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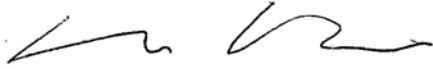
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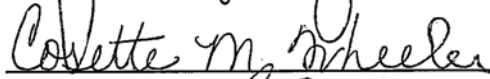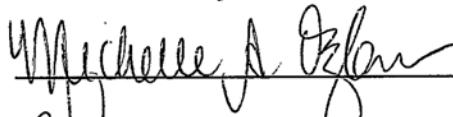
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_____, Chairperson _____

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_____ A. KAJON _____

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Dean, Graduate School

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Date

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MOLECULAR DYNAMICS OF ADENOVIRUS TYPE 3 AND 7 INFECTIONS IN MILITARY AND CIVILIAN POPULATIONS IN THE UNITED STATES

BY

LAURA M. DICKSON

B.S. BIOLOGY, SONOMA STATE UNIVERSITY 2004

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

December, 2009

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ABSTRACT OF THESIS

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ABSTRACT

Human adenoviruses (HAdV) are major causative agents of acute respiratory disease (ARD) worldwide. Military recruits and children are two of the populations most susceptible to HAdV infection in the United States. Infections by species B serotypes, including HAdV-3 and HAdV-7, are often associated with some of the most severe clinical manifestations of respiratory disease. In 1996, the production of the HAdV vaccine that was administered to US military recruits was discontinued. Since then, HAdV-associated ARD has reemerged in military training facilities nationwide compromising readiness and deployability of troops and causing significant financial burden to the Department of Defense (DoD). The HAdV vaccination protocol will be reassumed in 2010 with a formulation identical to that used as the original 1971 vaccine. For this study, we analyzed HAdV isolates from both military and civilian cases of respiratory disease to identify the most prevalent HAdV-3 and HAdV-7 genome types circulating in the last decade. Our goal was to determine if these genome types may

challenge vaccine efficacy and decide if there are possible common targets for intervention in the civilian population. We identified eleven HAdV-3 and six HAdV-7 genome types in circulation in military training camps and civilian communities, which include HAdV-3a, HAdV-3a2, HAdV-3a17, HAdV-3a*Bcl*I variant, HAdV-3a*Bst*EII variant, HAdV-3a*Bgl*II variant 1, HAdV-3a*Bgl*II variant 2, HAdV-3a*Bgl*II variant 3, HAdV-3a*Bcl*I2*Bst*EII variant, HAdV-3*Bam*HI variant and HAdV3*Bam*HI*Bgl*II variant, HAdV-7p, HAdV-7b, HAdV-7d, HAdV-7d2, HAdV-7h and HAdV-7*Bam*HI variant. Of the seventeen genome types identified, nine have not previously been described. Sequence data for the hexon and fiber genes revealed minor differences between the HAdV-7 genome types identified in the study and the vaccine strain, HAdV-7a3. Although some have suggested a cross-protection against HAdV-3 genome types, their implications on vaccine efficacy remain unclear. What is clear is the large prevalence of HAdV-3 which circulates in civilians. Continued surveillance in both populations is needed to identify the role that HAdV-3 genome types play in the etiology of ARD after the vaccine is reinstated in military recruits.

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Chapter One: Introduction

Human adenoviruses (HAdV) are major causative agents of respiratory infections worldwide. Historically, military recruits and children have been burdened with HAdV-associated respiratory disease. Since the discovery in 1953 (Rowe et al., 1953), 52 serotypes of HAdV have been identified. Serotypes are distinguished based on neutralization assays and have been grouped into species (A-G). Of these, species B, C and E HAdVs are most often associated with outbreaks of respiratory disease (Schmitz et al., 1983; Rubin, 1993). Species B and E HAdVs are frequently associated with respiratory infection in military recruits, whereas children are more likely to become infected with HAdVs of species B and C (Schmitz et al., 1983).

Military recruits are more susceptible to HAdV-associated respiratory illness than adult US civilians (Gray, 1995; Gray et al., 1999). In 1954, adenovirus was isolated from respiratory secretions collected during an outbreak of pneumonitis among recruits at a military training facility in Fort Leonard Wood, MO (Hilleman & Werner, 1954). Data collected during the 1950's showed that 20% of recruits had to be removed from training for over one week due to HAdV-associated illness (Hilleman et al., 1957). Further studies confirmed that HAdV was the causative agent of the majority of outbreaks of acute respiratory disease (ARD) or febrile respiratory illness (FRI) among military trainees (Hilleman, 1957; Grayston et al., 1959; McNamara et al., 1962). In 1971, a live enteric-coated vaccine was developed in order to reduce the disease and cost burden caused by HAdV respiratory infections in military trainees. The production of the highly efficacious vaccine was discontinued in 1996. Since the discontinuation of vaccination protocol, ARD caused by HAdV has increased dramatically causing thousands of

preventable HAdV infections (Gray et al., 2000). An estimated 10-20% of all military recruits currently become ill with febrile HAdV infection during their training (Russell et al., 2006). Recent data also show that out of the 22,000 cases of ARD in military recruits reported annually, over 15,000 cases are associated with HAdV (Russell et al., 2006). Once again, HAdV has reemerged as the major causative agent of ARD in military recruits, causing not only a financial burden due to the expense of medical bills and loss in training time, but also decreasing the lack of deployable troops.

HAdV is a causative agent of severe respiratory disease in young adults and children, with some serotypes associated with severe clinical presentations and occasionally fatal outcomes (Dudding et al., 1972; Centers for Disease Control and Prevention, 2001). HAdV is estimated to be responsible for up to 5% of upper respiratory tract infections in children under 5 years of age and 10% of pneumonias in children (Brandt et al., 1969; Mallet et al., 1966). During the 1960's and 1970's, the Virus Watch Program was established to increase awareness on natural occurrences and consequences of infection by respiratory viruses. The goal was to determine patterns of within-family spread, to identify clinical consequences of infection (frequency and spectrum of illness) and to investigate the relation of these to age and immunity (Elveback et al., 1966; Fox et al., 1969; Hall et al., 1971). The Virus Watch Program remains today, the only large-scale epidemiological study on HAdV infections in the pediatric population ever conducted in the United States. Although HAdV-associated disease is preventable with vaccination, a vaccine has never been licensed for civilian use. A current study to analyze the prevalent genome types of HAdV is needed to

determine whether common targets of intervention can be identified between military recruits and civilians.

There are currently 52 recognized serotypes of HAdV (HAdV-1 to HAdV-52). Historically, serotypes were identified by their resistance to neutralization by antisera against other known HAdV. Today the use of molecular techniques, such as polymerase chain reaction (PCR) and sequencing, as well as restriction enzyme analysis (REA), allows for an in depth characterization of different HAdV serotypes and reveals extensive intraserotypic diversity (Li & Wadell, 1986; Li & Wadell, 1988). REA is the only molecular approach that is capable of characterizing an adenovirus strain based on the entire genome at a relatively inexpensive cost. This technique is most frequently used to identify adenovirus genome types, also referred to as genomic variants. Genome types are distinguished based on their unique restriction profiles, as well as PCR and sequencing. REA is the most widely used technique for investigating the molecular epidemiology of HAdV infections.

Although HAdV-4 is responsible for the majority of ARD in military recruits, HAdV-3 and HAdV-7 also play a large role in the etiology of respiratory disease in this population (Gray et al., 2000; Ryan et al. 2002). An assessment of the genome types of HAdV-4 circulating in the post vaccination era was recently published (Kajon et al., 2007). However, the molecular characterization of HAdV-3 and HAdV-7 strains re-emerging after vaccine protocol cessation has not been examined. In 1997, a large epidemic outbreak of respiratory illness due to HAdV-3 and HAdV-7 was reported at the Naval Recruit Training Command in Great Lakes, Illinois (Ryan et al., 2002). This large outbreak included 541 confirmed cases of HAdV infection and was the first evidence of a

reemergence of HAdV-3 and HAdV-7 genome types in military trainees. Between October 1996 and June 1998 adenovirus was detected in 53.1% of throat swabs from symptomatic recruits in US military training camps, with HAdV-7 and HAdV-3 accounting for 25% and 9% of the total isolates analyzed, respectively (Gray et al., 2000). Although between 1999 and 2005 HAdV-4 was responsible for more than 95% of HAdV isolated from military recruits, a reemergence of HAdV-3 and HAdV-7 in association with ARD in military recruits was identified after 2005 (Metzgar et al., 2007). In 2001 a new manufacturer for the adenovirus vaccine was identified. Safety and efficacy trials are now underway (Lyons et al., 2008). Because of the impact HAdV-3 and HAdV-7 have on military recruits, the lack of updated information on the current circulating genome types of HAdV-3 and HAdV-7, and the testing of the adenovirus vaccine underway, an updated molecular epidemiology report is needed to understand the prevalence of these serotypes in the etiology of FRI in military recruits and how they may affect vaccine efficacy.

HAdV-3 and HAdV-7 are associated with severe respiratory illness worldwide in civilian populations (Mizuta et al., 2006; Moura et al., 2007; Chang et al., 2008; Wadell et al., 1980; Wong et al., 2008). HAdV-7 and HAdV-3 accounted for approximately 20% and 13%, respectively, of HAdV infections reported to the World Health Organization between 1967 and 1976 (Schmitz et al., 1983). In 1998 and 2005, outbreaks of HAdV-7 and HAdV-3, respectively, were identified in pediatric long-term care facilities in the United States (James et al., 2006; Gerber et al., 2001). A significant gap still exists in our understanding of the role of HAdV infections in the etiology of ARD in pediatric populations in the United States. The molecular characterization of HAdV-3 and HAdV-

7 strains currently circulating in US civilians is important for understanding the incidence and prevalence of different genome types and identifying possible targets to be considered for the development of a civilian vaccine.

An important aspect to performing epidemiological studies on HAdV infections in the United States is having a reliable resource to collect clinical samples. The Naval Health Research Center (NHRC) has maintained continuous surveillance of adenovirus infections in military recruits for the Department of Defense Global Emerging Infections Surveillance and Response System since the discontinuation of vaccine protocol in 1996. The Children's Hospital of Philadelphia (CHOP) has maintained surveillance of viral respiratory infections in the local pediatric population since 2001. Together, these facilities provide a wealth of epidemiological data on HAdV genome types associated with disease in the United States, allowing for molecular epidemiologists to investigate which genome types are responsible for this disease and whether or not the same genome types circulate nationwide. This information can also be used to identify whether the HAdV genome types circulating in the United States are the same as those affecting similar populations throughout the world.

To better understand the molecular dynamics of HAdV-3 and HAdV-7 in the United States, we analyzed HAdV strains isolated from military cases of ARD and civilian cases of HAdV-associated disease. In collaboration with the NHRC, HAdV-3 and HAdV-7 isolates from US military recruits representing 11 years of continuous sampling were genome typed by REA. In addition, civilian HAdV-3 and HAdV-7 isolates obtained from the collections of the CHOP and the University of Iowa (UI) were

characterized by the same procedure. Using these samples, we set out with the following aims:

(1) Identify the most prevalent genome types of HAdV-3 and HAdV-7 circulating in the United States over the last decade.

(2) Compare the most prevalent currently circulating HAdV genome types infecting military recruits and civilians to identify possible common targets for intervention.

(3) Analyze the occurrence of HAdV-3 and HAdV-7 genome types to identify geographical distribution and detect patterns of circulation.

Chapter Two: Background

Classification

Members of the family *Adenoviridae* are highly host species-specific and infect a broad range of vertebrates. HAdV are classified within the genus *Mastadenovirus*, which includes all mammalian adenoviruses. Currently, there are 51 HAdV serotypes distinguishable by neutralization assays using rabbit or horse reference sera (Hierholzer et al., 1991; Wadell, 1984; Wadell, 1994) and the recently identified HAdV-52 that was designated a new serotype based on genomic sequencing and phylogenetic analysis (Jones et al., 2007). HAdV serotypes are grouped into species (A-G). Species B is divided into two clusters of DNA homology, B1 and B2 (Wadell et al., 1980) (Table 1). Species classification is based on relative sequence homology, tissue tropism, restriction fragment length polymorphism (RFLP), GC content, and the genetic organization of the E3 region (International Committee on Taxonomy of Viruses, http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/).

Table 1. Species of HAdV and corresponding serotypes.

Species		Serotypes
A		12, 18, 31
	1	3, 7, 16, 21, 50
B	2	11, 14, 34, 35
C		1, 2, 5, 6
D		8, 9, 10, 13, 15, 17, 19, 20, 23-30, 32, 33, 36-39, 42-49, 51
E		4
F		40, 41
G		52

Virion Structure and Genome Organization

Adenoviruses are non-enveloped, icosahedral particles with fibers projecting from the twelve vertices of the capsid (Fig. 1). The 240 hexon capsomers, which make up the majority of the capsid surface, and the twelve penton capsomers, which are present at each of the vertices of the icosahedron, assemble to make the adenovirus capsid. The penton capsomer is a covalent complex of two proteins, the penton base and the fiber protein. These, along with the hexon, are known as the major capsid proteins. There are also minor capsid proteins that have a variety of functions. These include proteins IIIa, VI, VIII and IX. Proteins VIII and IX help stabilize the hexon proteins, protein VI helps disrupt the endosomal membrane during entry, and protein IIIa is important in assembly of virions (Boulanger et al., 1979; Lemay et al., 1980; Wiethoff et al., 2005).
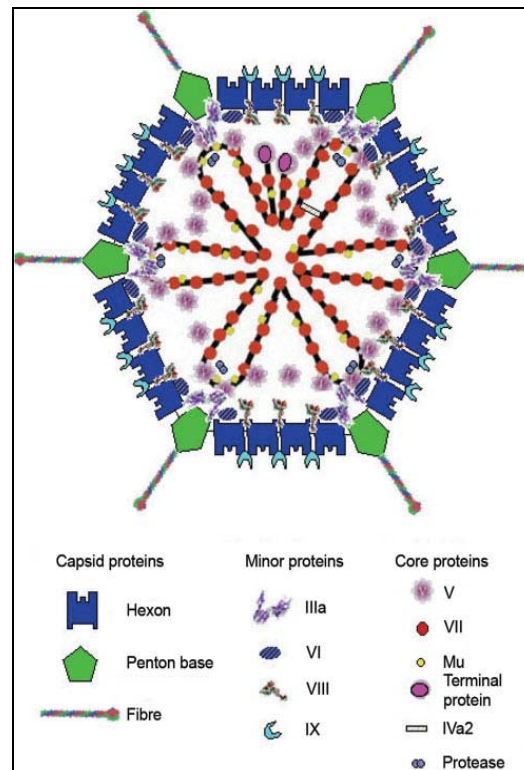
Figure 1. Schematic representation of a HAdV particle (Reproduced from Russell, 2009).

The hexon is a trimer composed of three molecules of the polypeptide II. Because the hexon is the major capsid component, it plays a large role in the induction of the host immune response (Russell, 2009). The fiber projects from the penton capsomer, and is a trimer formed by three monomers of polypeptide IV. The fiber contains three domains and each has an important function. The N-terminal domain binds to the penton base, the central shaft is flexible and is important for entry, and the globular C-terminal knob binds the primary receptor on host cells and plays an important role in adenovirus tropism (Louis et al., 1994; Stevenson et al., 1995).

Inside the capsid is the core of the virion, which contains the viral genome and several important proteins. Proteins V, VII, and μ facilitate the condensing of viral DNA into the core. Protein p23 cleaves protein precursors during assembly, maturation and disassembly, and aids in the escape from endosomes during infection, and protein IV makes contact with the capsid (Matthews & Russell, 1994; Matthews & Russell, 1995; Russell, 2009). The terminal protein is a core protein that is covalently attached to the 5' end of the viral DNA (Rekosh et al., 1977). This protein is important for genome replication.

The viral genome is linear, non-segmented, double-stranded DNA of approximately 36 Kbp. At the 5' and 3' ends of the genome are inverted terminal repeats that function as viral DNA replication origins. Once the strands have separated during the beginning of the replication process, the single-stranded DNA circularizes into a panhandle and creates an origin for the complimentary strand to be synthesized (Leegwater et al., 1988).

Fig. 2 is a linear map of the adenovirus genome detailing the coding regions and highlighting the location of some of the major genes of interest for this study. Adenovirus genes are temporally expressed in three major stages. The first stage is the expression of the early genes. Early genes (E1A, E1B, E2, E3 and E4) are encoded throughout the genome and transcripts are transcribed in the rightward (E1A, E1B and E3) and leftward (E2 and E4) directions. Early genes encode non-structural proteins that are responsible for a number of important processes. The E1A gene encodes two proteins that activate transcription and regulate the host cell cycle (Nevins, 1995). E1B encodes proteins that inhibit apoptosis (Burgert et al., 2002). Proteins encoded in the E2 region of the genome function in DNA replication and proteins encoded in the E3 region modulate host response to infection (Burgert et al., 2002). E4 products have a number of functions, including transcriptional and translational regulation, viral DNA replication, and virus assembly (Halbert et al., 1985; Falgout & Ketner, 1987).

The second stage of gene expression comprises the intermediate early genes. The intermediate early genes include IX, IVa2 and E2. The last stage involves expression of the major late genes. These genes encode the structural proteins of the virion such as the hexon, fiber and core proteins, as well as proteins that are important for assembly. All of these genes are transcribed in the rightward direction from one transcription unit with the same major late promoter (MLP). All HAdV genes are transcribed by cellular RNA polymerases, and transcripts are processed by alternative splicing.

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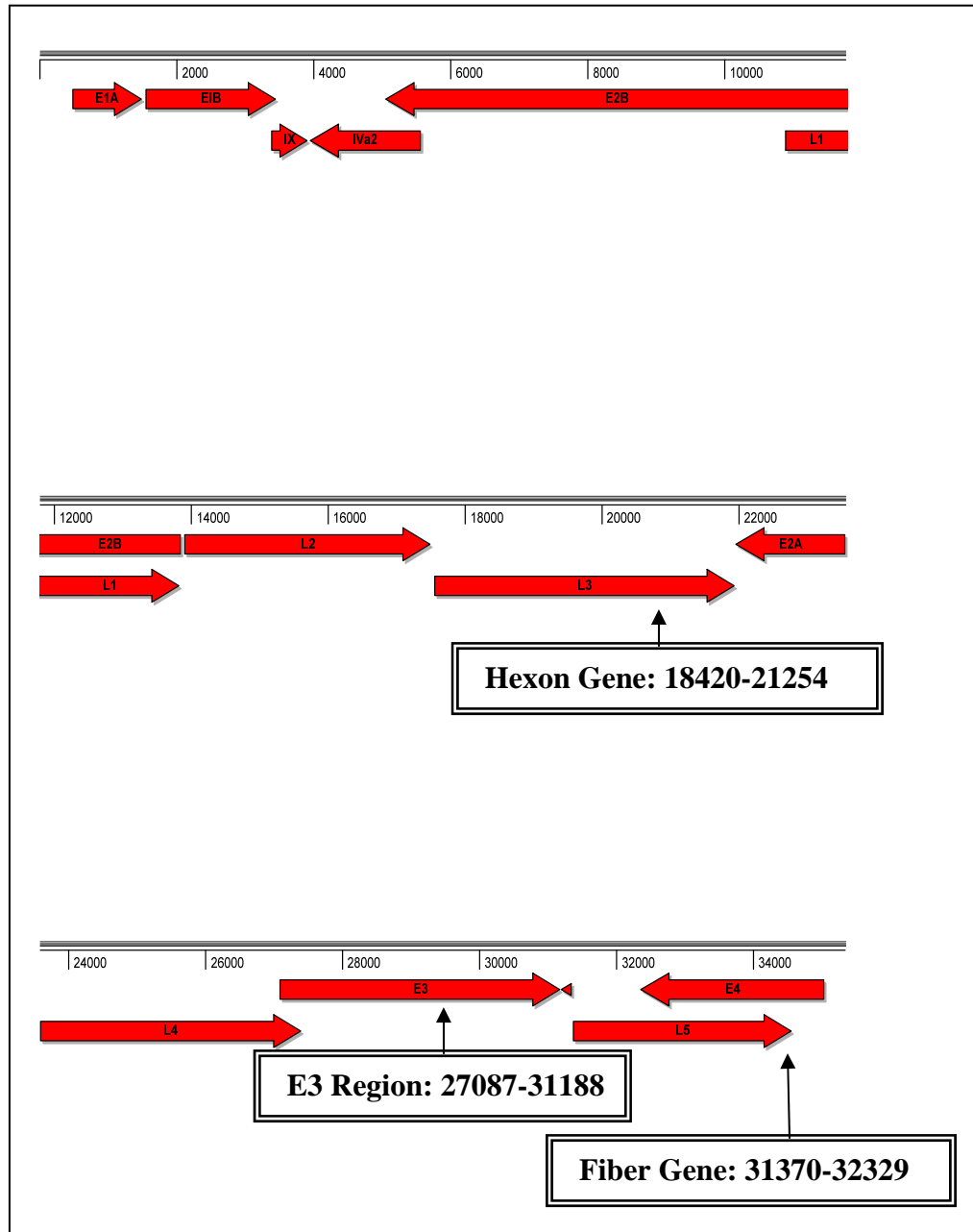
Figure 2. Linear map of the HAdV-3p genome (Genbank accession number AY599834), made with SeqBuilder (Lasergene 1.0; DNAStar) detailing the coding regions of the HAdV genes (displayed with red arrows).

Genetic Variability

Adenovirus DNA sequences are largely conserved for most virion structural proteins and proteins involved in replication and assembly. However, there are some areas of the genome that show extensive genetic variability. Early regions 1, 3, and 4 and small-virus associated RNA are not conserved. The E3 region of adenoviruses, which is only present in the genomes of the genera *Mastadenoviruses* and *Siadenovirus*, shows considerable intraserotypic variability (Burgert & Blusch, 2000; Kajon et al., 2005). Mammalian adenoviruses that display different degrees of virulence and host range exhibit differences in their E3 regions (Belak et al., 1986).

The hexon gene also exhibits areas of genetic variability. Seven hypervariable regions (HVR1-7) have been identified in the 5' end of the hexon gene that are associated with type-specific antigens (Crawford-Miksza & Schnurr, 1996), and at least one these HVRs constitutes the major neutralizing epitope (Pichla-Gollan et al., 2006). HVRs are located on two loops, L1 and L2, which project away from the surface of the virion (Fig. 3). L1 contains HVRs 1-6 and L2 contains HVR-7. HAdV serotype evolution appears to be driven by illegitimate recombination events occurring in these HVRs (Crawford-Miksza & Schnurr, 1996; Crawford-Miksza et al., 1999). Illegitimate recombination includes mutational events such as deletions, insertions, duplications and translocations. As mentioned previously, the fiber protein contains a globular C-terminal knob. This knob has a number of exposed loops, which contain variable amino acid sequences, providing a range of receptor binding sites (Xia et al., 1994). Together, the hexon and fiber proteins constitute the major neutralizing epitopes of adenoviruses and exhibit great genetic variability (Bailey & Mautner, 1994; Pichla-Gollan et al., 2006).
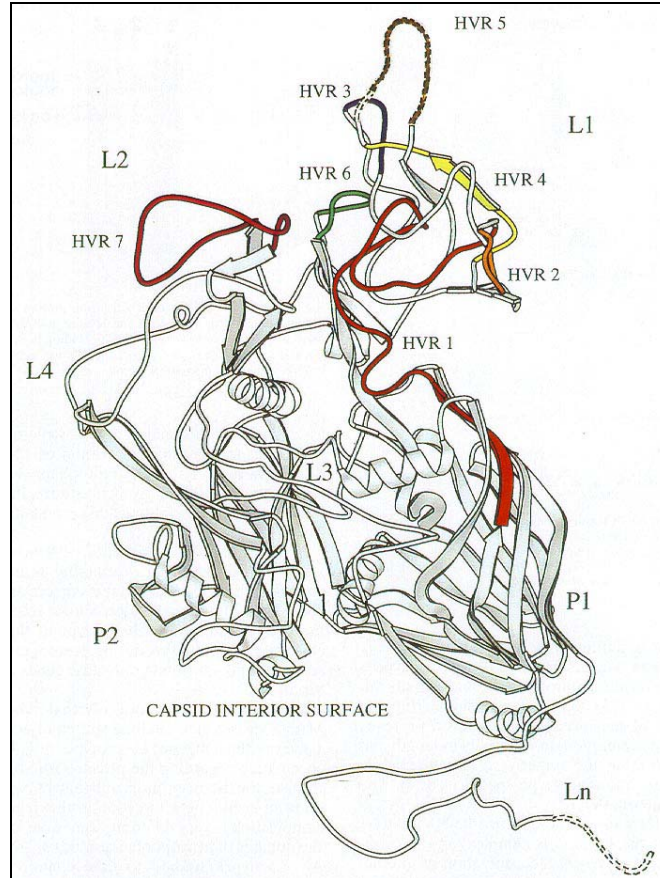
Figure 3. Ribbon diagram of the identified hypervariable regions and the serotype-specific residues (Reproduced from Crawford-Miksza and Schnurr, 1996). Loop 1 (L1) contains HVRs 1-6 and Loop 2 (L2) contains HVR7.

Intermediate variants are recombinants that exhibit antigenic properties of one serotype in the hexon gene and of another serotype in the fiber gene. Homologous recombination among closely related serotypes has been widely accepted as a common mechanism of adenovirus evolution (Sambrook et al., 1980). Intermediate variants were first isolated from civilian patients, mostly pediatric, between 1976 and 1978 (Hierholzer et al., 1980) and are very common among species D adenoviruses (Hierholzer & Rodriguez, 1981; Wigand & Adrian, 1989). However, they have also been identified within species B adenoviruses (Adrian & Wigand, 1986; Kajon & Wadell, 1996). For

example, a HAdV-3 strain isolated in 1966 in Takeuchi, Japan, was later identified as an HAdV-3-7 recombinant with a HAdV-3-like hexon and a HAdV-7-like fiber (Li & Wadell, 1988). HAdV-7h, a genomic variant of HAdV-7 originally detected in South America, was determined to be an intermediate variant with an HAdV-7-like hexon and an HAdV-3-like fiber (Kajon & Wadell, 1996).

HAdV-Associated Disease and Treatment

Disease associated with adenovirus infection varies depending on the species, serotype, route of infection, and immunocompetence of the host. Disease associated with species A serotypes (HAdV-12, HAdV-18 and HAdV-31) is mostly cryptic, but HAdV-31 has been associated with infant gastroenteritis and encephalitis (Adrian & Wigand, 1989; Schnurr et al., 1995). Species B serotypes (HAdV-3, HAdV-7, HAdV-11, HAdV-14, HAdV-16, HAdV-21, HAdV-34, HAdV-35 and HAdV-50) are predominately associated with respiratory disease in adults and children, although HAdV-3 has also been frequently associated with conjunctivitis, and HAdV-11, HAdV-34 and HAdV-35 have been associated with conjunctivitis and urinary tract infections (Martone et al., 1980; Itoh et al., 1999; James et al., 2007). Species C serotypes (HAdV-1, HAdV-2, HAdV-5 and HAdV-6) are causative agents of respiratory infections primarily in children under the age of five years. Species D includes 31 different serotypes. HAdV-8, HAdV-19, and HAdV-37 have been associated with ocular infection in healthy individuals; other species D serotypes have been isolated from immunocompromised individuals (Hierholzer et al., 1988; Schnurr & Dondero, 1993; Schnurr et al., 1995). Species E only contains one serotype, HAdV-4, which is often detected in association with respiratory

infections in children and young adults, but also frequently associated with conjunctivitis (Kolavic-Gray et al., 2002; Ren et al., 1985; Schepetuik et al., 1993; Tsuzuki-Wang et al., 1997). Species F consists of two serotypes, HAdV-40 and HAdV-41, which cause gastrointestinal infections. Species G includes the recently described HAdV-52, which is also an enteric pathogen (Jones et al., 2007).

HAdV-3, HAdV-7, HAdV-21 (species B), HAdV-1, HAdV-2, HAdV-5, HAdV-6 (species C) and HAdV-4 (species E) are well known causative agents of respiratory disease of variable severity (Hayashi & Hogg, 2007) (Table 2). Species B serotypes, HAdV-3, HAdV-7, HAdV-11, HAdV-14 and HAdV-21, have been associated with severe clinical manifestations of respiratory illness (Becroft, 1971; Binn et al., 2007; Hierholzer et al., 1974; Louie et al., 2008; Zhu et al., 2009). Severe respiratory illness includes pneumonia, bronchitis and bronchiolitis. Less severe respiratory disease includes pharyngitis and pharyngoconjuntival fever. Symptoms of HAdV infections can include sore throat, coughing, runny nose, wheezing, dyspnea, sinus tenderness, vomiting, abdominal pain and diarrhea (Itoh et al., 1999; Harley et al., 2001; Chen et al., 2004; Okamoto et al., 2004).

Table 2. HAdV-associated respiratory disease morbidity and the serotypes that are most often the causative agents of the disease.

Disease	Principle Serotypes
Acute febrile pharyngitis	1, 2, 3, 5, 6, 7
Pharynoconjunctival fever	3, 7
Acute respiratory disease of recruits (ARD)	3, 4, 7, 14, 21
Pneumonia	1, 2, 3, 4, 7, 14, 21

Adenoviruses also play an important role as opportunistic pathogens of immunocompromised individuals, especially stem-cell, bone marrow or organ transplant recipients (Hierholzer 1992; Hayashi & Hogg, 2007) and human immunodeficiency virus (HIV) positive individuals (Hierzholzer & Wigand, 1988; Leen & Rooney, 2005). HAdV-induced illness in the immunocompromised host is usually more severe, prolonged and frequently fatal. Certain HAdV serotypes that may not normally cause disease in healthy individuals can become opportunistic pathogens in immunocompromised individuals. For example, two serotypes, HAdV-31 (species A) and HAdV-49 (species D) were detected in an AIDS patient with encephalitis (Schnurr et al., 1995). These opportunistic infections are most likely due to the lack of T-cell immunity (Chakrabarti et al., 2002).

Treatment for adenovirus infections is mostly limited to symptomatic care. There are no antiviral drugs specific for adenoviruses. Current antivirals used to treat adenoviral infections include ribavirin and cidofovir, both of which have limitations (Ljungman, 2004). Ribavirin varies in its efficacy among different species of adenovirus, with group C viruses being most sensitive to treatment (Morfin et al., 2005). Cidofovir is a nucleoside analogue with efficacy against multiple DNA viruses. Reports of toxicity to the kidney have led to concern over treatment with cidofovir (Ljungman, 2004).

Currently no vaccine is available for civilian use to prevent HAdV infections. Between 1971 and 1996, a live enteric-coated vaccine formulated with HAdV-4 and HAdV-7 was administered to US military trainees. However, it was never licensed for use in civilians. This vaccine was efficient at preventing HAdV-associated disease in recruits but was discontinued in 1996 due to disagreements between the DoD and the

manufacturer. Since then, there has been a dramatic increase in adenovirus morbidity in US military recruits causing a significant financial burden to the DoD due to medical costs, loss of training, and the lack of deployable troops (Ryan et al., 2002; Russell et al., 2006; Kajon et al., 2007; Metzgar et al., 2007).

Laboratory Diagnosis of Adenovirus Infections

Most HAdV infections are difficult to distinguish clinically from other viral respiratory infections and even some bacterial infections. In order to detect adenovirus in clinical specimens, those specimens must be collected during the acute period of the infection (Hierholzer et al., 1988). However, in lymphoid tissues, such as the tonsils and adenoids, adenoviruses can become persistent and can be isolated intermittently for months or years in throat and stool samples (Fox et al., 1969).

Adenoviruses can be detected by a number of different methods. These include: immunofluorescence, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) or electron microscopy. The most common method for isolation of adenovirus in a research laboratory setting is by cell culture. Human cell lines used to culture adenovirus include: A549 (lung epithelial carcinoma), HeLa (cervical epithelial adenocarcinoma), KB (nasopharyngeal carcinoma) and HEp-2 (hepatocellular carcinoma). Adenoviruses cause distinct cytopathic effects (CPE) in cells (Fig. 4). Morphological changes include rounding and clumping, nuclear inclusions and netting.
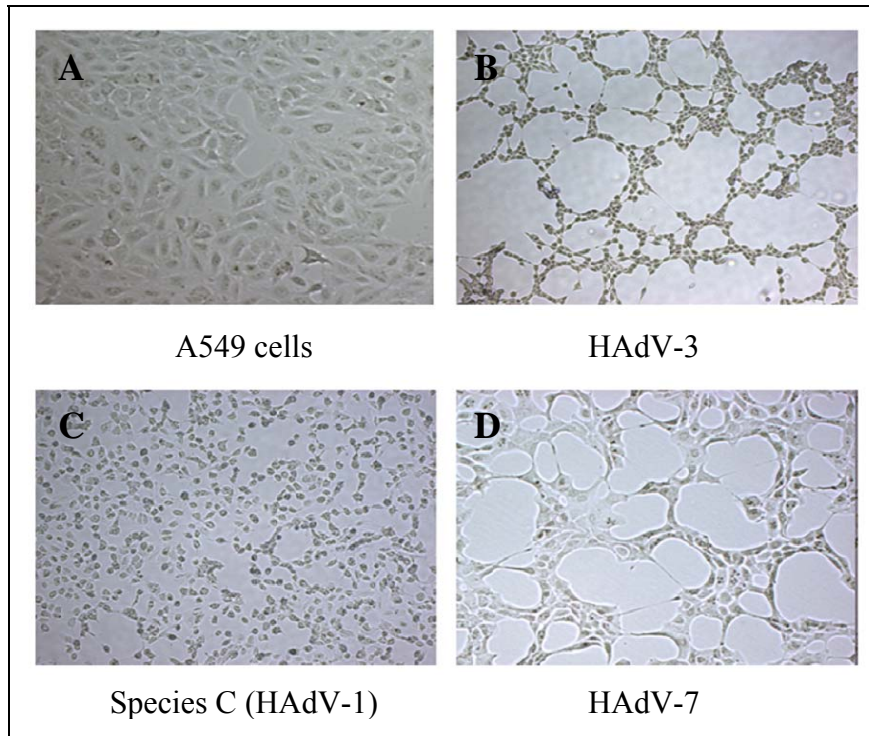
Figure 4. Typical HAdV CPE. A. Non-infected A549 cells. B. A549 cells infected with HAdV-3. C. A549 cells infected with HAdV-1. D. A549 cells infected with HAdV-7.

Serotypic identity of adenoviruses has been historically determined by seroneutralization (SN) assays using rabbit or horse reference sera and by hemagglutination inhibition (HAI) assays. However, newer molecular techniques have permitted rapid and objective type-specific identification of HAdV, and have replaced serotypic identification by SN or HAI assays.

Switching approaches from serological identification to identification by molecular techniques was possible because of one important study. In this study, the hexon protein sequences of fifteen serotypes of adenovirus were aligned for fine-mapping of the conserved and variable residues (Crawford-Miksza & Schnurr, 1996). From this alignment, seven HVRs were identified in the 5' end of the hexon gene. The high degree of variability in the HVR-7 allowed for the design of primers to produce PCR amplified

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DNA fragments that, when sequenced, were distinguishable for all the serotypes of adenovirus (Sarantis et al., 2004).

The fiber gene can also be analyzed to identify intermediate variants of adenoviruses. The traditional method for determining fiber serotype identity is to perform a HAI assay. In contrast to adenovirus neutralization, which is determined by epitopes present on both the hexon and fiber proteins, hemagglutination is an exclusive property of the fiber knob. Today, PCR amplification and sequencing of the fiber gene can establish fiber identity and often replace the traditional method described above.

Adenovirus can be detected without culturing the virus. The most common method of detection is PCR. Traditional PCR or real-time PCR assays allow for quick diagnosis, typing or quantification of viral load in various tissue specimens and body fluids. A commercial enzyme-linked immunoassay kit called Adenoclone (Meridian Biosciences, Inc., Ohio) has been approved by the US Food and Drug Administration and can be used to detect the presence of adenovirus directly from respiratory, ocular or stool specimens. The procedure can be performed in less than an hour, but requires large viral loads and is less sensitive than cell culture or IF staining. Therefore, negative results must be confirmed by cell culture methods. In most clinical settings, once a positive adenovirus diagnosis is made, further work is not conducted to identify the serotype or genetic variability.

Typing of Adenovirus Isolates

As mentioned above, there are a number of methods to detect adenoviruses. The serotype of an isolate is determined either by neutralization assay or by PCR

amplification of the hexon gene and sequencing followed by the Basic Local Alignment Search Tool (BLAST) comparison against the National Center for Biotechnology Information (NCBI) sequence database or similar text. REA is used to examine intraserotypic genetic variability. Viral DNA is extracted from infected human cells, digested with a panel of restriction endonucleases, and analyzed by gel electrophoresis. The profile of the DNA fragments visualized by gel electrophoresis is compared to other published profiles of adenovirus isolates in order to designate the genome type. REA is currently the most common way to analyze genetic variability among different strains of adenoviruses. Whole genome sequencing can also be used to look at intraserotypic genetic variability. However, whole genome sequencing of many HAdV isolates is costly and time consuming. The most effective way to identify the genome types of adenoviruses is to use a combination of REA and sequencing. REA is fast and efficient as well as affordable, and sequencing the hexon and fiber genes of isolates can verify the serotype and identify recombinants.

Genome types of HAdV can be grouped into genomic clusters. These genomic clusters are established based on the degree of pairwise comigrating restriction fragments (PCRFs). A degree of relation defined as 80% PCRFs was used to define the genomic clusters (Li & Wadell, 1986). Genome types with greater than 80% PCRFs are grouped into the same cluster, while those that have less than 80% PCRFs are placed in different clusters. HAdV-3 and HAdV-7 genome types are grouped into three genomic clusters (1-3) (Li & Wadell, 1986; Li & Wadell, 1988). Grouping genome types into genomic clusters helps estimate the degree of genetic relatedness within a serotype and sometimes among different serotypes.

Denomination Systems

Two systems for naming adenovirus DNA variants have been proposed. Quan-Gen Li and Goran Wadell created a denomination system in 1986 to delineate intraserotypic variation and called these variants genome types (Li & Wadell, 1986). Using REA, this system differentiates HAdV genomes based on their digestion profiles. Adenovirus DNA is first cut with *Bam*HI, which allows identification at the species level. *Bam*HI gives a unique profile from which subsequent restriction endonucleases can be chosen to identify different genome types. The profile for the prototype strain, the first strain discovered, is designated p (prototype). Other identified variants of a serotype distinguished by *Bam*HI are given letters, p, then a, b, c, etc., in the order of their discovery. Numerals are added after the letter to describe variation in profiles generated by additional restriction endonucleases (Li & Wadell, 1986).

T.H. Adrian and R. Wigand proposed a numerical code for denominating genome types in 1985. The numerical code requires REA to be performed with seven endonucleases. These restriction endonucleases are displayed in alphabetical order and include: *Bam*HI, *Bgl*II, *Bst*EII, *Eco*RI, *Hind*III, *Kpn*I and *Sma*I. The prototype restriction profile is designated 1 and each profile distinct for a given endonuclease is designated 2, 3, 4, etc., in chronological order of each new profile. For example, a strain may be designated AV6/3:1231121, indicating that it differs from the prototype strain with three (*Bgl*II, *Bst*EII, and *Kpn*I) of the seven restriction endonucleases (Adrian et al., 1985).

Although Li and Wadell's denomination system has been used more often recently, especially for the denomination of species B HAdV genome types, Adrian and

Wigand's system has been useful for naming genome types in species C adenoviruses. Presently, there is no universal reference database for HAdV restriction profiles. This presents a unique challenge when denominating new HAdV variants because both systems described above are in current use.

Epidemiology of HAdV Infections

There are a number of reasons why it is important to study the epidemiology of HAdV infections. First, it is important to understand the geographical distributions of pathogenic HAdV strains. This information can provide a guide for disease prevention and control. For example, it has been shown that certain genome types of HAdV-7 predominate in a geographic area for extended periods of time and then can be replaced by new genome types (Wadell et al., 1981; de Silva et al., 1989; Kajon & Wadell, 1994). The description of the geographic distribution of HAdV infections can reveal whether the pathogen has a wide or a limited area of circulation. Second, mapping the genetic similarities or differences among adenovirus strains is extremely important to identify candidate determinants of virulence and fitness. The severity of the associated disease also may differ among genome types. It has been shown that some adenoviruses that demonstrate different degrees of virulence and host range exhibit genetic variability in their E3 regions (Belak et al., 1986). Third, examining circulation patterns and occurrences of different genome types can identify whether or not a virus is fit enough to circulate frequently across a geographic area or circulate infrequently.

When studying the epidemiology of adenovirus infections, molecular techniques such as REA, PCR and sequencing have proven extremely useful to identify variation

among HAdV serotypes. REA is a powerful technique that allows for extremely detailed discrimination of HAdV genome types and is capable of revealing intraserotypic variability. It is one of the best ways to identify genome types of adenovirus and is a comprehensive way to detect variation throughout the entire HAdV genome. When performing large-scale epidemiological studies or determining the genome type for an outbreak, REA is the preferred method for genome typing.

PCR amplification of the hexon and fiber genes is extremely useful for distinguishing serotypes. However, it only analyzes a portion of the genome. Performing sequence analysis of the hexon and fiber genes as well as other regions of the genome that show variability, such as the E3 region, can reveal genetic diversity and identify intermediate variants of adenovirus.

Adenovirus Infections in Military Recruits

Respiratory illnesses have plagued US military recruits (Gray et al., 1999; Top, 1975). Adenovirus was first identified in the 1950s as one of the most prevalent causative agents of ARD in US military recruit training facilities (Hilleman, 1957; Grayston et al., 1959; McNamara et al., 1962). HAdV-4 and HAdV-7 were detected most frequently in association with ARD (Berge et al., 1955; Pierce et al., 1965; Dudding et al., 1973). The impact of adenovirus infections on military recruits identified the need for intervention, and soon, development of a vaccine against this etiological agent began. A formulation consisting of one enteric-coated tablet containing a live non-attenuated HAdV-4 strain and another containing live non-attenuated HAdV-7 strain was designed. This vaccine induced protective type-specific antibodies in two to three weeks after

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immunization (Couch et al., 1963). After this observation, a monotypic adenovirus vaccine was tested against HAdV-4 and shown to be very efficacious (Chanock et al., 1966; Edmonson et al., 1966). However, a second trial of this adenovirus vaccine showed a limitation due to the emergence of HAdV-7 (Buescher, 1967). It was clear that a monotypic HAdV-4 vaccine was not sufficient to control the burden of HAdV-associated disease. A surveillance program was instated at several of the basic combat trainee posts between 1966 and 1971. This surveillance provided the data necessary to justify the development of HAdV-4 and HAdV-7 vaccine for the trainee populations (Dudding et al., 1973).

Although adenovirus infections remained infrequent over the period of vaccine usage, vaccine production was discontinued by the sole manufacturer in 1996 due to disagreements with the DoD. The re-emergence of HAdV infections after the cessation of the vaccine prompted the development and implementation of a new surveillance program (Figure 5). The Naval Health Research Center (NHRC) in San Diego, California, instated a surveillance program at eight locations across the United States. Sites included: Coast Guard Training Center Cape May in New Jersey, US Army Fort Benning in Georgia, US Army Fort Leonard Wood in Missouri, US Army Fort Jackson in South Carolina, the Naval Recruit Training Center Great Lakes in Illinois, Lackland Air Force Base in Texas and the Marine Corps Recruit Depots in Parris Island, South Carolina and San Diego, California.

The military surveillance program has provided a large collection of viral isolates from recruit FRI cases since 1996. Between the years 1999 and 2004, a large-scale study was conducted to determine the etiology of FRI cases in the US military recruit

population. Among all FRI cases reported in this time period, depending on the military training facility, 52.3% to 76.4% were confirmed to be associated with adenovirus infections (Russell et al., 2006). This totaled over 73,000 cases at eight training centers across the United States. Another study was conducted between 2002 and 2006 to identify the most prevalent serotypes of adenoviruses infecting military recruits (Metzgar et al., 2007). Adenovirus isolate identification for this study was limited to serotyping by microneutralization assay or PCR.
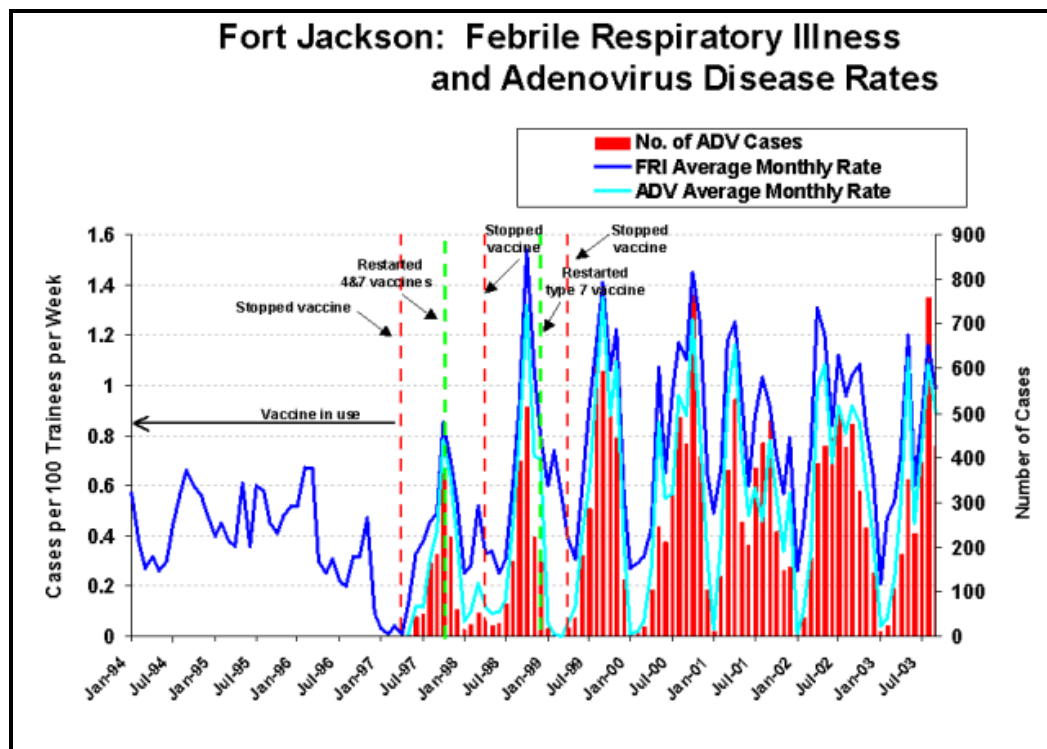
Figure 5. Rates of FRI after cessation of vaccination protocol at Fort Jackson US Army training camp in South Carolina (Courtesy of the Department of Respiratory Disease Research, Naval Health Research Center, San Diego, California, USA.)

Military recruits may be highly susceptible to infections because of their crowded or closed environments and possibly psychological stress (Sartwell, 1951; Cohen, 1995). Crowded environments create a setting in which viral infection can be spread easily from

person to person. In 2004, Russell and colleagues examined the prevalence of HAdV-4 in military barracks. On admission to training, 97% of incoming recruits were seronegative for HAdV-4. After completing training, 97% of trainees were seropositive for HAdV-4. Surfaces in the barracks were tested for adenovirus. Adenovirus was most commonly detected on fomites such as pillows, lockers and rifles (Russell et al., 2006). These fomites provided an optimal vehicle for spreading the pathogen among individuals. Because of the high prevalence of adenoviral infections in this type of environment it is important to understand which serotypes, as well as specific genome types, are responsible for causing disease.

Historically, few serotypes of adenovirus have been associated with respiratory illness in military recruits worldwide. These serotypes include types HAdV-3, HAdV-4, HAdV-7, HAdV-11, HAdV-14 and HAdV-21 (Chmielewicz et al., 2005; Jeon et al., 2007; Kajon et al., 2007; Kolavic-Gray et al., 2002; Metzgar et al., 2007). The most common serotypes detected in the US military are HAdV-4 and HAdV-7. A reemergence of species B adenovirus infections in US military recruits was detected by surveillance in 2006. This includes subspecies B1 HAdV-3, HAdV-7 and HAdV-21, and also subspecies B2 HAdV-14, which had previously never been detected in the United States (Metzgar et al., 2007).

Adenovirus Infections in Pediatric Populations

Like military recruits, children are also burdened with HAdV-associated respiratory illnesses. Diseases caused by adenovirus infection in children include tonsillitis, pharyngitis, pertussis-like syndrome, gastroenteritis, hepatitis, enchephilitis

and bronchiolitis. Adenovirus has been estimated to be responsible for up to 5% of upper respiratory tract infections, in children under five years of age (Brandt et al., 1969) and 10% of pneumonias (Mallet et al., 1966). Common symptoms include nasal congestion, fever, myalgia, headache, malaise and cough. Sequelae such as post-infectious bronchiolitis obliterans and bronchiectasis have also been linked to adenovirus respiratory infections (Becroft, 1971). Because the pediatric population is distinct from young adults in the military, studying the genome types that circulate in this population can be of interest. Comparing these two populations can give more insight into the epidemiology of adenovirus infections by allowing for identification of unique patterns of circulation and genome type distribution, and also the identification of common targets for intervention.

Species C adenoviruses are the most prevalent in the etiology of HAdV-associated respiratory disease in children (Schmitz et al., 1983; Moura et al., 2007). However, a few species B serotypes play a role in severe respiratory illness in the pediatric population. These include HAdV-3, HAdV-7 and HAdV-21 (Mizuta et al., 2006; Moura et al., 2007; Chang et al., 2008; Wadell et al., 1980; Wong et al., 2008). Occasionally, HAdV-4 genome types are isolated in the pediatric population. However, this occurs less frequently than in military recruits (Kajon et al., 1996; Chen et al., 2004; Lin et al., 2004). Although rare, reports in the literature show HAdV-21 was associated with bronchiectasis and other pulmonary sequelae, such as bronchiolitis obliterans, in young children during an epidemic of severe lower respiratory tract infections (Lang et al., 1969; Becroft, 1971).

HAdV-3 has been identified in association with severe respiratory disease in children (Kajon et al., 1990; Pingleton et al., 1978; Yurlova et al., 1986). Although high mortality rates are not usually associated with HAdV-3 infections, Kim and colleagues reported a 3.6% mortality rate among children in Seoul, Korea (Kim et al., 2003). Two fatal cases were reported in a Pediatric Intensive Care Unit in Hong Kong in association with HAdV-3 infection (Hon et al., 2008). Publications on ARD caused by HAdV-3 have shown that infections can result in pneumonia, bronchitis and bronchiolitis (Lin et al., 2004; Chang et al., 2008). HAdV-3 is often detected in community outbreaks involving children, especially in schools and swimming pool outbreaks (Martone et al., 1980; Harley et al., 2001).

HAdV-7 has been associated with the most severe clinical presentations of respiratory disease, disseminated diseases and mortality, especially in those who are immunocompromised (Beby-Defaux et al., 2001; Murtagh et al., 1993; Viquesnel et al., 1997). HAdV-7 was detected as a causative agent of outbreaks of ARD in several children's hospitals in 1980 (Straube et al., 1983). During the period between 1995 and 1999, an 18% mortality rate was associated with HAdV-7 respiratory illness in Seoul, Korea, as opposed to only a 3.6% mortality rate with HAdV-3 as the causative agent (Kim et al., 2003). In New South Wales, Australia, significant mortality was reported in association with HAdV-7b (de Silva et al., 1989). Between 1991 and 1994 in South America, HAdV-7h was associated with significantly higher mortality rates than other serotypes of adenovirus. The mortality rates for HAdV-7h were 16.8%, as opposed to 1.6% for the other serotypes (Kajon et al., 1996). During 1998, an outbreak in a chronic-

care pediatric facility in the United States occurred in which there were eight deaths associated with HAdV-7d2 infection (Gerber et al., 2001).

Species B serotypes are associated with outbreaks and severe disease in pediatric populations. There is a lack of a large scale epidemiological study in the United States documenting adenovirus infections in children. Because of this, examining HAdV-3 and HAdV-7 genome types can provide valuable information on the role certain HAdV genome types play in the US pediatric population.

Gap in Current Knowledge

A significant gap remains in epidemiological data collected from both the military and civilian populations in the United States. An abrupt reemergence of species B adenovirus infections in military recruit training centers was observed beginning in 2004 (Metzgar et al., 2007). This reemergence, which included HAdV-3 and HAdV-7, occurred towards the end of a period in which HAdV-4 was associated with over 95% of ARD in military recruits (Kajon et al., 2007). In 1997, an outbreak of HAdV-3 and HAdV-7 infection was reported at the US Navy's only basic training command in Great Lake, Illinois (Ryan et al., 2002). This large outbreak occurred only a year after the discontinuation of vaccination protocol by the DoD. These observations illustrate the importance that both HAdV-3 and HAdV-7 play in outbreaks of ARD in military recruits. Molecular typing the specific variants of HAdV-3 and HAdV-7 that have reemerged in military populations would allow for identification of the genome types that play a role in the etiology of ARD in military recruits and thus support the evaluation of vaccine efficacy once reinstated.

In the United States, there have been no recent studies to analyze the different variants of HAdV-3 and HAdV-7 circulating in civilian populations. Determining the currently prevalent genome types in US civilians may allow for identification of possible common targets for intervention to prevent HAdV-associated disease in both military recruits and civilians. The most comprehensive study on HAdV-3 genome types was conducted on strains circulating worldwide between the 1950s and 1960s (Li & Wadell, 1988). The most comprehensive surveillance study on adenovirus infections in the United States was conducted in the 1960s and 1970s with the Virus Watch Program (Elverback et al., 1966; Fox et al., 1969; Fox et al., 1977).

There is a lack of epidemiological studies on HAdV-associated disease in the United States. Recently there was an assessment of the HAdV-7 genome types associated with military and community outbreaks of adenovirus infection. The outbreaks occurred across the United States and in Eastern Ontario, Canada, between 1966 and 2000 (Erdman et al., 2002). Data from this study are a useful reference to help demonstrate the genome types of HAdV-7 that have circulated over the last 40 years, and along with other studies, show that the most prevalent circulating HAdV genome types change over time. It is also clear that HAdV-7 is associated with severe outbreaks of adenovirus infection and that the severity of the associated disease can change depending on the genome types of HAdV-7 (Kajon et al., 1996; Wadell et al., 1980). An update on HAdV-7 genomes circulating in the past nine years could complement the previous study conducted by Erdman and colleagues. Additionally, no data currently exist on the intraserotypic genetic variability of HAdV-3 strains infecting military recruits or civilians. A current study on HAdV-3 and HAdV-7 genome types associated with HAdV

infections would provide needed information on the current circulating genome types of HAdV in the United States and determine whether any of these genome types pose a challenge to vaccine efficacy.

Many important questions remain unanswered surrounding the epidemiology of HAdV morbidity in association with HAdV-3 and HAdV-7. What are the most prevalent genome types of HAdV-3 and HAdV-7 in the United States? Have the most prevalent genome types of HAdV-7 and HAdV-3 in the US military changed since the implementation and cessation of vaccination protocol? Are the same genome types of HAdV-3 and HAdV-7 circulating in US military and civilian populations? Do the genome types have a wide or restricted geographical distribution? Do the prevalent genome types circulate for limited or extended periods of time? Could any of the current circulating strains of HAdV-3 and HAdV-7 be recombinants? This study was proposed with the hope of answering some of these questions in order to obtain valuable information regarding vaccine efficacy, transmission, and the impact of HAdV-3 and HAdV-7 in the US military and civilian populations.

Chapter Three: Materials and Methods

Surveillance among military recruits and origin of HAdV isolates: The NHRC conducts active surveillance for respiratory pathogens among military recruits for the Department of Defense Global Emerging Infections Surveillance and Response System (DoD-GEIS; http://www.geis.org). The NHRC is accredited by the College of American Pathologists and has Clinical Laboratory Improvement Program certification (Ryan, Gray et al., 2000). Surveillance was established in October 1996 at eight US military training sites: CGTC Cape May, NJ; Fort Benning, GA; Fort Leonard Wood, MO; Fort Jackson, SC; NRTC Great Lakes, IL; Lackland Air force Base, TX; MCRD Parris Island, SC; and MCRD San Diego, CA. Military trainees are monitored for FRI. Recruits were diagnosed with ARD/FRI if they met two criteria: a fever (oral temperature $\geq 38°C/100.5°F$) and a respiratory symptom (cough, sore throat, runny nose, wheezing, dyspnea, sinus tenderness, or a physical exam consistent with respiratory tract infection). Recruits meeting the case definition were asked for permission to obtain a throat swab and asked to fill out a brief questionnaire.

Clinical samples were collected in viral transport medium, Micro Test^{TM} Multi-Microbe Media (REMEL, Lenexa, KS), frozen at -70C, then shipped to the NHRC laboratory and processed. Samples were screened for adenovirus, influenza A/B, parainfluenza 1-3 and respiratory syncytial virus by PCR. For adenovirus culture, throat specimens were inoculated into A549 cells and observed for evidence of virus replication and resulting CPE for a period up to fourteen days. Once viral CPE was observed, isolates were identified by PCR or immunofluorescence using identified monoclonal antibodies (CHEMICON International, Inc., Temecula CA) (Metzgar et al., 2005; Russell

et al., 2006). Adenoviral isolates were then serotyped by a microneutralization assay (Malasig et al., 2001), PCR, or both (Metzgar et al., 2005).

Only adenovirus-positive samples negative for other respiratory pathogens were included in this study. After identification of serotype at the NHRC, HAdV-3 and HAdV-7 isolates were sent to Lovelace Respiratory Research Institute (LRRI) for further characterization. Viral isolates chosen for genome typing were selected to include all sites and all times, rather than being a random subset of all samples collected. HAdV-3 and HAdV-7 strains isolated between 1997 and 2008 were included in this study.

Origin of civilian HAdV isolates: A collection of adenovirus strains isolated in the United States over the last decade was analyzed as a reference for HAdV-associated disease in civilian populations. Isolates were from the collections of two institutions: the Children's Hospital of Philadelphia and the University of Iowa. HAdV isolates from the CHOP, PA, were routinely collected beginning in 2000 from pediatric patients infected with respiratory viruses. Viral detection at the CHOP includes respiratory syncytial virus, influenza A, influenza B, parainfluenza 1, 2, and 3, adenovirus, rhinovirus and human metapneumovirus. Clinical samples were tested for the presence or absence of virus nucleic acid by PCR. Viral isolates were sent to LRRI for further characterization. The only information available for these isolates was the date of collection. All isolates sent to LRRI for further characterization were obtained from hospitalized pediatric patients (ages 0-18) with no obvious immunocompromising conditions. These isolates represent epidemiologically unrelated cases of HAdV-associated ARD diagnosed between 2001 and 2008.

Isolates from the collection of the University of Iowa represented fourteen sites and eleven states throughout the US (Gray et al., 2007).   Sites included: Laboratory Sciences of Arizona/Sonora Quest Laboratories, Tempe, AZ; The Children's Hospital, Denver, CO; Yale New Haven Hospital and Yale University, New Haven, CT; Indiana University School of Medicine and Clarian Health Partners, Indianapolis, IN; Baptist Medical Center, Jacksonville, FL; Children's Mercy Hospital, Kansas City and Louis Children's Hospital, St. Louis, MO; SUNY Upstate Medical University, Syracuse and North Shore University Hospital, Manhasset, NY; Vanderbilt University School of Medicine, Nashville, TN; Texas Children's Hospital, Houston, TX; Children's Hospital and Regional Medical Center, Seattle WA; and Medical College of Wisconsin, Milwaukee, WI. Samples were collected and sent to the University of Iowa's Center for Emerging Infectious Disease for genetic typing.   HAdV-3 and HAdV-7 viral isolates were sent to LRRI for further characterization with only the date and site of collection. Diagnostic specimens were obtained from patients of all ages, however 76.8% were less than seven years old (Gray et al., 2007). These isolates represent epidemiologically unrelated cases and include HAdV-3 and HAdV-7 strains circulating between 2004 and 2006.

Note: In the body of the text, the term civilian population is used to describe the patient population from the CHOP and the UI collections. If referring to the CHOP collection of samples only, the use of the term pediatric will be used.

Human Subject Protection: Because all viral isolates sent to the LRRI from the NHRC, the CHOP and the UI are free of any identifiers that can link them to the patient, this

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study has met the conditions for IRB exemption under 45 CFR 46.101(b)(4). This exemption states, "Research, involving the collection or study of existing data, documents, records, pathological specimens, if these sources are publicly available or if the subject information is recorded by the investigator in such a manner that the subject cannot be identified, directly or through identifiers linked to the subjects." Date of approval, August 19th, 2008.

Cell lines: Virus isolates were cultured in A549 cells, a human lung carcinoma cell line (ATCC #CCL-185). Cells were grown in tissue culture flasks with Eagle's Modified Essential Medium (EMEM), containing 8% newborn calf serum. After infection with HAdV, cells were replenished with maintenance medium: EMEM containing 2% newborn calf serum.

Adenovirus DNA purification and REA: Viral isolates from the NHRC, CHOP and UI were passed once in A549 cells to verify typical adenovirus cytopathic effect. In brief, samples were freeze-thawed, clarified by centrifugation (1000 rpm), and 100μL of the supernatant were used to infect monolayers of A549 cells in 75-cm^2 flasks for viral DNA extraction. Intracellular viral DNA was isolated using a modified version (Kajon and Erdman, 2007) of the extraction method developed by Shinagawa and colleagues (Shinagawa et al., 1983).

To perform REA, 1 μg of the viral DNA was digested using *Bam*HI, following manufacturer's instruction, and further analyzed with *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Hind*III or *Sma*I (Promega, Madison, WI). DNA fragments were separated by horizontal gel

electrophoresis in 0.8%, 1.0 % or 1.2% agarose gels using 1X Tris borate-EDTA buffer (0.09M Tris-Borate and 0.002M EDTA, pH 8). Gels were stained with ethidium bromide (0.6μg/mL) for 10 minutes and destained with water for 15 minutes. Fragments were visualized using UV transillumination, at 303 nm, with a Gel Doc imaging system (Bio-Rad, Temecula, CA).

All HAdV-3 and HAdV-7 isolates were digested using the restriction endonucleases *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Hind*III and *Sma*I. If the isolates were collected from an outbreak scenario (several from the same site/location and date), then only a random set of isolates were digested to determine the genome type. However, if any restriction pattern differences were detected among isolates from the same cluster of cases, all isolates were cut with the panel of endonulceases.

Restriction sites were mapped and fragment sizes calculated using SeqBuilder software (Lasergene, DNAStar, Inc. Madison, WI) and the following whole genome sequences available from Genbank: HAdV-3 prototype strain GB (HAdV-3p GB, Genbank accession number AY599834), a HAdV-3 field strain isolated in 1997 in Great Lakes, genome typed in this study (NHRC 1276, Genbank accession number AY599836) and two Chinese field strains isolated in Southern China in 2004 and 2005 (Genbank accession numbers DQ105654 and DQ099432), HAdV-7 prototype strain (HAdV-7p, Gomen, Genbank accession number AY594255), HAdV-7 vaccine strain (Genbank accession number AY594256) and a HAdV-7 field strain isolated in 1997 in Great Lakes, genome typed in this study (NHRC 1315, Genbank accession number AY601634), using SeqBuilder software (Lasergene, DNAStar, Inc. Madison, WI).

Genome type denomination: Genome types were designated using the system proposed by Li and Wadell (Li and Wadell, 1986). Genome types were initially discriminated based on their distinct *Bam*HI profile and designated p for prototype, or a, b, c, etc., for subsequent variants. Additional restriction endonucleases were used for final determination of the genome type. An extensive literature search was conducted to identify already described genome types of adenovirus. If a novel profile was identified, it was tentatively designated based on the restriction endonuclease with which variation was observed. For example, if a novel profile was identified using *Bam*HI, it was tentatively designated, HAdV-3*Bam*HI or HAdV-7*Bam*HI. However, if the isolate had a recognizable *Bam*HI pattern, but showed a novel pattern using another endonuclease, it was named as a variant for the genome type that the *Bam*HI profile displayed, then for the restriction endonulcease that showed variation. For example, an isolate that had a HAdV-3a *Bam*HI profile, but a novel *Bgl*II profile was tentatively designated HAdV-3a*Bgl*II.

PCR amplification and sequencing of the hexon and fiber genes: Fragments spanning the HVR1-7 of the hexon gene and the entire fiber gene were generated by PCR using a high fidelity Taq Polymerase (iProof, Bio-Rad, Temecula, CA). Cycling conditions were as follows: a preliminary denaturation at 94°C for 2 min., followed by 49 cycles of denaturation at 94°C for 1 min., annealing temperatures and times listed below, primer extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Primers to amplify HVR1-6 of species B adenoviruses were: Forward (5' AATTAGGCAGTTCATCC 3') and Reverse (5' GGCTGAGTTGCTTTC 3') annealed at

49°C for 45 seconds. Internal primers were needed for sequencing and included: Forward (5' AACATATCAGCCAGAGC 3') and Reverse (5' ATGATTCTTCTCCAACTTG 3'). Primers to amplify the HVR-7 of species B adenovirus were: Forward (5'-CTGATGTACTACAACAGCACTGGCAACATGGG-3') and Reverse (5'-GCGTTGCGGTGGTGGTTAAATGGGTTTAC-3') annealed at 51°C for 30 seconds. Primers for the fiber gene of species B adenoviruses were: Forward (5'-CATACTTTCTCCACAC-3') and Reverse (5'-GCAAAATAACTACTCG-3') annealed at 42°C for 45 seconds or Forward (5'-CTACCAGCAGCACCTC-3') and Reverse (5'-TAAAGCTGTGCGTGG-3') annealed at 52°C for 45 seconds. Some instances required internal primers to complete the sequence for both strands. These included: Forward (5'-GGAGGAATTGTTAATGGA-3') and Reverse (5'-TCCATTAACAATTCCTCC-3'). PCR products were visualized by agarose gel electrophoresis, then purified using Montage-PCR columns (Millipore, Billerica, MA) or QIAquick PCR purification kit (QIAGEN Sciences, Maryland, USA) as recommended by the manufacturers.

Purified PCR products were sent for sequencing to DNA Research Services in the Pathology Department of the University of New Mexico. Sequence data were edited and aligned using Lasergene software (Lasergene, DNAStar, Inc. Madison, WI). Data were compared to available sequences for the hexon and fiber genes using NCBI Genbank and BLAST. To determine the percent of sequence identity, sequences were aligned using Clustal W (Lasergene, DNAStar, Inc. Madison, WI) to known sequences for similar genome types to identify variability at both the nucleotide and amino acid levels.

Chapter Four: Results

Contribution of HAdV-3 and HAdV-7 to total HAdV infections

In order to determine the prevalence of HAdV-3 and HAdV-7 relative to other serotypes of HAdV in military and civilian populations, the number of HAdV-3 and HAdV-7 isolates was compared to the total number of HAdV isolates typed by the NHRC, the CHOP or the UI over the surveillance period. Surveillance data from the NHRC show that between 1997 and 2008, 11,455 HAdV isolates were recovered from military recruits with HAdV-associated FRI (D. Metzgar, personal communication, August 2009). Of those, 361 (3.2%) and 553 (4.8%) were identified as HAdV-3 and HAdV-7, respectively (Fig. 6). HAdV-4 accounted for 78.8% (n=9,027) of all cases of HAdV infection during this period. In our study we analyzed 108 HAdV-3 isolates and 120 HAdV-7 isolates representing all sites and detected associated outbreaks.
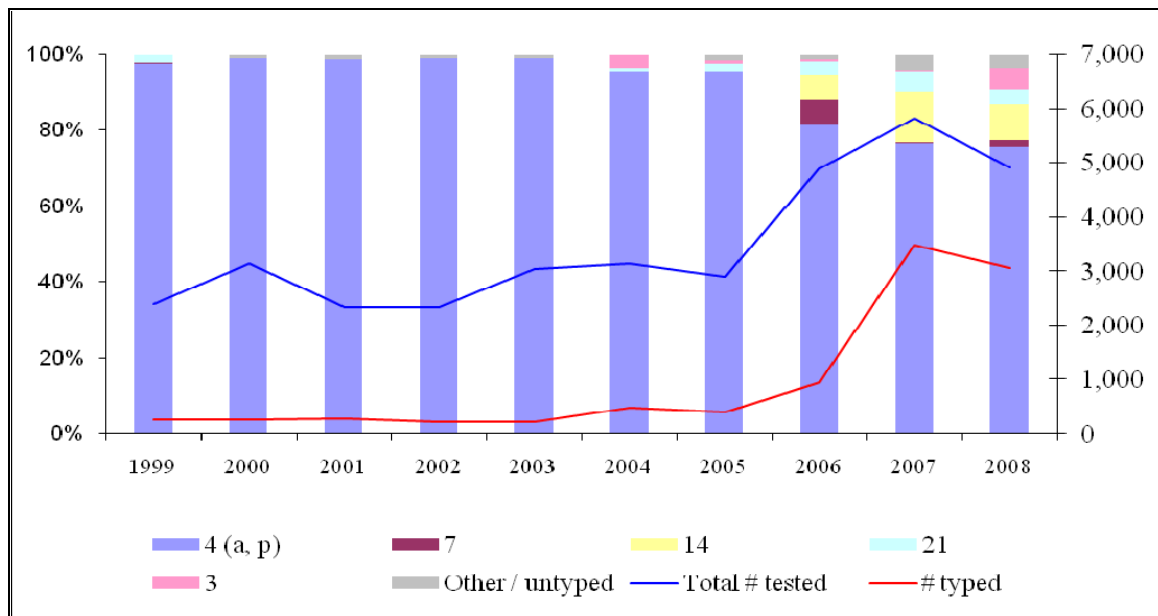
Figure 6. HAdV serotypes associated with FRI in military recruits from 1999-2008 (courtesy of D. Metzgar, NHRC).

A collection of 782 civilian HAdV isolates was provided for our study by the CHOP (n=731) and the UI (n=51). Of the 731 CHOP isolates processed, 24.4% were identified as HAdV-3 (22.8%, n=167) or HAdV-7 (1.6%, n=12), and 1.9% as other species B serotypes (HAdV-11 and HAdV-21), 66.4% as species C and 3.0% as species E (Fig. 7).
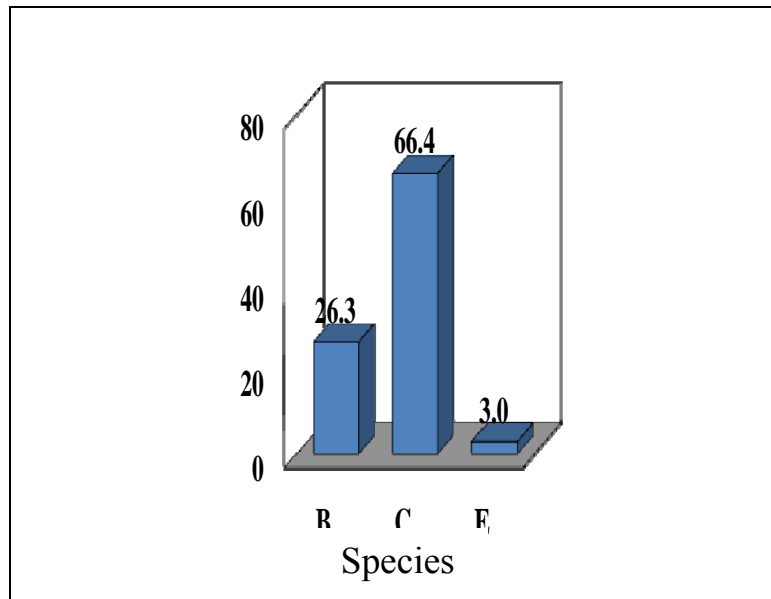
Figure 7. Percentage of isolates from the Children's Hospital of Philadelphia corresponding to species B, C and E.

The HAdV-3 and HAdV-7 isolates that were genome typed in this study represented a fraction (3.2%) of the isolates examined between 2004 and 2006 by Dr. Gray and colleagues (Gray et al., 2007). Of the total isolates (n=1608) analyzed in his prospective study (Gray et al., 2007), HAdV-3 represented 34.6% (n=556) and HAdV-7 represent 3% (n=48).

Genetic variability of HAdV-3 isolated in the US from military recruits and civilians

Eleven genome types of HAdV-3 were identified in the examined collection of isolates (Table 3). Restriction patterns for each of the HAdV-3 genome types identified with restriction endonucleases (*Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Hind*III and *Sma*I) are shown in Figures 8 and 9. Restriction site maps and estimated restriction-fragment sizes for each endonuclease and each individual genome type were determined (Tables 4 and 5). Of the eleven genome types, ten were identified as variants of genome type HAdV-3a (Li and Wadell, 1988). Three genome types had been previously described: HAdV-3a (Wadell, 1984), HAdV-3a2 (Li and Wadell, 1988), and HAdV-3a17 (Kim et al., 2003). The remaining eight HAdV-3 genome types were identified as novel. These novel genome types were tentatively designated HAdV-3a*Bcl*I variant, HAdV-3a*Bgl*II variant 1, HAdV-3a*Bgl*II variant 2, HAdV-3a*Bgl*II variant 3, HAdV-3a*Bst*EII variant, HAdV-3a*Bcl*I2*Bst*EII variant, HAdV-3*Bam*HI variant and HAdV-3*Bam*HI*Bgl*II variant.

All previously described HAdV-3 isolates detected in US military recruits and civilians belonged to genomic cluster 3 (Li and Wadell, 1988) and were determined to be variants of HAdV-3a genome types, based on their *Bam*HI restriction profile (Fig. 8, *Bam*HI profile I). Based on the percentage of co-migrating bands, all eight novel HAdV-3 genome types identified belong to genomic cluster 3.

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Table 3. Restriction enzyme analysis of HAdV-3 genome types isolated among military recruits (blue) and civilian populations (black) in the United States. Roman numerals in shown in red are novel profiles. "*" Denotes genome types found in both the military and civilian populations.

Genome Type	*Bam*HI	*Bcl*I	*Bgl*I	*Bgl*II	*Bst*EII	*Hind*III	*Sma*I
*3a	I	I	I	I	I	I	I
*3a2	I	I	I	I	I	I	II
*3a17	I	I	II	II	I	I	I
*3a*Bcl*I variant	I	II	I	I	I	I	II
*3a*Bst*EII variant	I	I	I	I	II	I	II
3a*Bcl*I2*Bst*EII variant	I	III	I	I	II	I	II
3a*Bgl*II variant 1	I	I	I	III	I	I	II
*3a*Bgl*II variant 2	I	I	I	IV	I	I	II
3a*Bgl*II variant 3	I	I	I	VI	I	I	II
3*Bam*HI*Bgl*II variant	II	I	I	V	III	I	II
3*Bam*HI variant	II	I	III	II	I	I	II

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Figure 8. Restriction enzyme analysis of HAdV-3 strains isolated in the United States between 1997 and 2008 in both military and civilian populations. HAdV-3 strains were characterized by digestion with *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Hind*III and *Sma*I. Lane M, molecular weight markers (1kb + 100bp ladders; BioRad). Roman numerals designate unique profiles for each enzyme.
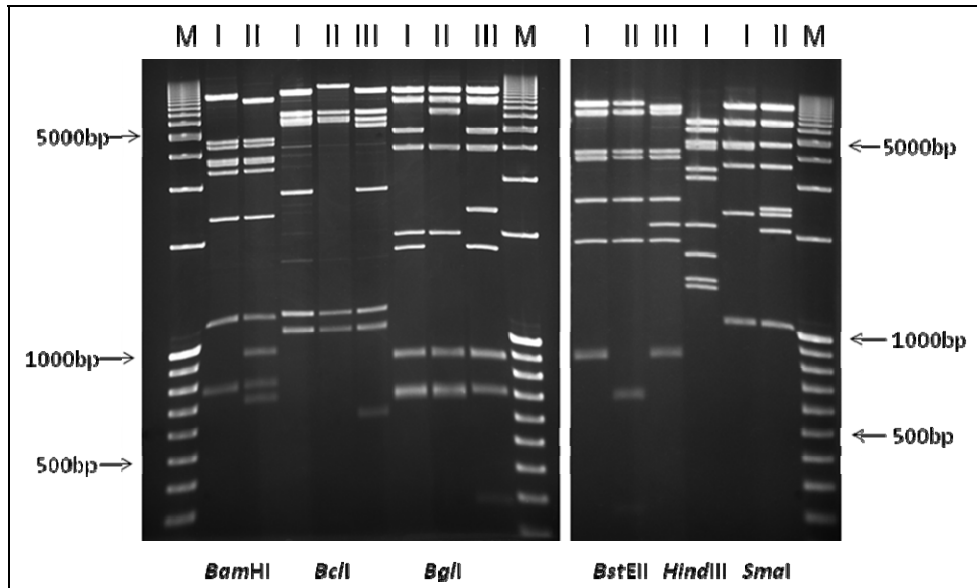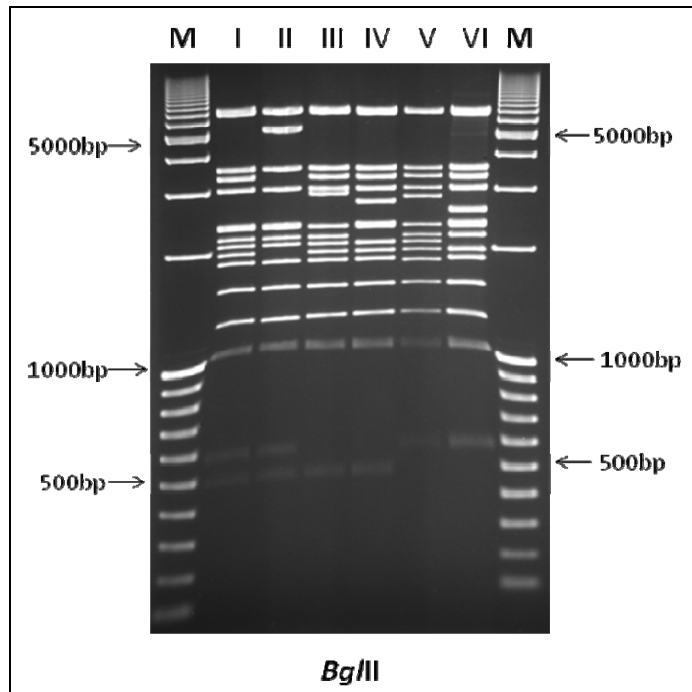

Figure 9. Restriction enzyme analysis of HAdV-3 strains isolated in the United States between 1997 and 2008 in both military and civilian populations. HAdV-3 strains were characterized by digestion with *Bgl*II. Lane M, molecular weight markers (1kb + 100bp ladders; BioRad). Roman numerals designate unique profiles for the enzyme.

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Novel HAdV-3 genome types

Of the eight novel HAdV-3 genome types identified, three were detected in military and civilian populations; HAdV-3a*Bcl*I variant, HAdV-3a*Bgl*II variant 2, and HAdV-3a*Bst*EII variant. As their name suggests, these variants had novel profiles with one restriction endonuclease. The other five novel HAdV-3 genome types were detected only in isolates from the CHOP or the UI. HAdV-3a*Bcl*I2*Bst*EII variant was detected in the collection of isolates from the UI and had the same *Bst*EII profile as HAdV-3a*Bst*EII variant (Fig. 8, *Bst*EII profile II). In addition to the novel *Bst*EII pattern, it also had a unique *Bcl*I pattern (Fig. 8, *Bcl*I profile III). HAdV-3a*Bgl*II variant 1 had a novel *Bgl*II pattern (designated variant 1 based on the isolation date) (Fig. 9, profile III), while HAdV-3a*Bgl*II variant 3 had a *Bgl*II restriction profile identical to that previously described for genome type HAdV-7h (Fig. 9, profile VI) (Kajon and Wadell, 1994).

HAdV-3*Bam*HI variant and HAdV-3*Bam*HI*Bgl*II variant shared a novel *Bam*HI profile (Fig. 8, *Bam*HI profile II). Sequences generated for the HVR7 of the hexon gene and the fiber gene were analyzed and revealed 99% sequence identity with HAdV-3. Both of these novel genome types yielded previously described profiles when digested with the other restriction endonucleases, except for HAdV-3*Bam*HI*Bgl*II variant, which also had a novel *Bgl*II profile (Fig. 9, Profile VI).

Table 4. Location of *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Hind*III and *Sma*I restriction sites in the genomes of HAdV-3 genome types. *Estimated based on electrophoretic mobility.

Restriction Enzyme, Genome Type	Number of Sites	Site Location, nt
***Bam*HI**		
3p	8	747, 1911, 3574, 13013, 21117, 24571, 29165, 31596
3a, 3a2, 3a17, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bcl*I2*Bst*EII variant	8	747, 1909, 13005, 16733, 21112, 24552, 29145, 31518
3*Bam*HI variants	10	747, 1911, 2858, 3574, 13005, 16733, 21112, 24552, 29145, 31518
***Bcl*I**		
3p	4	11558, 14394, 20313, 29369
3a, 3a2, 3a17, 3a*Bst*EII variant, 3a*Bam*HI variants	6	11550, 14386, 20308, 27082, 28156, 29349
3a*Bcl*I variant	5	14386, 20308, 27082, 28156, 29349
3a*Bcl*I2*Bst*EII variant	7	11550, 14386, 20308, 27082, 28156, 29349, *34649
***Bgl*I**		
3p	8	10367, 11055, 11947, 16016, 16040, 17844, 22751, 23473
3a17	8	8355, 10356, 11047, 11939, 16008, 16032, 22747, 23466
3a, 3a2, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bcl*I2*Bst*EII variant, 3*Bam*HI*Bgl*II variant	9	8355, 10365, 11054, 11946, 16013, 16037, 17844, 22752, 23471
3*Bam*HI variant		8355, ~10765, 11054, 11946, 16013, 16037, 17844, 22752, 23471
***Bgl*II**		
3p	14	1565, 3898, 7474, 8754, 9795, 11669, 14760, 22189, 24312, 24810, 27197, 27783, 30022, 32006
3a, 3a2, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bam*HI variant	14	1563, 3896, 7472, 8752, 9793, 11668, 14759, 22190, 24298, 24796, 27182, 27768, 29950, 31933
3a17	13	1563, 3896, 7472, 8743, 9784, 11661, 14752, 22185, 24293, 24791, 27177, 27763, 29945
3a*Bgl*II variant (7h)	13	1563, 3898, 7474, 8754, 9795, 11618, 14709, 22094, 24718, 27103, 27690, 29890, 31874
3*Bam*HI*Bgl*II variant	13	1563, 3898, 7474, 8754, 9795, 11669, 14760, 22189, 24312, 27197, 30022, 32006
3a*Bgl*II variant 1	13	1563, 3896, 7472, 8752, 9793, 11668, 14759, 22190, 24298, 24796, 27768, 29950, 31933
3a*Bgl*II variant 2	13	1563, 3896, 7472, 8752, 9793, 11668, 14759, 22190, 24298, 24796, 27182, 29950, 31933
***Bst*EII**		
3p, 3a, 3a2, 3a*Bcl*I variant, 3a*Bgl*II variant	6	12405, 15061, 24457, 26355, 30608, 34514
3a*Bst*EII variant, 3a*Bcl*I2*Bst*EII variant	6	12405, 15061, 24457, 26355, 30608, 34514, *34714 or *35164
3BamHI variants	7	10235, 12355, 15011, 24366, 26264, 30477, 34370
***Hind*III**		
3p, 3a, 3a2, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bgl*II variant, 3a*Bcl*I2*Bst*EII variant, 3*Bam*HI variants	10	1385, 2714, 6135, 10633, 17725, 17763, 22501, 24671, 27878, 29589
***Sma*I**		
3p	7	4617, 6705, 16396, 21398, 23805, 27481, 34300
3a, 3a17	6	4615, 9177, 21393, 23786, 27461, 34222
3a2, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bgl*II variant, 3a*Bcl*I2*Bst*EII variant, 3*Bam*HI variants	7	4615, 6705, 9186, 21398, 23791, 27466, 34227

Table 5. Molecular size of DNA fragments of HAdV-3 genome types generated by digestion with 7 endonucleases. * Estimated based on electrophoretic mobility.

Restriction Enzyme, Genome Type	Number of Fragments	Fragment Size, bp
*Bam*HI		
3p	9	747, 1164, 1663, 9439, 8104, 3454, 4594, 2431, 3749
3a, 3a2, 3a17, 3a*Bcl*I variant, 3a*Bst*EII variant	9	747, 1162, 11096, 3728, 4379, 3440, 4593, 2373, 3747
3*Bam*HI variants	11	747, 1164, 947, 716, 9431, 3728, 4379, 3440, 4593, 2373, 3747
*Bcl*I		
3p	5	11558, 2836, 5919, 9056, 5976
3a, 3a2, 3a17, 3a*Bst*EII variant, 3a*Bam*HI variants	7	11550, 2836, 5922, 6774, 1074, 1193, 5916
3a*Bcl*I variant	6	14386, 5922, 6774, 1074, 1193, 5916
3a*Bcl*I2*Bst*EII variant	8	14386, 5922, 6774, 1074, 1193, * 5300, * 600
*Bgl*I		
3p	9	10367, 688, 892, 4069, 24, 1804, 4907, 722, 11872
3a17	9	8355, 2001, 691, 892, 4069, 24, 6715, 719, 11799
3a, 3a2, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bcl*I2*Bst*EII variant, 3*Bam*HI*Bgl*II variant	10	8355, 2010, 689, 892, 4067, 24, 1807, 4908, 719, 11798
3*Bam*HI variant		8355, *2400, *300, 892, 4067, 24, 1807, 4908, 719, 11798
*Bgl*II		
3p	15	1565, 2333, 3576, 1280, 1041, 1874, 3091, 7429, 2123, 498, 2387, 586, 2239, 1984, 3339
3a, 3a2, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bam*HI variant	15	1563, 2333, 3576, 1280, 1041, 1875, 3091, 7431, 2108, 498, 2386, 586, 2182, 1983, 3336
3a17	14	1563, 2333, 3576, 1271, 1041, 1877, 3091, 7433, 2108, 498, 2386, 586, 2182, 5320
3a*Bgl*II variant (7h)	14	1563, 2333, 3576, 1280, 1041, 1875, 3091, 7431, 2624, 2386, 586, 2182, 1983, 3336
3*Bam*HI*Bgl*II variant	14	1563, 2333, 3576, 1280, 1041, 1875, 3091, 7431, 2108, 2884, 586, 2182, 1983, 3336
3a *Bgl*II variant 1	14	1563, 2333, 3576, 1280, 1041, 1875, 3091, 7431, 2108, 498, 2972, 2182, 1983, 3336
3a*Bgl*II variant 2	14	1563, 2333, 3576, 1280, 1041, 1875, 3091, 7431, 2108, 498, 2386, 2768, 1983, 3336
*Bst*EII		
3p, 3a, 3a2, 3a*Bcl*I variant, 3a*Bgl*II variant	7	12405, 2656, 9396, 1898, 4253, 3906, 831
3a*Bst*EII variant	7	12405, 2656, 9396, 1898, 4253, 3906, *650, *200
3*Bam*HI variant, 3*Bam*HI*Bgl*II variant	8	10235, 2120, 2656, 9355, 1898, 4213, 3893, 828
*Hind*III		
3p, 3a, 3a2, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bgl*II variant, 3*Bam*HI variant, 3*Bam*HI*Bgl*II variant	11	1385, 1329, 3421, 4498, 7092, 38, 4738, 2170, 3207, 1711, 5756
*Sma*I		
3p	8	4617, 2088, 9691, 5002, 2407, 3676, 6819, 1045
3a, 3a17	7	4615, 4562, 12216, 2393, 3675, 6761, 1043
3a2, 3a*Bcl*I variant, 3a*Bst*EII variant, 3a*Bgl*II variant 2, 3*Bam*HI variant, 3*Bam*HI*Bgl*II variant	8	4615, 2088, 2483, 12212, 2393, 3675, 6761, 1042

Genetic variability of HAdV-7 isolated in the US from military recruits and civilians

Six genome types of HAdV-7 were identified in the examined collection of isolates (Table 6). Restriction endonuclease patterns for each of the identified HAdV-7

genome types are shown in Figure 8. Restriction site maps were determined and restriction-fragment sizes were calculated for each endonuclease and each individual genome type (Tables 7 and 8). Of the six genome types detected, five have been previously described and include HAdV-7p (Li and Wadell, 1986), HAdV-7b (Li and Wadell, 1986), HAdV-7d (Li and Wadell, 1986), HAdV-7d2 (Azar et al., 1998) and HAdV-7h (Kajon and Wadell, 1994). One of the genome types was identified as novel and was tentatively designated HAdV-7*Bam*HI variant.

Table 6. Restriction enzyme analysis of HAdV-7 genome types associated with febrile respiratory illness in recruits (blue) in the United States from 1997-2008 and infections in civilian populations (black) in the United States from 2001-2008. Roman numerals in shown in red are novel profiles. "*" Denotes genome types found in both the military and civilian populations.

Genome Type	*Bam*HI	*Bcl*I	*Bgl*II	*Bst*EII	*Sma*I
HAdV-7p	I	I	I	I	I
*HAdV-7b	II	I	II	II	II
HAdV-7d	III	I	II	II	II
*HAdV-7d2	III	I	II	III	II
*HAdV-7h	IV	II	III	II	II
HAdV-7*Bam*HI variant	V	II	III	IV	I

The HAdV-7 genome types detected belonged to two clusters of homology (Li and Wadell, 1999). Genome type HAdV-7p detected in only one isolate belongs to cluster 1 and the rest of the HAdV-7 genome types identified belong to cluster 2. Five of the genome types of HAdV-7 were isolated from military recruits. These included

HAdV-7p, HAdV-7b, HAdV-7d, HAdV-7d2 and HAdV-7h. HAdV-7p and HAdV-7d were not identified among the isolates from the civilian population.
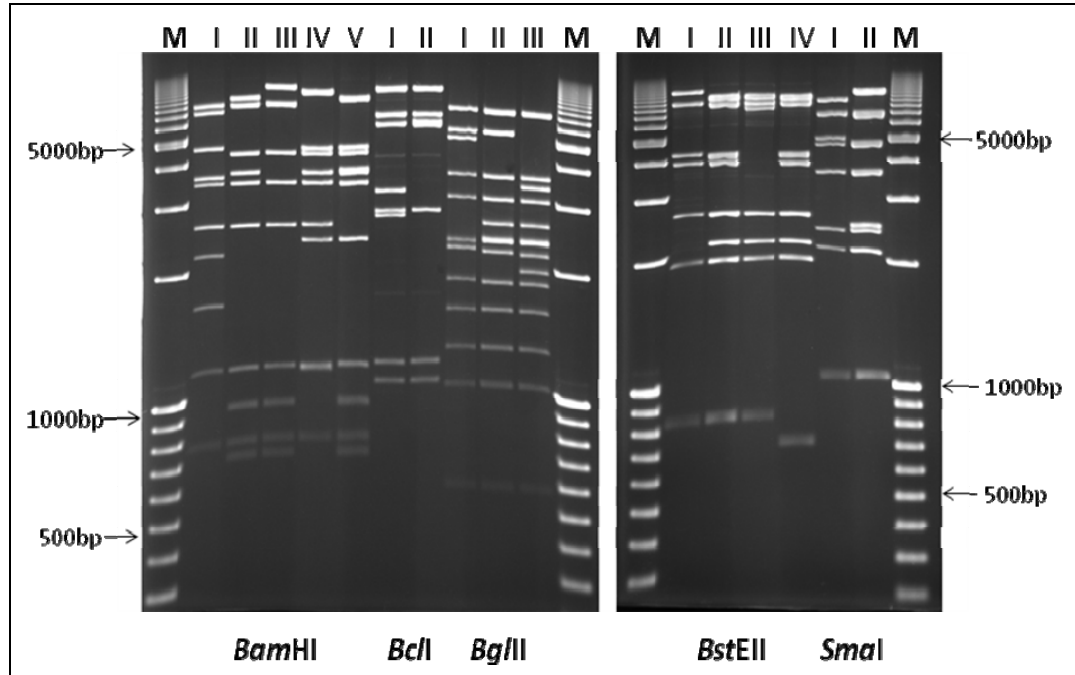
Figure 10. Restriction enzyme analysis of HAdV-7 strains isolated in the United States during the period 1997-2008 in both military and civilian populations. HAdV-7 strains were characterized by digestion with *Bam*HI, *Bcl*I, *Bgl*II, *Bst*EII and *Sma*I. Lane M, molecular weight markers (1kb + 100bp ladders; BioRad). Roman numerals represent unique profiles for each enzyme.

Novel HAdV-7 genome types

The novel HAdV-7 genome type, HAdV-7*Bam*HI variant, was detected in one civilian isolate, but was not detected in military recruits (Table 6). The novel *Bam*HI profile was identical to the profile identified in the HAdV-3*Bam*HI variants. However, when the fiber gene and the HVR7 of the hexon gene were PCR-amplified and sequenced,this isolate shared over 98% sequence identity with other HAdV-7 genome types in cluster 2. Digestion with *Bcl*I, *Bgl*I, *Bgl*II and *Sma*I identified HAdV-7-like

patterns as well (Fig. 10). However, digestion with *Bst*EII identified a novel HAdV-7

profile (Fig. 10, *Bst*EII profile IV).

Table 7. Location of *Bam*HI, *Bcl*I, *Bgl*II, *Bst*EII and *Sma*I restriction sites in the genomes of HAdV-7 genome types HAdV-7p, HAdV-7b, HAdV-7d, HAdV-7d2, HAdV-7h, HAdV-7*Bam*HI variant. *Estimated based on electrophoretic mobility.

Restriction Enzyme, Genome Type	Number of Sites	Site Location, nt
***Bam*HI**		
7p	9	748, 1911, 3575, 5766, 13013, 21095, 24550, 29143, 32703
7b	9	747, 1911, 2858, 3574, 12963, 16689, 21020, 24480, 32596
7d, 7d2	8	747, 1911, 2858, 3574, 16689, 21020, 24480, 32596
7h	8	747, 1911, 12963, 16689, 21020, 24480, *26980 or *28980, 32596
7*Bam*HI variant	10	747, 1911, 2858, 3574, 13005, 16733, 21112, 24552, 29145, 31518
7a3 vaccine	9	747, 1911, 2858, 3574, 12988, 21059, 24519, 29112, 32637
***Bcl*I**		
7p, 7b, 7d, 7d2, 7*Bam*HI variant,	7	11558, 14394, 20291, 27080, 28154, 29347, 32112
7h	6	11558, 14394, 20291, 27080, 28154, 29347
7a3 vaccine	7	11533, 14369, 20255, 27049, 28123, 29316, 32047
***Bgl*II**		
7p	11	1565, 3899, 7476, 8756, 9797, 11669, 14760, 22168, 27175, 27761, 29998
7b, 7d, 7d2, 7*Bam*HI variant	12	1565, 3898, 7475, 8755, 9796, 11619, 14710, 22095, 24719, 27105, 27691, 29891
7h	13	1565, 3898, 7475, 8755, 9796, 11619, 14710, 22095, 24719, 27105, 27691, 29891, extra site
7a3 vaccine	12	1565, 3898, 7475, 8755, 9796, 11644, 14735, 22134, 24758, 27144, 27730, 29931
***Bst*EII**		
7p	6	12405, 15061, 24436, 26334, 30583, 34477
7b, 7d, 7h	7	10235, 12355, 15011, 24366, 26264, 30477, 34370
7d2	6	10235, 12355, 15011, 24366, 26264, 34370
7a3 vaccine	7	10235, 12380, 15036, 24405, 26303, 30518, 34411
7*Bam*HI variant	7	10235, 12355, 15011, 24366, 26264, 30477, 34370
***Sma*I**		
7p	7	4619, 6707, 16393, 21376, 23784, 27459, 34263
7b, 7d, 7d2, 7h, 7*Bam*HI variant	7	4618, 6706, 9189, 21301, 23714, 27389, 34156
7a3 vaccine	8	4618, 6706, 9189, 16367, 21340, 23753, 27428, 34197

Table 8. Molecular size of DNA fragments of HAdV-7 genome types generated by digestion with selected endonucleases. *Estimated based on electrophoretic mobility.

| Restriction Enzyme, Genome Type	Number of Fragments	Fragment Size, bp
***Bam*HI**		
7p	10	748, 1163, 1664, 2191, 7247, 8082, 3455, 4593, 3560, 2603
7b	10	747, 1164, 947, 716, 9389, 3726, 4331, 3460, 8116, 2602
7d, 7d2	9	747, 1164, 947, 716, 13115, 4331, 3460, 8116, 2602
7h	9	747, 1164, 11052, 3726, 4331, 3460, *4500, *2500, 2602
7*Bam*HI variant	11	747, 1164, 947, 716, 9431, 3728, 4379, 3440, 4593, 2373, 3747
7a3 vaccine	10	747, 1164, 947, 716, 9414, 8071, 3460, 4593, 3525, 2603
***Bcl*I**		
7p, 7b, 7d, 7d2, 7BamHI variant	8	11558, 2836, 5897, 6789, 1074, 1193, 2765, 3194
7h	7	11558, 2836, 5897, 6789, 1074, 1193, 5959
7 vaccine	8	11533, 2836, 5886, 6794, 1074, 1193, 2731, 3193
***Bgl*II**		
7p	12	1565, 2334, 3577, 1280, 1041, 1872, 3091, 7408, 5007, 586, 2237, 5308
7b, 7d, 7d2, 7*Bam*HI variant	13	1565, 2333, 3577, 1280, 1041, 1823, 3091, 7385, 2624, 2386, 586, 2200, 5307
7h	14	1565, 2333, 3577, 1280, 1041, 1823, 3091, 7385, 2624, 2386, 586, 2200, 1984, 3339
7a3 vaccine	13	1565, 2333, 3577, 1280, 1041, 1848, 3091, 7399, 2624, 2386, 586, 2201, 5309
***Bst*EII**		
7p	7	12405, 2656, 9375, 1898, 4249, 3894, 829
7b, 7d, 7h	8	10235, 2120, 2656, 9355, 1898, 4213, 3893, 828
7d2	7	10235, 2120, 2656, 9355, 1898, 8106, 828
7a3 vaccine	8	10235, 2145, 2656, 9369, 1898, 4215, 3893, 829
7*Bam*HI variant	8	10235, 2120, 2656, 9355, 1898, 4213, 3893, *750
***Sma*I**		
7p	8	4619, 2088, 9686, 4983, 2408, 3675, 6804, 1043
7b, 7d, 7d2, 7h, 7*Bam*HI variant	8	4618, 2088, 2483, 12112, 2413, 3675, 6767, 1042
7a3 vaccine	9	4618, 2088, 2483, 7178, 4973, 2413, 3675, 6769, 1043
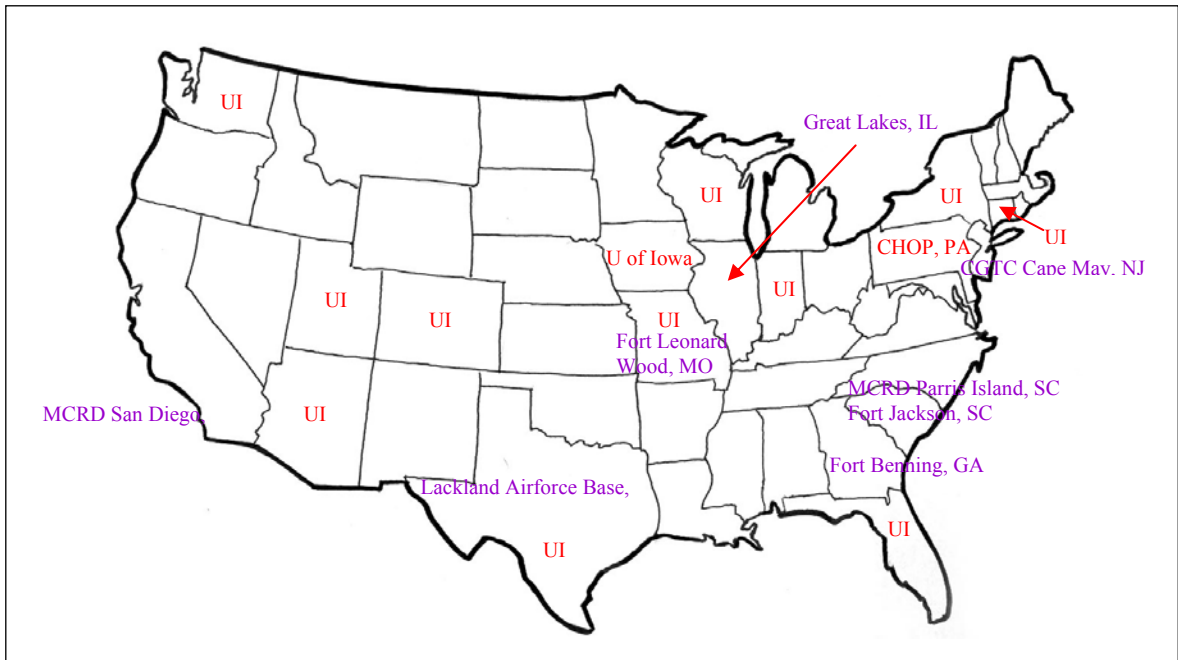
Figure 11. **Locations of adenovirus isolates collected across the United States, including military sampling sites (Purple) and civilian sampling sites (Red).**

Geographical distribution and temporal occurrence of HAdV-3 and HAdV-7 genome types

Sampling sites for the NHRC, the CHOP and the UI are shown in Figure 11. Isolates were obtained throughout the United States in order to identify the geographical distribution and temporal occurrence of HAdV-3 and HAdV-7 genome types during the surveillance period. An overview of all genome types and their place and period of isolation in either military or civilian populations are shown in Table 9. Samples were collected between 1997 and 2008 in US military recruits, between 2001 and 2008 in pediatric patients from the CHOP and between 2004 and 2006 for the collection from the UI.

Table 9. Overview of individual genome types detected at each location (M=military, C=Civilians).

Genome Type	Population	Location in US	Years
HAdV-3a	M, C	CA, PA	1997, 2002 and 2004
HAdV-3a2	M, C	GA, CA, MI, SC, PA, CT, FL, KT, UT, NY, TN, WI	2002-2008
HAdV-3a17	M, C	GA, CA, MO, SC, PA, NY	1997, 2001-2006
HAdV-3a*Bcl*I variant	M, C	SC, TX, NJ, PA, AZ, CO, CT, NY, TX	2002- 2008
HAdV-3a*Bst*EII variant	M, C	GA, CA, MI, MO, IN, UT, TX	2003-2005
HAdV-3a*Bcl*I2*Bst*EII variant	C	TX	2004
HAdV-3a*Bgl*II variant 1	C	PA	2005
HAdV-3a*Bgl*II variant 2	M, C	MI, SC, PA	2006-2008
HAdV-3a*Bgl*II variant 3	C	WA	2004, 2005
HAdV-3a*Bam*HI*Bgl*II variant	C	PA	2002
HAdV-3*Bam*HI variant	C	CO	2004
HAdV-7p	M	MCRD SD	1997
HAdV-7b	M, C	GA, MI, MO, PA, AZ	1997, 1999, 2004, 2005
HAdV-7d	M	MI	1997
HAdV-7d2	M, C	GA, MI, MO, SC, TX, PA, AZ, CT, IN, NY, WI	1997, 2002, 2005-2008
HAdV-7h	M, C	MI, CO	1997, 2005
HAdV-7*Bam*HI variant	C	TX	2004

Geographical distribution and temporal occurrence of HAdV-3 and HAdV-7 genome types associated with FRI outbreaks in military training facilities

In order to identify patterns of circulation, we investigated the geographical distribution and temporal occurrence of HAdV-3 and HAdV-7 genome types in association with FRI outbreaks in military recruit training centers (Fig. 12). HAdV-3a was detected in MCRD San Diego, CA, in August 1997. HAdV-3a17 was detected in Fort Jackson, SC, in July 1997, in Great Lakes, IL, between August and November 1997, in Fort Benning, GA, in August 2002 and in MCRD San Diego, CA, in October 2003. HAdV-3a2 was detected in Fort Benning, GA, Fort Leonard Wood, MO, MCRD San Diego, CA, and Fort Jackson, SC, between January 2004 and April 2008.
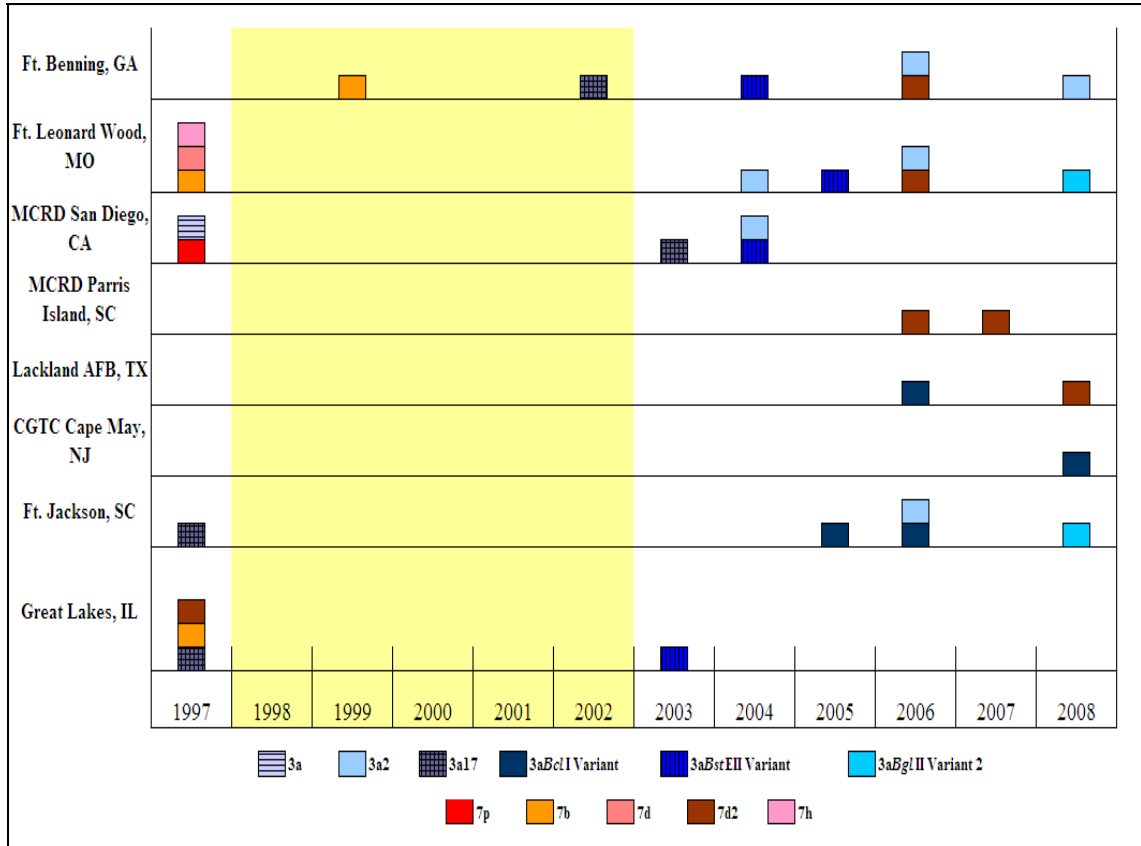
52

Figure 12. Geographical distribution and temporal occurrence of HAdV-3 and HAdV-7 genome types in US military training sites (1997-2008). Each square represents an cluster of cases of HAdV-associated FRI (size of outbreak is not identified). Area in yellow represents a period of almost complete HAdV-4 domination.

HAdV-3a*Bst*EII variant was detected in Great Lakes, IL, in 2003, in Fort Benning, GA, in 2004, in MCRD San Diego, CA, in 2004, and in Fort Leonard Wood, MO, in 2005. HAdV-3a*Bcl*I variant was detected in military recruits training at Fort Jackson, SC, in February 2005, Lackland Air Force Base, TX, in May 2006 and Cape May, NJ, between April and July 2008. HAdV-3a*Bgl*II variant 2 was detected in outbreaks at Fort Leonard Wood, MO, and Fort Jackson, SC, in 2008 (Fig. 12).

HAdV-7d2 was detected in 90 out of the 120 (75%) HAdV-7 isolates analyzed. In the collection of isolates from the US military, HAdV-7d2 was detected in outbreaks

in 1997 and in outbreaks between 2006 and 2008. In 1997, HAdV-7d2 was detected at the Great Lakes training facility in Illinois. In 2006, HAdV-7d2 was detected at three training sites: Fort Benning, GA, Fort Leonard Wood, MO, and MCRD Parris Island, SC. In 2007, HAdV-7d2 was detected in MCRD Parris Island, SC and in 2008 it was detected at Lackland AFB in Texas. HAdV-7b was detected in outbreaks in Fort Leonard Wood, MO, and Great Lakes, IL, in 1997 and in Fort Benning, GA, in 1999. HAdV-7d was detected infrequently in 1997 in Fort Leonard Wood, MO. HAdV-7p was only indentified in one isolate in April 1997 in MCRD San Diego, CA. HAdV-7h was only detected in one isolate at Fort Leonard Wood, MO, in August 1997.

Geographical distribution and temporal occurrence of HAdV-3 and HAdV-7 genome types isolated from civilians

The geographical distribution and temporal occurrence of HAdV-3 and HAdV-7 genome types isolated from civilian cases of disease were also investigated (Figs. 13 and 14). HAdV-3a was detected in the collection of isolates from the CHOP, PA, during 2002 and 2004. This genome type was also detected in isolates from the UI in September 2004 in Tennessee. HAdV-3a17 was detected in the collection from CHOP, PA, between January 2001 and October 2006 and in the isolates from the UI collection recovered from cases in August 2005 in Syracuse, New York. HAdV-3a2 was detected between May 2002 and June 2008 in the CHOP, PA, collection and was detected between November 2004 and June 2006 in the UI collection in Connecticut, Florida, Missouri (St. Louis and Kansas City), New York (Syracuse), Tennessee and Wisconsin (Figs. 13 and 14).
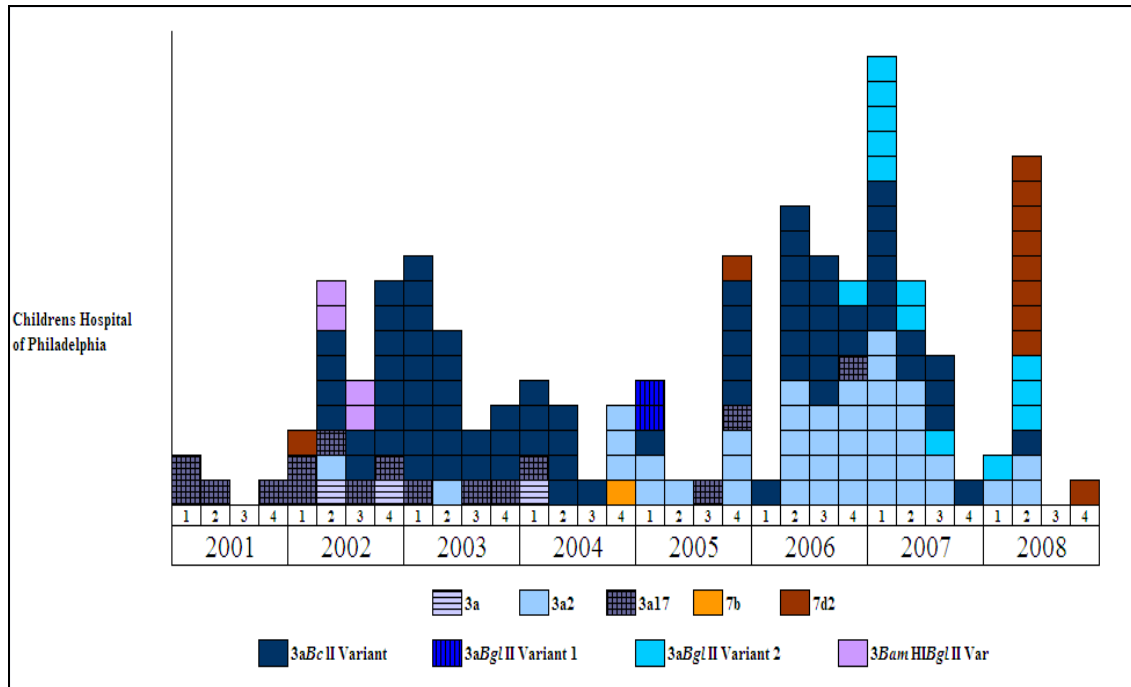
Figure 13. Temporal occurrence of HAdV-3 and HAdV-7 genome types identified in the pediatric isolates from the Children's Hospital of Philadelphia (2001-2008). Each square represents an individual case of infection. 1= Jan-Mar, 2= Apr-Jun, 3= Jul-Sep, 4= Oct-Dec.

HAdV-3a*Bcl*I variant was detected in the collection of isolates from the CHOP, PA, between May 2002 and May 2008 and between November 2004 and January 2006 in isolates from the UI recovered from cases in Arizona, Colorado, Connecticut, New York (Manhasset) and Texas (Figs. 13 and 14). HAdV-3a*Bst*EII variant was detected in three isolates between 2003 and 2005 in Indiana, Missouri and Texas (Fig. 14). Several *Bgl*II variants were identified in civilians. HAdV-3a*Bgl*II variant 1 was detected in two isolates from CHOP, PA, during January and March 2005 (Fig. 13). HAdV-3a*Bgl*II variant 2 was first identified in December, 2006 and was identified through the end of the study period in 2008 in the collection of the CHOP, PA (Fig. 13). HAdV-3a*Bgl*II variant 3 was detected in Washington during December 2004 and May 2005 (Fig. 14). HAdV-3a*Bcl*I2*Bst*EII variant was detected in Texas in April 2004 (Fig. 14). HAdV-3*Bam*HI

variant was detected in only one isolate from Colorado in 2004 (Fig. 14) and HAdV-3*Bam*HI*Bgl*II variant was detected in four cases in Pennsylvania during 2002 (Fig. 13).
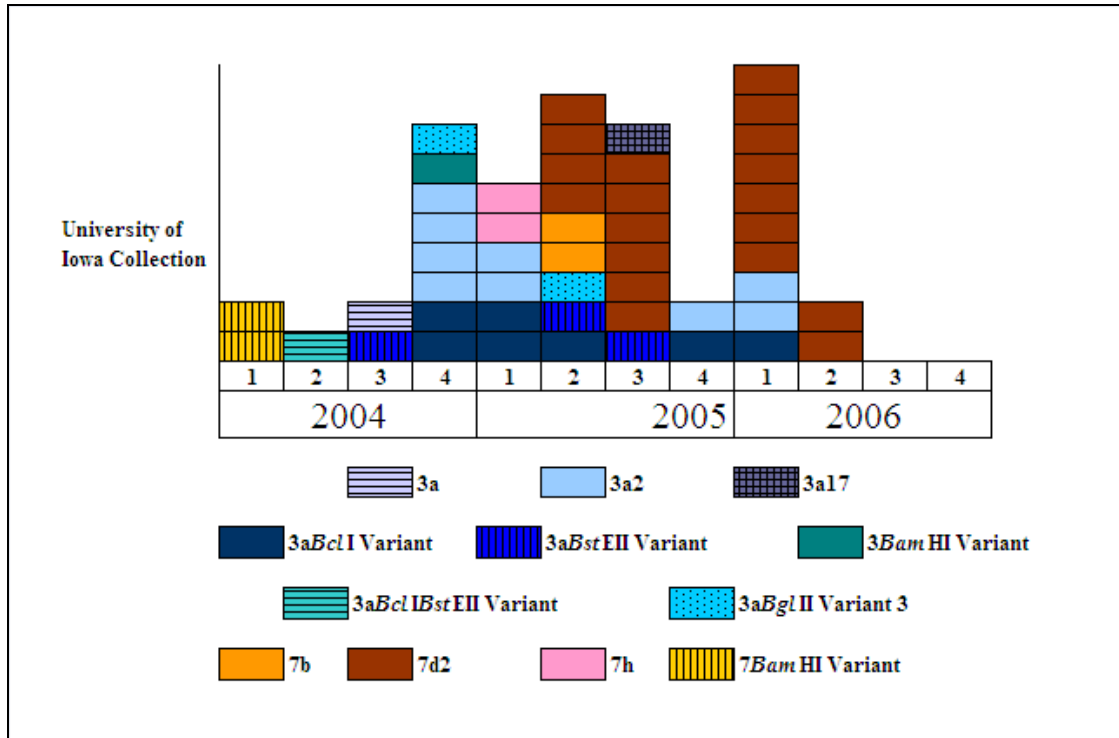

Figure 14. Temporal occurrence of HAdV-3 and HAdV-7 genome types identified in civilian isolates from the collection of the University of Iowa (2004-2006). Each square represents an individual case of infection. 1= Jan-Mar, 2= Apr-Jun, 3= Jul-Sep, 4= Oct-Dec.

HAdV-7d2 was detected in the majority of the HAdV-7 isolates from the CHOP and the UI collections, accounting for 31 of the 38 (81.6%) HAdV-7 isolates analyzed. HAdV-7d2 was detected in Indiana, Connecticut, New York (Syracuse) and Arizona in 2005 and 2006 (Fig. 14). In the collection of isolates from the CHOP, PA, HAdV-7d2 was detected in 2002, 2005, 2006 and 2008 (Fig. 13). HAdV-7b was detected in one isolate from the CHOP, PA, in October 2004 and the UI collection in Arizona in May 2005 (Figs. 13 and 14). HAdV-7h was detected in two isolates from 2005, one from

Colorado and one from Indiana (Fig. 14). HAdV-7*Bam*HI variant was detected in two isolates from the UI collection. Both isolates were from cases detected in Texas in March 2004 (Fig. 14).

PCR amplification and sequencing of the HVR1-7 of the hexon gene and the fiber gene of HAdV-3 and HAdV-7 genome types

In order to confirm the serotype of the isolates analyzed, identify intermediate variants and examine variation in the regions of the genes encoding the major neutralizing epitopes, the hexon and fiber genes were sequenced. The HVR1-7 of the hexon gene and the fiber gene sequences were determined and analyzed for all previously described genome types (HAdV-3a, HAdV-3a2, HAdV-3a17, HAdV-7p, HAdV-7b, HAdV-7d, HAdV-7d2 and HAdV-7h) and for the novel genome types detected in both the military and civilian isolates (HAdV-3a*Bcl*I variant, HAdV-3a*Bgl*II variant 2, HAdV-3a*Bst*EII variant). All sequences were analyzed using BLAST to confirm serotype. HAdV-7 strains were compared to HAdV-7a3, the vaccine strain, to identify differences with potential implications for vaccine efficacy.

The nucleotide and amino acid alignments of the HVRs 1, 2, 4, 5 and 6 of the hexon gene of HAdV-3 genome types showed no variation (Figs. 15 and 16). HVR-3 showed variation in one amino acid among the HAdV-3 genome types. At this location (aa position 199), HAdV-3a17 and HAdV-3a have valine substitution where as all other genome types had a glycine (Fig. 15).

Figure 15: Amino acid sequences for the HVR1-3 in the hexon gene of HAdV-3 and HAdV-7 genome types isolated in military recruits. HVRs1-3 are highlighted in green and those residues that are different from HAdV-7 vaccine strain are highlighted in yellow.

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HVR 4

HVR 5

HVR 6

Figure 16: Amino acid sequences for the HVR4-6 in the hexon gene of HAdV-3 and HAdV-7 genome types isolated in military recruits. HVR4-6 are highlighted in green and those residues that are different from HAdV-7 vaccine strain are highlighted in yellow.

The L2 portion of the hexon gene (aa positions 405-454), which includes the HVR-7 (aa positions 415-443), for each HAdV-3 genome type was compared to the prototype strain, HAdV-3p (Fig. 17). Among the HAdV-3 genome types, four amino acid changes were identified. Genome types HAdV-3a2, HAdV-3a17, HAdV-3a*Bgl*II variant 1, HAdV-3a*Bgl*II variant 2, HAdV-3a*Bcl*I2*Bst*EII variant and HAdV-3*Bam*HI*Bgl*II variant showed variation within HVR7. At position 417, these genome types encode a histidine instead of an asparagine. At position 429, they have an alanine instead of a threonine. At position 439 they have an aspartic acid instead of a threonine. At position 418, all genome types in the HAdV-3a cluster of homology have an arginine, while the prototype of HAdV-3 has a threonine. Predicted secondary structures using Chou-Fasman and Garnier-Robson methods in PROTEAN (Lasergene, DNAStar, Inc. Madison, WI) showed modified alpha, beta, turn and coil regions, as well as, modified antigenic indexes and surface probability plots in the genome types with these amino acid substitutions. These predicted alterations may affect protein folding and therefore have implications on epitope binding.

The nucleotide and amino acid alignments of the fibers of the HAdV-3 genome types identified in both the military and civilian populations showed little variation. The only variation identified at the amino acid level occurred in the isolates typed as HAdV-3a. The predicted fiber polypeptide of these isolates encoded an asparagine instead of aspartic acid at position 72, which is located in the shaft of the HAdV-3 fiber (Signas et al., 1985). Otherwise, all genome types in the HAdV-3a cluster of homology had identical fiber proteins.

Majority

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      390                 400                 410
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G H R Y   Majority

D S Y D P D V R I I E N H G V E D E L P N Y C F P L D G I G P G N K Y   Ad7 Vaccine Strain AY594256
D S Y D P D V R I I E N H G I E D E L P N Y C F P L N G I G P G H T Y   Ad3p GB AY599834
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G N R Y   Ad3a NHRC 1276 AY599836
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G N R Y   Ad3a UI
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G H R Y   Ad3aBglII variant 2 CHOP
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G H R Y   Ad3a2 NHRC
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G H R Y   Ad3a17 NHRC
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G N R Y   Ad3aBclI variant UI
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G N R Y   Ad3aBstEII variant NHRC
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G H R Y   Ad3aBclI2BstEII variant UI
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G H R Y   Ad3aBglII variant UI
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G N R Y   Ad3BamHI variant UI
D S Y D P D V R I I E N H G I E D E L P N Y C F P L D G I G P G H R Y   Ad3BamHIBglII variant CHOP
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Majority

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 420                 430                 440                 450
Q G I K V K T D D T N G W E K D A N V A T A N E I A I G N N L A M E I   Majority

Q G I K P R - - D T - A W E K D T K V S T A N E I A I G N N L A M E I   Ad7 Vaccine Strain AY594256
Q G I K V K T D D T N G W E K D A N V A P A N E I T I G N N L A M E I   Ad3p GB AY599834
Q G I K V K T D D T N G W E K D A N V A T A N E I A I G N N L A M E I   Ad3a NHRC 1276 AY599836
Q G I K V K T D D T N G W E K D A N V A T A N E I A I G N N L A M E I   Ad3a UI
Q G I K V K T D D A N G W E K D A N V D T A N E I A I G N N L A M E I   Ad3aBglII variant 2 CHOP
Q G I K V K T D D A N G W E K D A N V D T A N E I A I G N N L A M E I   Ad3a2 NHRC
Q G I K V K T D D A N G W E K D A N V D T A N E I A I G N N L A M E I   Ad3a17 NHRC
Q G I K V K T D D T N G W E K D A N V A T A N E I A I G N N L A M E I   Ad3aBclI variant UI
Q G I K V K T D D T N G W E K D A N V A T A N E I A I G N N L A M E I   Ad3aBstEII variant NHRC
Q G I K V K T D D A N G W E K D A N V D T A N E I A I G N N L A M E I   Ad3aBclI2BstEII variant UI
Q G I K V K T D D A N G W E K D A N V D T A N E I A I G N N L A M E I   Ad3aBglII variant UI
Q G I K V K T D D T N G W E K D A N V A T A N E I A I G N N L A M E I   Ad3BamHI variant UI
Q G I K V K T D D A N G W E K D A N V D T A N E I A I G N N L A M E I   Ad3BamHIBglII variant CHOP
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Figure 17. Amino acid sequences for the HVR7 in the hexon gene in HAdV-3 genome types from both military and civilian isolates. HVR7 region is highlighted in green and those residues that are different from HAdV-3a genome type are highlighted in yellow.

Figure 18. **Amino acid sequences for the HVR7 in the hexon gene in HAdV-7 genome types from both military and civilian isolates. HVR7 is highlighted in green and those residues that are different from HAdV-7 vaccine strain are highlighted in yellow.**

The nucleotide and amino acid alignments of HVR1-6 (Figs. 15 and 16)   and the
HVR-7 (aa 408-433) (Fig. 18) of the hexon of HAdV-7 genome types identified in
military recruits showed little variation compared to HAdV-7a3, the vaccine strain.
Except for HAdV-7p, all other genome types identified in military recruits, HAdV-7b,
HAdV-7d, HAdV-7d2 and HAdV-7h, showed no difference in HVR1-6 compared to the
vaccine strain of HAdV-7 (Figs. 15 and 16).   Amino acid alignments of HVR-7 sequence
data from isolates detected in both military and civilian populations were performed (Fig.
18). Again, the HAdV-7p isolate was the only isolate to show variation in this region.

The nucleotide and amino acid alignments of the fibers of the HAdV-7 genome
types identified in both the military and civilian populations showed some variation from
the HAdV-7a3 vaccine strain. Five amino acid substitutions were identified in the fiber
gene sequence data. Three of these were identified in the HAdV-7p genome type, one
was in HAdV-7d and HAdV-7d2 genome types in the shaft of the fiber, and one was in
HAdV-7d2 and HAdV-7b genome types in the fiber knob. The sequence data for the
HAdV-7h isolates showed a perfect alignment with HAdV-3 fiber sequence data. It has
previously been shown that HAdV-7h is a 7-3 recombinant with a HAdV-7-like hexon
and a HAdV-3-like fiber (Kajon & Wadell, 1996).

Chapter Five: Discussion

HAdV-3 and HAdV-7 prevalence in US military and civilian populations

Little epidemiological data on HAdV-3 and HAdV-7-associated morbidity worldwide are available. According to the World Health Organization, HAdV-3 infections accounted for 13% of all HAdV morbidity reported in the 1980's, while HAdV-7 infections accounted for 20% (Schmitz et al., 1983). Data from continuous surveillance of FRI among US military recruits between October 1996 and June 1998 identified HAdV-3 and HAdV-7 infections in 9% and 25% all HAdV positive cases of FRI, respectively (Gray et al., 2000). The Seoul National University Children's Hospital reported epidemics of HAdV-3 and HAdV-7 every one to two years since the late 1990's (Lee et al., 2005). A study of all HAdV-associated cases of lower respiratory tract infection between 1990 and 1998 in Korea reported that HAdV-3 and HAdV-7 were responsible for 15% and 41%, respectively, of total HAdV morbidity (Hong et al., 2001).

Although the HAdV-3 and HAdV-7 genome types are not responsible for the majority of the cases of HAdV infection in US military recruits or US civilians, they are important in both populations because their association with severe disease (Beby-Defaux et al., 2001; Kajon et al., 1990; Kim et al., 2003; Murtagh et al., 1993; Pingleton et al., 1978; Viquesnel et al., 1997; Yurlova et al., 1986). Our results from the characterization of the CHOP collection of isolates indicated that HAdV-3 was a more frequent causative agent of disease in children than HAdV-7. These data are in agreement with the article published by Gray and colleagues, in which they identified HAdV-3 more frequently than HAdV-7 in clinical specimens between 2004 and 2006. This is in contrast to what others

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have published on the prevalence of HAdV-3 and HAdV-7 in civilian populations where HAdV-7 was identified more frequently in the pediatric population (Schmitz et al., 1983; Fu, et al., 1989; Kajon et al., 1996).

It is important to understand the role other species of HAdV play in the etiology of ARD. Since the discovery of HAdV as the major causative agent of ARD in military recruits, HAdV-4 (Species E) has been the most frequently detected serotype (Van Der Veen et al., 1969; Barranza et al., 1999; Gray et a. 2000; Kolavic-Gray et al., 2002). There has been some suggestion that the pre-existing immunity of incoming recruits affects the ability of certain serotypes to spread within the population (Metzgar et al., 2007). Further studies to test this hypothesis are needed. Previously published data depict the role that species C adenoviruses play in HAdV-associated respiratory disease in the pediatric population in the United States (Fox et al., 1977; Hall et al., 1971). Our data confirm that species C adenoviruses are the most prevalent HAdVs infecting children (Fig. 7). Species E and species C genome types could potentially affect the circulation of HAdV-3, HAdV-7 and their corresponding variants.

Genome localization of variable restriction sites

Determining the location of variation within the genome using REA identifies genes, that when altered, could possibly have an effect on the fitness or virulence of the virus. Our study identified mutations across the entire genome with no specific clustering to any particular coding region. Figures 19 and 20 show the regions of the genome where genetic variability was identified for each of the HAdV-3 genome types. Mutations map to the DNA polymerase, hexon, fiber, and several genes for which protein function has

yet to be determined. Restriction patterns for HAdV-3a, HAdV-3a2, HAdV-3a17 and several of the newly circulating variants of HAdV-3, have a number of different restriction fragments in comparison to the prototype strain of HAdV-3 (Table 4). This illustrates the high degree of genetic variability between the prototype-like strains of HAdV-3 circulating in the 1950's and the current circulating strains of HAdV-3.

All HAdV-3 isolates identified in this study belong to the same cluster of homology that includes genome types HAdV-3a, HAdV-3c, HAdV-3d and HAdV-3-7 and their corresponding variants (Li & Wadell, 1988). HAdV-3a, HAdV-3a2 and HAdV-3a17 are closely related genome types as revealed by the high percentage of co-migrating fragments when examined by REA. The location of lost or new restriction sites in HAdV-3a2 and HAdV-3a17 genomes is illustrated in comparison to the HAdV-3a genome in Figure 17. HAdV-3a2 differs from HAdV-3a only when digested with *Sma*I. The distinguishing restriction site maps to the coding region for the DNA polymerase (Fig. 19). HAdV-3a17 showed variation with both *Bgl*I and *Bgl*II. The lost *Bgl*I restriction site in the HAdV-3a17 genome mapped to the ORF for the protein VI precursor. Protein VI is a minor capsid protein that helps disrupt the endosomal membrane during entry (Wiethoff et al., 2005). The loss of a *Bgl*II restriction site in HAdV-3a17 genomes mapped to the coding region of the fiber protein (Fig. 19).

Few differences were identified among HAdV-7 genome types. HAdV-7b and HAdV-7d only differ when digested with one (*Bam*HI) of the twelve original restriction endonucleases Li and Wadell used to distinguish HAdV-7 genome types (Li & Wadell, 1986). The only difference between HAdV-7b and HAdV-7d is one restriction site

located in a region that encodes the protein IIIa precursor. Protein IIIa is a minor capsid

protein that binds to the penton base of the fiber (Vellinga et al., 2005).

Figure 19. Linear map of HAdV-3a genome (Genbank accession number AY599836), generated with SeqBuilder (Lasergene 1.0; DNAStar) showing the coding regions and genes (displayed with red arrows) as well as the mutational changes for HAdV-3a2 and HAdV-3a17 genome types.

There is also little difference between genome types HAdV-7d and HAdV-7d2. These genome types differ from one another at a single *Bst*EII restriction site mapping to the E3 region. This restriction site maps to the ORF encoding the 14.9 KDa protein, which is part of the RID complex. This complex down-regulates the apoptosis receptor CD95 from the cell surface of HAdV infected cells, causing the cells to remain viable for viral replication (Shisler et al., 1997).

In figure 20 a linear map of the adenovirus genome shows the location of the changes for all nine HAdV-3 novel genome types. HAdV-3a*Bst*EII variant was identical to HAdV-3a2 when digested with all restriction endonucleases except for *Bst*EII (Fig. 8, *Bst*EII profiles I and II). An additional restriction site is located at the 3' end of the HAdV-3a*Bst*EII variant genome. The area of variation maps to the E4-encoded 13.9 KDa protein. This protein shares similar functions with other E4 proteins, including modulation of cell functions such as transcription, the cell-cycle, cell-signaling and DNA repair, and has been shown to augment viral DNA replication, late viral protein synthesis, shut-off of host protein synthesis and production of virus progeny (Tauber & Dobner, 2001; Weitzman, 2005).

HAdV-3a*Bcl*I variant shared identical restriction profiles with HAdV-3a2 with all restriction endonucleases except *Bcl*I (Fig. 8, profile II). The lost *Bcl*I restriction site is located in the open reading frame (ORF) for the 55 kDa protein in the L1 region. This protein has been shown to function in the encapsidation of DNA (Gustin & Imperiale, 1998). HAdV-3a*Bgl*II variant 2 differs from HAdV-3a2 when digested with *Bgl*II. *Bgl*II has proven to be a powerful restriction endonuclease to discriminate HAdV-3 genomic variants in this study. Of the eleven HAdV-3 genome types identified in isolates

collected from both the military and civilian populations, six showed variation with *Bgl*II, most of which mapped to the 3' end of the genome (Table 4). HAdV-3a*Bgl*II variant 2 is missing a *Bgl*II restriction site that maps to the ORF 16 KDa in the E3 region. Although the function encoded in ORF 16 KDa has not been elucidated, the protein is only encoded by species B adenoviruses (Burgert & Blusch, 2000).

The rest of the novel genome types identified were detected only in isolates from the CHOP and the UI. There were three more genome types identified with novel *Bgl*II profiles. HAdV3a-*Bgl*II variant 1 is identical to the genome type HAdV-3a2 in all restriction endonucleases except for *Bgl*II (Fig. 8, profile III), the distinct site maps to the E3 promoter. The second genome type with a novel *Bgl*II profile is the HAdV-3a*Bgl*II variant 3, which is identical to HAdV-3a2 with all other restriction endonucleases (Fig. 8, profile VI). This genome type has profiles identical to HAdV-3a2 in all other restriction endonucleases as well. However, it has a *Bgl*II profile like a HAdV-7h (Fig. 8, profile VI and Fig. 9, profile III). The last genome type to show variation with *Bgl*II is HAdV-3*Bam*HI*Bgl*II variant. This variant has the unique *Bam*HI profile (Fig. 8, profile II), as well as a novel *Bgl*II profile (Fig. 8, profile V). The lost *Bgl*II restriction site in this variant is located in the coding region for the 100 KDa hexon-assembly associated protein.

There were three genome types identified with a novel *Bam*HI profile (HAdV-3*Bam*HI variant, HAdV-3*Bam*HI*Bgl*II variant and HAdV-7*Bam*HI variant). The four restriction sites for *Bam*HI, located along the first 3,574 bp on the 5' end of the genome, are identical to HAdV-7b and HAdV7d or HAdV-7d2 (Table 7). The remaining

restriction sites are identical to the HAdV-3a *Bam*HI sites, which begin at nucleotide

position 13,005 and continue to the 3' end of the genome (Table 4).
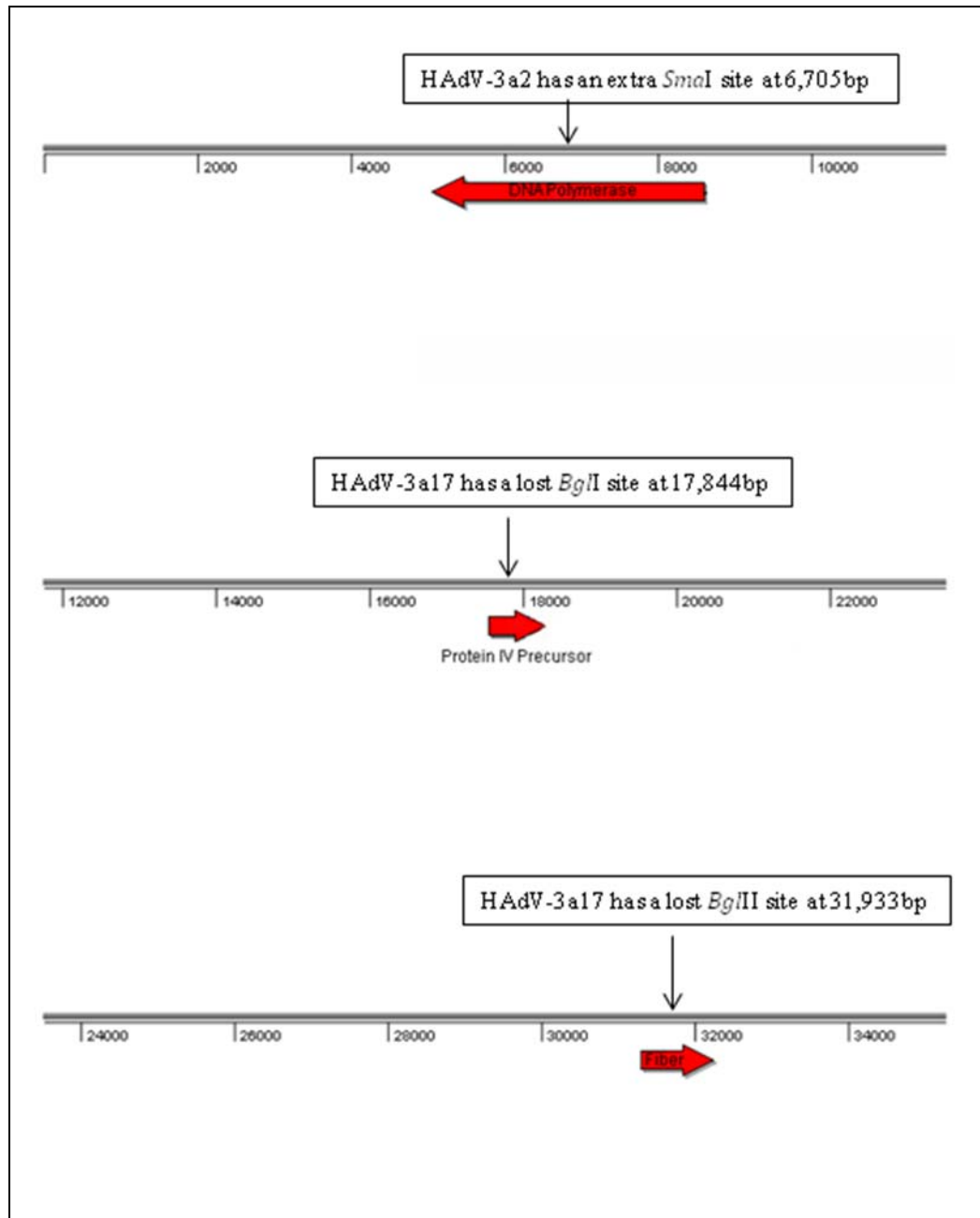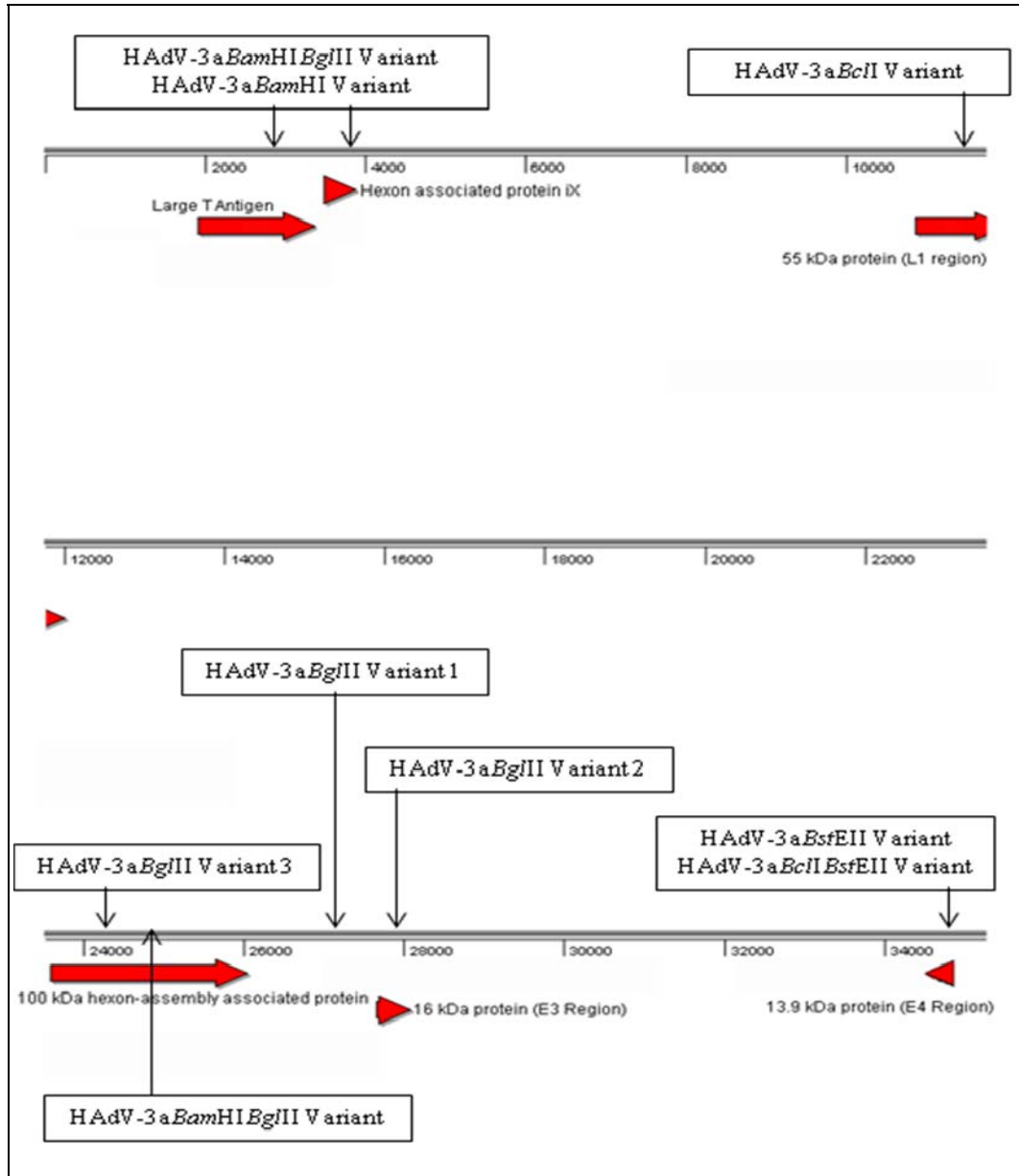

Figure 20. **Linear map of HAdV-3a genome (Genbank accession number AY599836), made with SeqBuilder (Lasergene 1.0; DNAStar) detailing the coding regions and genes (displayed with red arrows) as well as the sites where the mutational changes were identified in HAdV-3a variant genomes.**

Another novel genome type detected in the civilian population was HAdV-3a*Bcl*I2*Bst*EII variant. It shares the same novel profile for *Bst*EII as HAdV-3aBstEII variant. However, along with the novel profile for *Bst*EII, it also has a novel profile for *Bcl*I (Fig. 8, profile III). This novel genome type has an additional *Bcl*I restriction site at the 3' end of the genome (Table 4). The exact location could not be determined, but based on the size of the fragments generated from this extra site, the mutational change is likely to map to either the end of the E3 region or the E4 region of the genome.

Geographical distribution of HAdV-3 and HAdV-7 genome types

HAdV-3 genome types vary in their geographic area of circulation (Li & Wadell, 1988). While HAdV-3a and HAdV-3p have been detected worldwide, including the United States, Canada, Japan, Holland, Australia, China and South Africa, other genome types of HAdV-3 are restricited to specific geographic areas (Li & Wadell, 1988; Kajon et al 1990; Guo et al., 1988; Itoh et al., 1999). Although genome typing data is limited, HAdV-3a and the corresponding variants are the only genome types of HAdV-3 identified recently in the United States. A prototype-like strain of HAdV-3 was detected in the United States in 1959; however, the circulation of prototype-like strains has not been documented since (Li & Wadell, 1988).

HAdV-3a2 was first detected in 1962 in Beijing and became the most prevalent genome type of HAdV-3 in China during the 1980s (Zheng et al., 1994). HAdV-3a2 was responsible for an outbreak of respiratory illness in Taiwan in 2004 and 2005 and in Illinois at a pediatric long-term care facility in 2005 (James et al., 2007; Chang et al., 2008). Our data show the widespread circulation of HAdV-3a2 in both military recruits and civilians.

Other previously described HAdV-3a variants detected in our study include HAdV-3a and HAdV-3a17. HAdV-3a was first detected in the United States in 1977, but also circulated in Canada, Japan and Holland in the late 1970s and early 1980s (Li & Wadell, 1988). HAdV-3a17 was first described in Seoul, Korea, in children between November 1998 and February 1999 (Kim et al., 2003). The detection of HAdV-3a and HAdV-3a17 in all three collections of isolates shows that the geographical distribution across the United States is not restricted to a specific population or location.

Most of the novel genome types detected in the civilian populations were less frequently identified as causative agents of HAdV-associated respiratory disease and showed a very restricted geographical distribution. However, the three novel genome types that circulated in both the military and civilian populations were identified more frequently during the study period and had a wider geographical distribution across the United States.

A diversity of HAdV-7 genome types have been detected worldwide (Li & Wadell, 1986; Kajon et al., 1996; Azar et al., 1998; Noda et al., 2002; Ryan et al., 2002; Kim et al., 2003). In community outbreaks of HAdV-associated respiratory disease over the past 20 years, the most prevalent genome types have been HAdV-7b in the United States and Taiwan (Calder et al., 2004; Erdman et al., 2002; Lin et al., 2004), HAdV-7b1 in China (Li et al., 1996), HAdV-7d in Korea (Kim et al., 2003; Choi et al., 2006), HAdV-7d2 in Israel, the United States and Asia (Azar et al., 1998; Gerber et al., 2001; Noda et al., 2002; Ikeda et al., 2003; Blasiole et al., 2004) and HAdV-7h in South America (Kajon & Wadell, 1994; Carballal et al., 2002; Moura et al., 2007).

Although HAdV-7b and HAdV-7d2 have been detected most frequently in the United States recently, HAdV-7p, HAdV-7h and HAdV-7a have also been detected (Erdman et al., 2002). Our data show only a single isolate of HAdV-7p in the military. HAdV-7p was first isolated in the United States in 1954 (Berge et al., 1955; Li & Wadell, 1986), and although it dominated in the United States during the 1950s, by the late 1960s and early 1970s it was apparently replaced by HAdV-7b. Since then, HAdV-7p has only been detected sporadically worldwide (Wadell et al., 1985; Adrian et al., 1989; Adrian et al., 1989; Erdman et al., 2002).

Like HAdV-7p, HAdV-7h was detected infrequently during the study period. HAdV-7h was first identified in the 1980's in South America where it replaced the previously dominant variant, HAdV-7c, in 1986 (Kajon & Wadell, 1994). Since then, HAdV-7h has primarily circulated in South America. However, cases of respiratory infection in association with HAdV-7h have been reported in the US, South America and Japan (Moraes et al., 1997; Erdman et al., 2002; Ikeda et al., 2003). In our study, HAdV-7h was identified in both the military and civilian populations. However, it was only detected in one outbreak in US military recruits and in two epidemiologically unrelated cases of infection in the collection of isolates from the UI. Why is this genome type so prevalent in South America, but not in the United States? It has been shown that certain adenovirus genome types occupy certain geographical niches (Wadell et al., 1981; de Silva et al., 1989; Kajon & Wadell, 1994). HAdV-7h appears to dominate South America, while HAdV-7d2 is dominating the United States.

Our data also document the first cases of HAdV-7d infection in the United States. HAdV-7d was first detected in China, in isolates from 1981 (Wadell et al., 1985). Since

then, HAdV-7d has become extremely prevalent in Japan, China and Korea (Fu et al., 1989; Zheng et al., 1994). However, this genome type has been fairly restricted to Asia. Our data show that HAdV-7p, HAdV-7h and HAdV-7d have restricted geographical distribution in the United States throughout our surveillance period.

HAdV-7b and HAdV-7d2 have been more frequently associated with HAdV respiratory disease in the United States (Gerber et al. 2001; Erdman et al., 2002; Calder et al. 2004). Our data confirm this role. HAdV-7b was first detected in an HAdV-7 epidemic in Paris during 1956 (Chany et al., 1958; Li & Wadell, 1986), but by the 1980's was the dominant genome type in the United States, Europe, Brazil and Australia (Wadell et al., 1985; Wadell et al., 1980; Moraes et al., 1997). In the United States, HAdV-7b continued to cause ARD through the 1980's and 1990's (Erdman et al., 2002). The reemergence of respiratory illness in association with HAdV-7b was reported on in Taiwan between 1999 and 2001 (Lin et al., 2004).

HAdV-7d2 was first identified in Israel in 1992 (Azar et al., 1998) and in the United States in Maryland and New York in 1993 (Erdman et al., 2002). Since its first detection in the United States, HAdV-7d2 has been detected in numerous outbreaks involving both civilians and military recruits nationwide (Erdman et al., 2002; Gerber et al., 2001; Gray et al., 2005), and data from our study show HAdV-7d2 most frequently associated with respiratory infections in both populations.

Temporal occurrence of HAdV-3 and HAdV-7 genome types

The surveillance period (1997-2008) has been an interesting time to study the epidemiology of adenovirus in military recruits. When administration of the vaccine

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ceased in 1996, an intervention was removed, and respiratory illness caused by adenovirus increased dramatically (Russell et al., 2006). The incidence of HAdV-associated FRI doubled, and between 1999 and 2005, HAdV-4 reemerged as the dominant serotype (Russell et al., 2006). The discontinuation of vaccine protocol provided a unique opportunity to study HAdV-3 and HAdV-7. It has also been interesting to study HAdV-3 and HAdV-7 genome types in the civilian population. Epidemiological data on US civilian populations are scarce and limited to outbreaks or severe infection, and a study including detailed molecular characterization of isolates like this one has never been performed.

The analysis of the temporal occurrences of HAdV-3 genome types identified unique patterns of circulation. A shift was observed in both military and the pediatric population from the CHOP, from HAdV-3a and HAdV-3a17 to HAdV-3a2 and other novel HAdV-3a variants. Novel genome types of HAdV-3 were detected throughout the study period in civilian populations (2001-2008), but were only detected after 2003 in the military population. HAdV-3a*Bcl*I variant was detected in three outbreaks in military recruits and identified in 43% of the HAdV-3 isolates in the pediatric patients from the CHOP. It was the only novel genome type detected in all three collections of isolates. HAdV-3a*Bgl*II variant 2 and HAdV-3a*Bst*EII variant emerged after 2004 in both populations. Although they were more frequently detected in association with ARD in military recruits and civilians than the rest of the novel genome types, they were not identified in all three collections.

A shift in prevalent genome types was also identified for HAdV-7. In the military the only genome type to emerge after the period of HAdV-4 domination (1999-2005) was

HAdV-7d2. All the other genome types of HAdV-7 detected in US military recruits circulated before 2000. Data from our study suggest that HAdV-7d2 is replacing the other HAdV-7 genome types as the most prevalent, which is consistent with previously published data (Erdman et al., 2002). Although HAdV-7 was detected less frequently in the collection of isolates from the CHOP, the number of HAdV-7d2 isolates detected since 2006, also suggests a similar shift in the pediatric population.

Genetic variability of the HVR1-7 of the hexon gene and the fiber gene

REA is an extremely powerful tool for epidemiologists to perform molecular typing of HAdV. However, it must be used in combination with sequence data generated for the hexon and fiber genes. While sequence data lacks the ability to analyze the antigenetic properties of HAdV, sequencing PCR amplicons can predict changes in antigenecity. Today, determining the serotype is still the primary designation for HAdV strains. Therefore, PCR amplification and sequencing of the hexon and fiber genes are crucial in identifying HAdV genome types.

Sequence analysis for the hexon and fiber genes of the identified HAdV-3 and HAdV-7 genome types detected little intraserotypic variation. No differences were found between military and civilian isolates corresponding to the same genome type. One interesting finding from sequence data for the HVR7 was the identification of two different serotypes from isolates with the same *Bam*HI profile (HAdV-3*Bam*HI variant, HAdV-3*Bam*HI*Bgl*II variant and HAdV-7*Bam*HI variant). This illustrates the need for REA to be performed in combination with neutralization assays or PCR and sequencing to determine the serotype of a genome type.

With clinical trials underway to reinstate the HAdV vaccine in military recruits, studying the genomic similarities and differences of currently circulating HAdV-7 strains in comparison to the HAdV-7 vaccine strain is critical to evaluate whether the vaccine will still induce protective antibodies against the newly emerged genome types. A large study was conducted in 1998 on strains circulating between 1963 and 1997 to assess the magnitude of intraserotypic genetic variability between these HAdV-7 strains and the HAdV-7a3 strain used in the vaccine, which circulated in the 1950's and 1960's (Crawford-Miksza et al., 1999). The authors identified all isolates collected between 1963 and 1997 as the same genome type as the HAdV-7 vaccine strain, distinct from that of the prototype, based on sequence data from the hexon gene. Although the genome types identified in our study are not HAdV-7a-like, they are in this same cluster of homology as the current HAdV-7 vaccine strain.

Antigenic differences were not determined by neutralization tests in our study, but sequence data for the hexon and fiber genes of HAdV-7 genome types reveal little variation between the vaccine strain and the current circulating genome types. This suggests that the vaccine strain of HAdV-7 may confer protection against the current circulating genome types of HAdV-7 (Crawford-Miksza et al., 1999).

HAdV vaccines

HAdV-associated ARD is vaccine preventable. Data from previously published studies show the need for the HAdV vaccine to be reinstated into the military recruit population (Gray et al., 1999; Kajon et a. 2007; Russell et al., 2006). Our study has shown that many of the same genome types that play a role in the etiology of disease in

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the military population also circulate in the civilian population. Although clinical data were not assessed in this study, we can hypothesize that similar associated disease will be identified in the civilian population. We have also identified the circulation of several new genome types whose impact on vaccine efficacy needs to be evaluated once the vaccine is reinstated.

When the military vaccination studies were conducted during the 1950s and 1960s, HAdV-4 and HAdV-7 were recognized as the most common causative agents of respiratory disease in military recruits (Berge et al., 1955; Dudding et al., 1973; Pierce et al., 1965). Although HAdV-3 was not included in the vaccine formulation, some antigenic similarity between HAdV-3 and HAdV-7 has been suggested (Moraes et al., 1998). It is possible that the heterotypic antibody responses elicited by the vaccine could potentially offer cross-protection against HAdV-3. Little surveillance HAdV-associated respiratory disease was conducted in the US military while the vaccine was in place. Our data show that after the discontinuation of the vaccine in 1996, HAdV-3a genome types were already present, and suggest that monitoring after the vaccine is reinstated will be necessary in order to understand whether vaccination confers cross-protection. It is possible that HAdV-3 genome types have circulated during vaccine administration; however, their frequency in association with ARD was minimal and therefore ignored.

Since the increase in the burden of HAdV-associated ARD in the military due to the loss of the vaccine, the Department of Defense has been working to reinstate the vaccine (Lyons et al., 2008). However, this vaccine that will soon be reinstated has an identical formulation to the one released in 1971, which contained a HAdV-4p1 strain (Li and Wadell, 1988) and a HAdV-7a3 (Li and Wadell, 1986) strain that circulated in the

1950s and 1960s. It is important to stress the need for surveillance to continue once the vaccine is reinstated. ARD associated with HAdV-4 and HAdV-7 is expected to diminish with vaccination. However, the impact the vaccination will have on infections by other serotypes, such as HAdV-3, HAdV-14 and HAdV-21, remains unclear. Previous studies reported that after administration of the HAdV vaccine in 1971, no other respiratory pathogens emerged to replace HAdV-4 and HAdV-7 as major causative agents of ARD (Dudding et al., 1972). If surveillance and genome typing continue, we will be able to determine if this remains true.

The HAdV vaccine has never been licensed for use in the civilian population. The most prevalent circulating serotypes detected in the pediatric population belong to species C; however, species B serotypes represented almost 25% of the isolates included in the collection from the CHOP (Fig. 7). Infections by species B serotypes historically have resulted in severe clinical presentations requiring longer hospitalizations, and occasionally in pulmonary sequelae (Kajon et al., 1990; Pingleton et al., 1978; Yurlova et al., 1986; Lang et al., 1969; Becroft, 1971; Beby-Defaux et al., 2001; Murtagh et al., 1993; Viquesnel et al., 1997). Should a vaccine be considered for use in the civilian population? The current vaccine formulation would most likely be inadequate because it does not target the serotypes most frequently associated with pediatric ARD in the United States.

Further directions and limitations

With the completion of this study came the identification of future experiments to perform. There are also limitations in the field of molecular typing of adenoviruses that

should be addressed.   It has been suggested that different genome types of a given serotype may cause disease of variable severity (Kajon et al., 1996; Wadell et al., 1980). Although host determinants of susceptibility cannot be examined easily, the analysis of disease associated with specific HAdV genome types could yield valuable information to identify more virulent candidate genome types. A study should be conducted on the pediatric samples collected from the CHOP, identifying clinical presentation and/or disease associated with the specific genome types of HAdV-3 or HAdV-7, as well as the age distribution, sex and identification of pre-existing immunocompromising conditions for the patients. This could identify whether infections caused by different genome types of HAdV-3 and HAdV-7 result in disease of variable severity and whether certain genome types could be tentatively recognized as more virulent.

Since the HAdV vaccine is close to reinstatement in US military recruit populations, it would be interesting to investigate whether the vaccine could induce protective immunity against HAdV-3 genome types. There has been some evidence to suggest that anti-HAdV-7 serum reacts weakly with the prototype of HAdV-3 (Noda et al., 2002). It would be interesting to conduct neutralization assays using antisera against the HAdV-7 and/or HAdV-4 vaccine strains against several of the HAdV-3 genome types detected in this study to determine if the vaccine offers cross protection against HAdV-3 genome types.

Another interesting area to explore would be to compare growth phenotypes of HAdV-7 and HAdV-4. HAdV-4 has been detected most frequently in military recruits since the loss of the vaccine (Gray et al., 2000; McNeill et al., 2000). Why did HAdV-7 not dominate like HAdV-4? Although incidence of HAdV-7-associated FRI did not

reach the extent of that of HAdV-4, HAdV-7 prevalence did increase after the vaccine was discontinued, and since 2005 there has been a reemergence of HAdV-7 in US military recruit training facilities (Metzgar et al., 2007). It would be interesting to compare growth curves and perform endpoint assays to assess virulence for the different genome types of HAdV-7 and HAdV-4 to help determine what makes HAdV-4 more commonly detected in US military recruits. We know that fewer recruits entering the training facilities have antibodies to HAdV-4 than to HAdV-7 (Russell et al., 2006). This could definitely have an effect on the susceptibility to infections by these serotypes.

In continuation of this epidemiological study, it would be interesting to perform serological studies to investigate why genome types occupy certain geographical niches? Determining the seroprevalence of anti-HAdV-3 and anti-HAdV-7 in a given population may help to identify why certain genome types circulate in that population. For example, if a given population has antibodies to HAdV-3 because of exposure to this serotype during childhood, that may explain why the population has a greater number of infections associated with HAdV-4 in adulthood. This is somewhat evident from experiments in US military recruits when the monotypic HAdV-4 vaccine that was first tested. Those who were given the vaccine for only HAdV-4 became ill due to infections caused by HAdV-7 (Buescher, 1967).

Further studies should be conducted to identify which of the HAdV-3 and HAdV-7 genome types are most fit. Viral fitness refers to the replicative adaptability of an organism to its environment (Flint et al., 2004). A growth curve assay, which measures the infectious virus yields over a given time course, could give insight as to which genome types replicate and disseminate faster in cell culture. It would also be

informative to identify which genome types produce the highest infectious yields. Inoculating cell monolayers with the same multiplicity of infection (MOI) and harvesting virus at the same time post-infection, can allow for identification of genome types that replicate and produce virus faster. It is also possible to identify infectious virus from environmental samples. HAdV-4 was commonly detected on pillows, lockers and rifles in military recruit training centers (Kajon et al., 2006). Analysis of HAdV-3 and HAdV-7 in environmental samples could also be informative. All of these studies could help us to determine which genome types of HAdV-3 and HAdV-7 are most fit.

The biggest problem to overcome in the field of molecular epidemiology of adenovirus infections is the lack of a unified system to type and designate novel genome types. A database with restriction profiles or sequence data files and a clear consensus in the field on the criteria to denominate novel genome types of adenovirus would be incredibly useful. Because two denomination systems were created (Adrian et al., 1985; Li and Wadell, 1986), there can be confusion in naming newly detected genome types. The lack of a reference database for restriction profiles makes searching for previously described genome types difficult. If there was a universal database that profiles could be uploaded to and stored in for anyone to have access to, there would be no need to search through years of literature to determine whether or not a candidate new genome type has been detected before. This database could also be used to upload sequencing data for either whole or partial genomes. A system such as this could be useful for the Center of Disease Control and other public health laboratories.

However, before a database such as this could even exist, there would have to be an agreement on which denomination system should be used, as well as other criteria for

describing new genome types. There is a lack of consensus on which restriction endonucleases should be used to identify new genomes by REA. Some researchers use several restriction endonucleases to identify a new variant, while others use only a few. A more clear definition of which endonucleases should be used in a typing panel when identifying genome types would help dramatically.

In summary, HAdV-3 and HAdV-7 genome types circulating in military and civilian populations in the United States have changed since the implementation and discontinuation of initial vaccine protocol in the 1970s. Although the circulating HAdV-7 genome types are closely related (same cluster of homology) to the vaccine strain and are therefore not expected to pose a major challenge to vaccine efficacy, the impact that HAdV-3 genome types will have on vaccine efficacy, once reinstated, is unknown. The prevalence of HAdV-3 in civilian populations raises concern on the implementation of the bivalent HAdV-4 and HAdV-7 vaccine which ignores HAdV-3. HAdV-3 genome types could emerge as the major causative agent of ARD after the new vaccine is in place in military recruits. Continued surveillance is necessary in both populations to determine the impact of vaccination on the dynamics of HAdV-associated ARD and further studies should be performed to evaluate the cost-effectiveness of a civilian vaccine to help reduce preventable HAdV-associated respiratory disease.

Appendix A: Acronyms

HAdV	Human Adenovirus
FRI	Febrile Respiratory Illness
ARD	Acute Respiratory Disease
NHRC	Naval Health Research Center
CHOP	Children's Hospital of Philadelphia
UI	University of Iowa
LRRI	Lovelace Respiratory Research Institute
HVR	Hypervariable Region
REA	Restriction Enzyme Analysis
DoD	Department of Defense
CGTC	Coast Guard Training Center
NRTC	Naval Recruit Training Center
MCRD	Marine Corps Recruit Depot

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