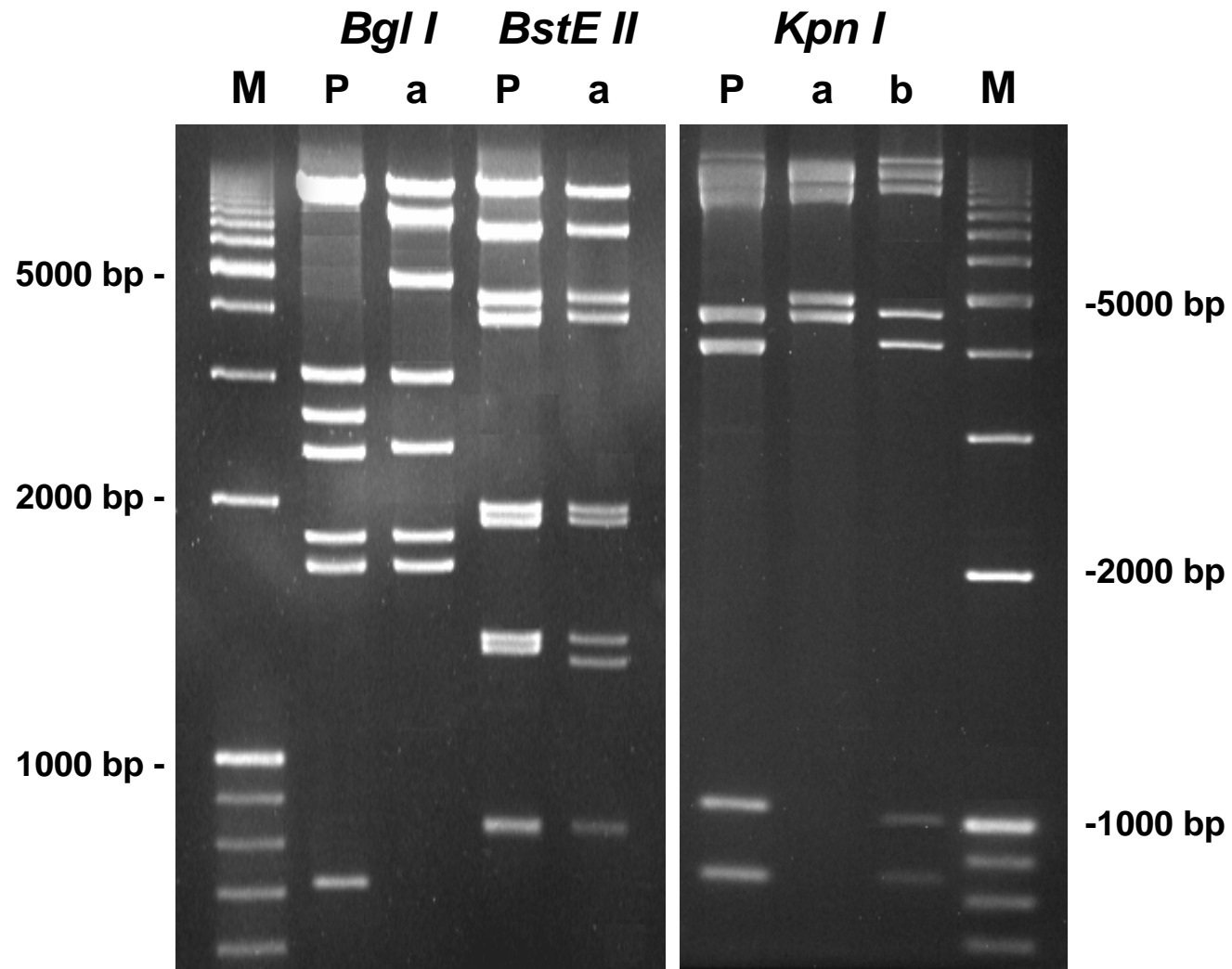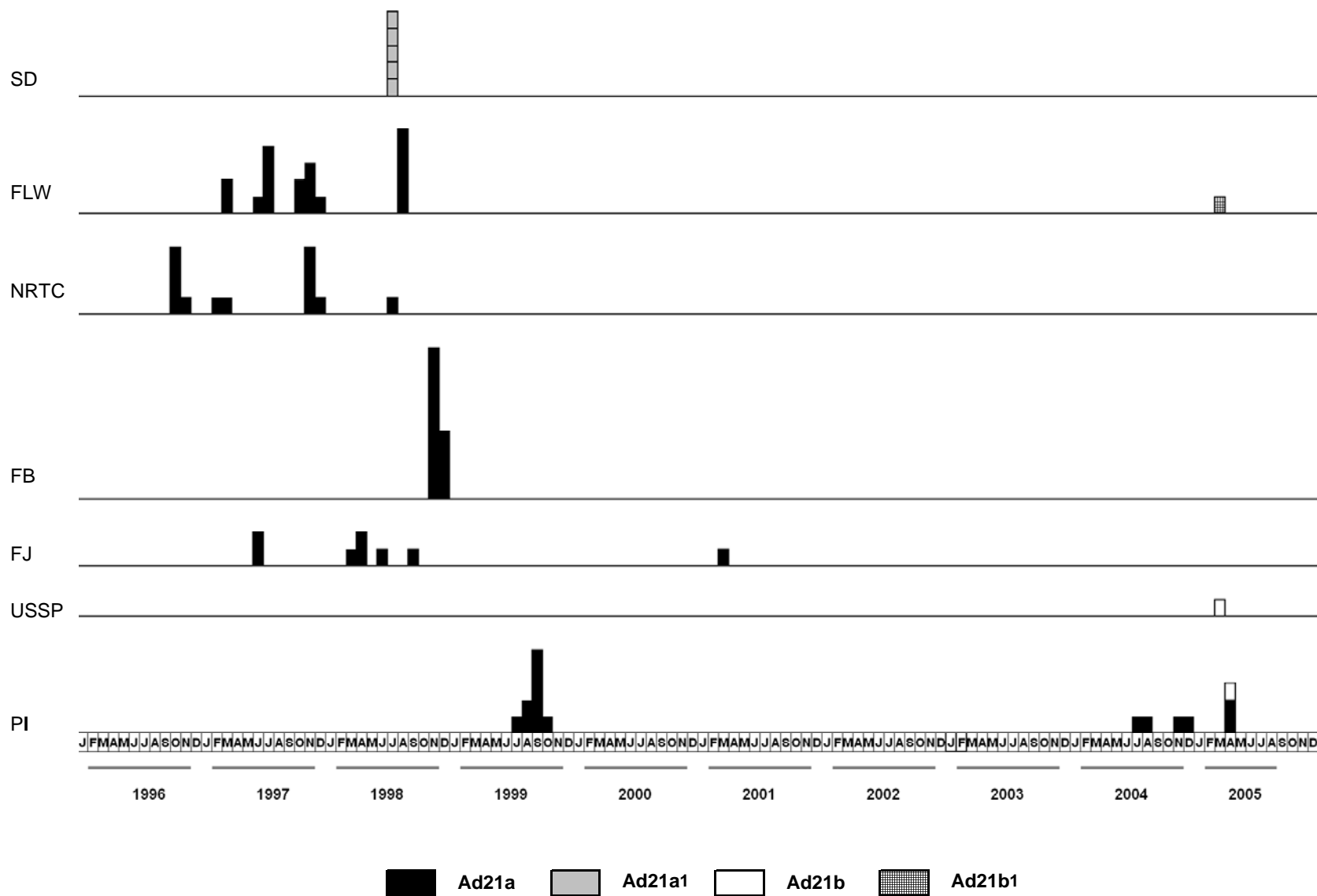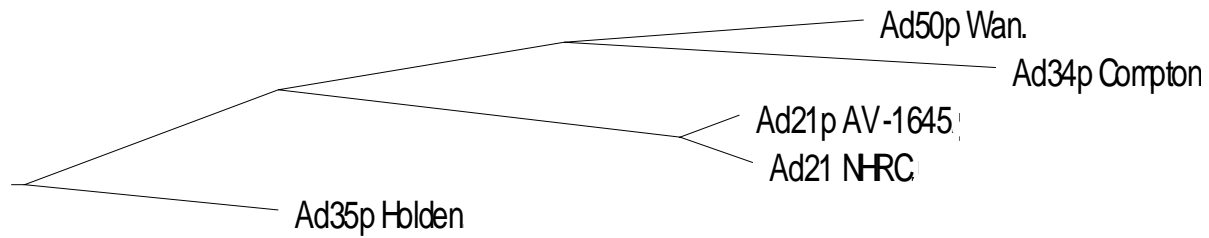
Figure 6

Hexon polypeptide		Percent identity				
		1	**2**	**3**	**4**	**5**
Ad21p-AV1645	**1**	■	98.1	85.0	83.3	85.8
Ad21 NHRC	**2**	■	■	84.6	82.9	86.2
Ad50p Wan	**3**	■	■	■	87.7	82.9
Ad34p Compton	**4**	■	■	■	■	84.4
Ad35p Holden	**5**	■	■	■	■	■

Figure 7

Tail ·· Shaft ──────────

```
AV-1645 fiber.pro      MTKRVRLSDSFNPVYPYEDESTSQHPFINPGFISPNGFTQSPDGVLTLNCLTPLTTTGGPLQLKVGGGLI  70
Ad21 NHRC fiber.pro    ...........................................K..............................T  70
Ad50p Wan fiber.pro    .........................................................................  70
Ad34p Compton fiber.pro ..........................................K...........S.........T  70
Ad35p Holden fiber.pro ..........................................K...........S.........T  70
```

──────────────────────────────── Knob

```
AV-1645 fiber.pro      VDDTDGTLQENIRATAPITKNNHSVELSIGNGLETQNNKLCAKLGNGLKFNNGDICIKDSINTLWTGIKP  140
Ad21 NHRC fiber.pro    ...........................................................................  140
Ad50p Wan fiber.pro    ....................V......................................................  140
Ad34p Compton fiber.pro ......................................................................N.  140
Ad35p Holden fiber.pro ......................................................................N.  140
```

─ ─

```
AV-1645 fiber.pro      PPNCQIVENTDTNDGKLTLVLVKNGGLVNGYVSLVGVSDTVNQMFTQKSATIQLRLYFDSSGNLLTDESN  210
Ad21 NHRC fiber.pro    ...........................................................................  210
Ad50p Wan fiber.pro    ...........................................................................  210
Ad34p Compton fiber.pro ..........N...........................................T.N....................D  210
Ad35p Holden fiber.pro ..........N...........................................T.N..............E..D  210
```

─ ─

```
AV-1645 fiber.pro      LKIPLKNKSSTATSEVLQPAEAFMPSTTAYPFNTTTRDSENYIHGICYYMTSYDRSLVPLNISIMLNSRT  280
Ad21 NHRC fiber.pro    ....................AATSSK.............................................H.  280
Ad50p Wan fiber.pro    ....................AATSSK.................................................  280
Ad34p Compton fiber.pro ....................TVASSK...........................F...........M  280
Ad35p Holden fiber.pro ....................TVASSK...........................F...........M  280
```

─ ─

```
AV-1645 fiber.pro      ISSNVAYAIQFEWNLNAKESPESNIATLTTSPFFFSYIREDDN  323
Ad21 NHRC fiber.pro    ..........................................  323
Ad50p Wan fiber.pro    ..........................................  323
Ad34p Compton fiber.pro .....................S.................T....  323
Ad35p Holden fiber.pro .....................S.................T....  323
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REPORT DOCUMENTATION PAGE

1. Report Date (DD MM YY) 02 05 06	2. Report Type Technical Report	3. DATES COVERED (from - to) October 1998-present

4. TITLE AND SUBTITLE
Genomic Characterization of Adenovirus 21 Strains Associated With Outbreaks of Febrile Respiratory Illness in United States Military Recruit Training Centers Between 1996 and 2005

5a. Contract Number:
5b. Grant Number:
5c. Program Element:
5d. Project Number:
5e. Task Number:
5f. Work Unit Number: 60501

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9. PERFORMING ORGANIZATION REPORT NUMBER
Report No. 06-15

10. Sponsor/Monitor's Acronyms(s)
NMRC/NMSC

11. Sponsor/Monitor's Report Number(s)

12 DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT (maximum 200 words)

Adenovirus type 21 (Ad21) is a well-known causative agent of acute respiratory disease among both military recruits and children. In an effort to characterize the molecular epidemiology of Ad21 infections in the military environment, genome-typing work was carried out on a collection of 75 Ad21 strains isolated from the pharyngeal swabs of military recruits presenting with symptoms of febrile respiratory illness between 1996 and 2005 at eight US training centers. One further strain from an ill serviceperson deployed at sea was also characterized. Restriction enzyme analysis with *Bam*HI discriminated two distinct DNA variants, Ad21a and Ad21b. Further analysis with *Bgl*I, *Bgl*II, *Bst*EII, *Hind*III, *Kpn*I, and *Sma*I discriminated two new subtypes, Ad21a1 and Ad21b1. Ad21a was the most prevalent genome type, accounting for 69 of the 76 strains examined. Genome type Ad21a1 was identified only among the strains isolated at the Marine Corps Recruit Depot, San Diego, CA. Genome types Ad21b and Ad21b1 were identified among strains isolated in 2005 and seem to have emerged after a 4-year (1999–2002) disappearance of all Ad21 genome types. After the reintroduction of the Ad4/Ad7 vaccine in 2008, Ad21 is expected to become a predominant adenovirus serotype in US recruit training centers once again. Knowledge of circulating genome types and their epidemic behavior will be of significant value to ongoing surveillance efforts in these highly susceptible and impacted populations.

15. SUBJECT TERMS
adenovirus, strain typing, surveillance

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT UNCL	b.ABSTRACT UNCL	c. THIS PAGE UNCL	UNCL	37	Commanding Officer

19b. TELEPHONE NUMBER (INCLUDING AREA CODE)
COMM/DSN: (619) 553-8429

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39-18