



Abrupt Emergence of Diverse of Species B Adenoviruses at US Military Recruit Training Centers

***D. Metzgar
M. Osuna
A. E. Kajon
A. W. Hawksworth
M. Irvine
K. L. Russell***



Naval Health Research Center

Report No. 07-12

. Approved for public release: distribution is unlimited.

***Naval Health Research Center
140 Sylvester Road
San Diego, California 92106***

Abrupt Emergence of Diverse Species B Adenoviruses at US Military Recruit Training Centers

David Metzgar,¹ Miguel Osuna,¹ Adriana E. Kajon,² Anthony W. Hawsworth,¹ Marina Irvine,¹ and Kevin L. Russell¹

¹Respiratory Disease Laboratory, Department of Defense Center for Deployment Health Research, Naval Health Research Center, San Diego, California; ²Infectious Disease Program, Lovelace Respiratory Research Institute, Albuquerque, New Mexico

(See the editorial commentary by Binn et al., on pages 1436–7.)

Background. Adenoviruses (Ads) cause continuous outbreaks of acute respiratory disease (ARD) in US military training facilities. In 1996, the loss of vaccines targeting the dominant recruit-associated serotypes precipitated the reemergence of Ads in these populations. Between 1999 and 2002, serotype 4 accounted for >95% of Ads isolated from recruits and for >50% of ARD cases in training facilities (15,000 cases/year).

Methods. Ads ($n = 1867$) collected between 2002 and 2006 from recruits with ARD at 8 military training facilities in the United States were serotyped by serum neutralization and polymerase chain reaction.

Results. The dominance of Ad4 continued through 2005, followed by a simultaneous emergence of diverse species B serotypes at the majority of sites. This included the subspecies B1 serotypes 3, 7, and 21 and the subspecies B2 serotype 14. Ad14 was the most prevalent species B serotype, appearing in high numbers at 3 sites and becoming dominant at 1.

Conclusions. Subspecies B2 Ads have rarely been associated with ARD, and only in Eurasia. This survey represents the first report of AdB2-associated ARD in the Western Hemisphere. The simultaneous emergence of several species B Ads suggests a common external source (the civilian population) and a decrease in preexisting immunity to species B Ads.

Adenoviruses (Ads) were discovered in 1953 [1]. The diverse human pathogens in this family are categorized by hemagglutination into species A–F. They are further characterized by serum neutralization, and there are 51 currently recognized serotypes (Ad1–51) [2–4]. Higher resolution is offered by whole-genome restriction enzyme analysis (genome typing) [5]. Ad genomes are stable and recombinations are rare, allowing inference of species and serotype from single-locus polymerase chain reaction (PCR) or sequence data [6–9].

Human Ads of species B (specifically, subspecies B1), C, and E are globally distributed pathogens responsible for outbreaks of respiratory disease and conjunctivitis [10, 11]. Species C Ads are ubiquitous and endemic and are primarily responsible for childhood illness. Healthy adults are more likely to suffer from respiratory disease caused by AdE and AdB1 [10], against which a larger proportion of adults lack protective antibodies [11, 12]. Subspecies B1 Ads, most commonly serotypes 3 and 7, cause recurring epidemics of febrile acute respiratory disease (ARD) in civilian populations, with symptoms ranging from influenza-like fever and discomfort to pneumonia and death [6, 10, 11]. These serotypes, along with the AdB1 serotype 21 and the AdE serotype 4, are also responsible for essentially continuous epidemics of ARD at military recruit training facilities worldwide [12–15]. Prevalence data from US military training facilities showed that 20% of all trainees had to be removed from training for a week or more for recovery from Ad-associated illness, 40% became ill, and 80% were infected [16]. Current data, collected since the loss of the Ad4 and Ad7 vaccines previously used by the US

Received 17 April 2007; accepted 11 May 2007; electronically published 31 October 2007.

Potential conflicts of interest: none reported.

Financial support: Department of Defense Global Emerging Infections Surveillance and Response System under research work unit 60701 (Naval Health Research Center report 07-12).

The views expressed in this work are those of the authors and do not reflect the official policy or position of the Department of the Navy, Department of the Army, the Department of Defense, or the US government.

Reprints or correspondence: Dr. David Metzgar, Naval Health Research Center, PO Box 85122, San Diego, CA 92186 (david.metzgar@med.navy.mil).

The Journal of Infectious Diseases 2007; 196:1465–73

This article is in the public domain, and no copyright is claimed.

0022-1899/2007/19610-0008

DOI: 10.1086/522970

military, reflect similar trends (6.4% removed from training, 25% ill, 98% of those initially seronegative seroconverted) [17]. US recruits suffer 22,000 cases of reported febrile ARD per year, 15,000 of which are associated with Ads [18]. Many more Ad infections go unreported in this population. Studies have shown that less than half of recruits with ARD seek medical care [17, 18].

Between 1971 and 1996, the United States used Ad4 and Ad7 vaccines to successfully control adenoviral illness in recruit populations, decreasing ARD by 50%–60% and decreasing Ad-associated ARD by >95% [11, 13, 18]. Other subspecies B1 Ads, including Ad3 and Ad21, appeared in vaccinated recruit populations, although they did not cause a return to prevaccination levels of ARD [14, 19, 20].

Subspecies B2 Ads are generally associated with sporadic diseases of the kidney and urinary tract [3]. The literature has, however, recorded occasional respiratory disease outbreaks associated with 2 AdB2 serotypes, Ad11 and Ad14 [21, 22].

The US military has conducted population-based ARD surveillance at 8 US training facilities since the loss of vaccine production in 1996, through the subsequent gradual reduction in vaccination between 1997 and 1999, and through the following period of no vaccination [13, 18, 20]. We present here data describing the serotype distributions at those facilities during the years 2002–2006. As part of ongoing population-based surveillance at these sites, 14,548 samples were collected and tested for Ad. The resulting numbers, paired with data on population size, were used to generate estimates of Ad-associated ARD rates. From the resulting set of cultured isolates, 1867 samples were randomly chosen and typed by either serotype-specific antibody neutralization (through March 2004) or serotype-specific PCR (beginning in April 2004; a PCR flow chart is shown in figure 1). The resulting serotype identifications were verified in a subset of cases by sequencing the primary antigenic determinant [8] and by genome typing [5].

To address the epidemiological consequences of serotype turnover events, rate data are presented for 1 site at which a particularly sharp serotype emergence occurred. Serotyping results are discussed in relation to both general Ad epidemiology and in relation to the current effort to reinstitute the Ad4 and Ad7 vaccines.

MATERIALS AND METHODS

ARD case definition and sample collection. Collaborators and Naval Health Research Center (NHRC) staff at each site monitor trainee populations for symptoms of febrile respiratory illness. A case of febrile respiratory illness is defined as occurring when a recruit presents for medical care and meets the following 2 criteria: fever with oral temperature $\geq 38^{\circ}\text{C}$ (100.5°F) and a respiratory symptom (cough or sore throat). Clinical specimens are obtained as pharyngeal swabs in viral transport medium (Remel) from a subset of recruits who seek medical care. These samples are then sent on dry ice to the NHRC to be tested for respiratory pathogens.

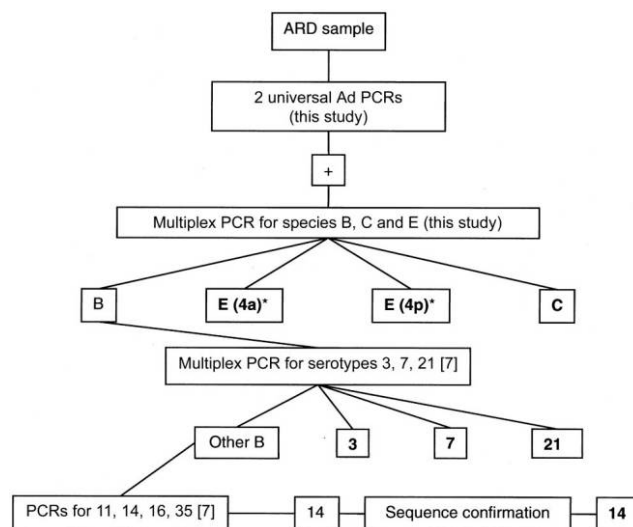


Figure 1. Flow chart representing the series of polymerase chain reactions (PCRs) used to identify adenovirus (Ad) strains in this study. The PCR indicated by the asterisks discriminates the highly divergent genome types Ad4a and Ad4p on the basis of an amplicon length polymorphism (see table 1). ARD, acute respiratory disease.

Origin of virus strains. Serotyping work was done on a collection of 1867 Ad strains isolated from the pharyngeal swabs of military trainees presenting with symptoms of ARD between 2002 and 2006 at 8 training sites in the United States: Fort Jackson, South Carolina; Fort Benning, Georgia; Fort Leonard Wood, Missouri (all Army); Naval Recruit Training Command, Great Lakes, Illinois; Lackland Air Force Base, San Antonio, Texas; Coast Guard Training Center, Cape May, New Jersey; Marine Corps Recruit Depot (MCRD) Parris Island, Parris Island, South Carolina; and MCRD San Diego, San Diego, California. Initial identifications were made in the College of American Pathologists–accredited NHRC diagnostic microbiology laboratory either by culture/immunofluorescence in A549 cell monolayers or by PCR. PCR identifications were followed by culture/immunofluorescence to verify the result and produce high-titer stocks for molecular serotype analysis.

Serotype determination. The serotype identifications presented in this study were done using a microneutralization assay [23], PCR, or both. PCR primers, as well as the source of the primers and references to previous validations in the hands of the original authors, are shown in table 1. PCR methods have been used as a primary means of serotype identification since April 2004, when type-specific serum became increasingly difficult to obtain [6]. These methods have also been retrospectively applied to thousands of archived samples collected before 2003, which were initially serotyped by traditional neutralization (by both ourselves [6, 20, and unpublished data] and others [7, 9]) to validate the ability of PCR to predict serotype. All such validations have shown an exceptionally high level of correlation between different methods.

Table 1. Primers.

Test, primer	Sequence (5'→3')	Target	Amplicon, bp	Source
CAP Ad				
AdUniv α F	CTGAACTAICAIAACGACCACAGC	Ad penton	165	This article ^a
AdUniv α R	ACTCGTTCACITTIGGCATGTTGG	Ad penton	165	This article ^a
AdUniv β F	CCTGCTCTCACAGATCACGG	Ad penton	122	This article ^a
AdUniv β R	GACTATGCCAGGGCCTTG	Ad penton	122	This article ^a
BCE multiplex				
Ad(E1A)B F	CGGAGCT(G/T)CCTGGACATG	AdB E1A	319	This article
Ad(E1A)B R	GCTTAGGCTTCACAGGAATG	AdB E1A	319	This article
Ad(E1A)C F	GGTGATCGATCTTACCTGC	AdC E1A	410	This article
Ad(E1A)C R	CTCAGGAGGTGTGTTAGAAGG	AdC E1A	410	This article
Ad(E1A)E F	CTGCACGATTTGTATGATCTGG	AdE E1A	222 (Ad4a)	This article
Ad(E1A)E R	CCTGCTCGTICTCATCATCG	AdE E1A	255 (Ad4p)	This article
Ad3/Ad7/Ad21 multiplex				
Ad3 F	GGTAGAGATGCTGTTGCAGGA	Ad3 hexon	502	[7]
Ad3 R	CCCATCCATTAGTGCATCGGT	Ad3 hexon	502	[7]
Ad7 F	GGAAAGACATTACTGCAGACA	Ad7 hexon	311	[7]
Ad7 R	AATTCAGGCGAAAAAGCGTCA	Ad7 hexon	311	[7]
Ad21 F	GAAATTACAGACGGCGAAGCC	Ad21 hexon	237	[7]
Ad21 R	AACCTGCTGGTTTTCGCGTTG	Ad21 hexon	237	[7]
Ad11				
Ad11 F	GAAGTTTCAGATGAAGAAAGTA	Ad11 hexon	293	[6]
Ad11 R	AGGACTTAAGTTTGTCTCTGC	Ad11 hexon	293	[6]
Ad14				
Ad14 F	AAATGCTAATCTTGGACAGCAGTC	Ad14 hexon	324	[6]
Ad14 R	AGCCGTCAGTGGAACAGTAGT	Ad14 hexon	324	[6]

^a These primers and the reported assays are diagnostic polymerase chain reactions accredited by the College of American Pathologists for use as in-house diagnostics at the Naval Health Research Center. Ad, adenovirus.

We performed a validation of PCR relative to serum neutralization by using PCR to reanalyze isolates previously serotyped by neutralization, including all serotypes that had been collected by the laboratory during the previous 5 years of recruit surveillance activity. Initially, PCR identifications of species B and E were made using the B/E portion of the A–F species multiplex [9], as described elsewhere [6]. Later, we developed the BCE multiplex (described below) in order to capture all respiratory species (B, C, and E) while simultaneously differentiating the 2 very distinct genome types of Ad4, 4a and 4p [24]. Both methods were validated using the same set of cultured clinical specimens, and both yielded identical results. Results are shown only for the BCE multiplex. A similar validation was performed for the published Ad3/Ad7/Ad21 serotype-specific multiplex [7] used in this study. The numbers of clinical isolates used in each validation, along with the resulting sensitivity and specificity measures, are shown in table 2.

The primers used to test for Ad11 and Ad14 [6] were developed in-house using all available sequence data and tested for sensitivity against appropriate control strains from the American Type Culture Collection. These serotypes had never been seen before in US recruit ARD surveillance; therefore, clinical samples previously identified as Ad11 or Ad14 were unavailable

for validation. Positive identifications made using these primers were verified by secondary analysis of a subset of samples, including 1 each from San Diego, Great Lakes, and Fort Benning, by sequencing hypervariable region (HVR) 7 of the hexon gene [8]. BLAST analysis [25] of these sequences by means of GenBank allowed sequence-based assignment of serotype. The analyzed gene includes the primary serotype determinants and, hence, offers the most direct opportunity for molecular inference of serotype [8].

All PCRs were subjected to specificity tests against control samples of a wide variety of respiratory pathogens, commensals, and environmentally common bacteria (40 bacteria and 50 viruses), including several serotypes of Ad. No cross-reactions were detected (data not shown). Specificity testing also included a large number of culture-negative patient specimens, as indicated in table 2.

Secondary verification of the serotype of all Ad3, Ad7, Ad14, and Ad21 isolates and >100 Ad4 isolates was performed at the Lovelace Respiratory Research Institute by traditional whole-genome restriction enzyme analysis with *Bam*HI (genome typing) [24].

PCR. PCRs were done as shown in the flow chart (figure 1). Both monoplex and multiplex PCRs were formulated as described elsewhere [6], under essentially standard conditions with the addition of the solvent betaine (Q-Solution; Qiagen).

Table 2. Performance of polymerase chain reaction (PCR)–based adenovirus (Ad) identification, species typing, and serotyping with respect to culture/immunofluorescence and serum neutralization.

Result	BCE multiplex			Ad3/Ad7/Ad21 multiplex			CAP Ad (universal), any type
	B	C	E	3	7	21	
Positive, no.	90	27	345	42	39	15	413
Negative, no.	408	471	150	84	81	114	174
Sensitivity, %	98.9	100	98.84	100	100	100	93.7
Specificity, %	100	100	98.04	100	100	100	96.5

NOTE. All comparisons were made using infected tissue culture fluid from A549 cells inoculated with clinical throat swab samples—that is, under conditions identical to those used to collect the data in this study. Positive and negative nos. reflect the no. of traditionally typed specimens used to evaluate the PCR tests. For the multiplexed typing tests (BCE and Ad3/Ad7/Ad21), negative samples were primarily those that were positive for the other targeted serotypes in the test. The universal Ad PCR is considered to be positive only when both universal primer sets yield positive results.

The only exception to the previously described conditions was for the BCE multiplex, which used 0.1 $\mu\text{mol/L}$ concentration of each primer instead of 0.2 $\mu\text{mol/L}$.

Cycling conditions included a 10-min final extension at 72°C and a final hold at 4°C. Regarding other parameters, reactions were cycled as shown in table 3.

All products were analyzed by standard agarose gel electrophoresis and ethidium bromide/UV visualization, as described elsewhere [6].

Development of a new PCR test for respiratory Ads. The novel BCE PCR test used in the present work was developed to target the relevant species (B, C, and E) by use of a gene (E1A) that is very well conserved within species but that is highly divergent between species while simultaneously discriminating between the 2 primary genome types of species E, Ad4a and Ad4p. Discrimination of Ad4a and Ad4p is based on a 30-bp insertion/deletion mutation in the E1A gene. This PCR was briefly described in an earlier work [24], in which it was used to confirm the results of another test.

Sequence analysis of hexon genes. Fragments of 599 bp spanning HVR7 of the hexon gene of Ad14 were amplified and sequenced using the primers HVR7f (5'-GTCTTATGTACTAT-AACAGTACTGG-3') and HVR7r (5'-GTGGTTGAATGGGTT-GAC-3'). Amplification reactions contained 1.5 mmol/L MgCl₂,

1 U of iProof *Taq* polymerase (Bio-Rad Laboratories), 200 $\mu\text{mol/L}$ dNTP, 1 $\times$ iProof high-fidelity buffer, and 1 $\mu\text{mol/L}$ each primer in a total volume of 50 μL . Cycling conditions consisted of denaturation for 30 s at 94°C followed by 50 cycles of 30 s at 94°C, 45 s at 51°C, and 45 s at 72°C. A final extension for 5 min at 72°C was added. Amplicons were purified with Montage PCR columns and Micropure-EZ columns (Millipore). Sequencing reactions used the same primers and standard conditions. Sequencing services were contracted from the DNA Research Services of the University of New Mexico Health Sciences Center.

Sequence data analysis was done using SeqMan software for contig assembly and MegAlign software for sequence alignments (Lasergene 7.0.0; DNASTar). Ad14 hexon sequences were deposited in GenBank under accession numbers EF503568, EF503569, and EF503570.

Ethics. This research has been conducted in compliance with all applicable federal and international regulations governing the protection of human subjects in research (Department of Defense protocol NHRC.2005.0017).

RESULTS

Serotype data are shown in figure 2. Ad4 (species E) was identified in >95% of recruit ARD samples collected from 2002

Table 3. Polymerase chain reaction (PCR) conditions.

PCR step	Cycles, no.	AdUniv α	AdUniv β	BCE	Ad3/Ad7/Ad21	Ad14	Ad11
Initial denaturation	1	5:00 (95)	5:00 (95)	15:00 (95)	15:00 (95)	5:00 (95)	5:00 (95)
Denaturation	35	1:00 (94)	1:00 (94)	0:30 (94)	0:30 (94)	1:00 (94) ^a	1:00 (94) ^a
Annealing	35	1:00 (57)	1:00 (56)	1:30 (58)	1:30 (56)	1:00 (63) ^a	1:00 (63) ^a
Extension	35	2:00 (72)	2:00 (72)	1:00 (72)	1:30 (72)	1:30 (72) ^a	1:30 (72) ^a
Final extension	1	10:00 (72)	10:00 (72)	7:00 (72)	10:00 (72)	10:00 (72)	10:00 (72)

NOTE. All conditions are shown as time (temperature), with the time shown as minutes:seconds and the temperature in Celsius. All reactions have a final cool down to 4°C.

^a Forty cycles instead of 35.

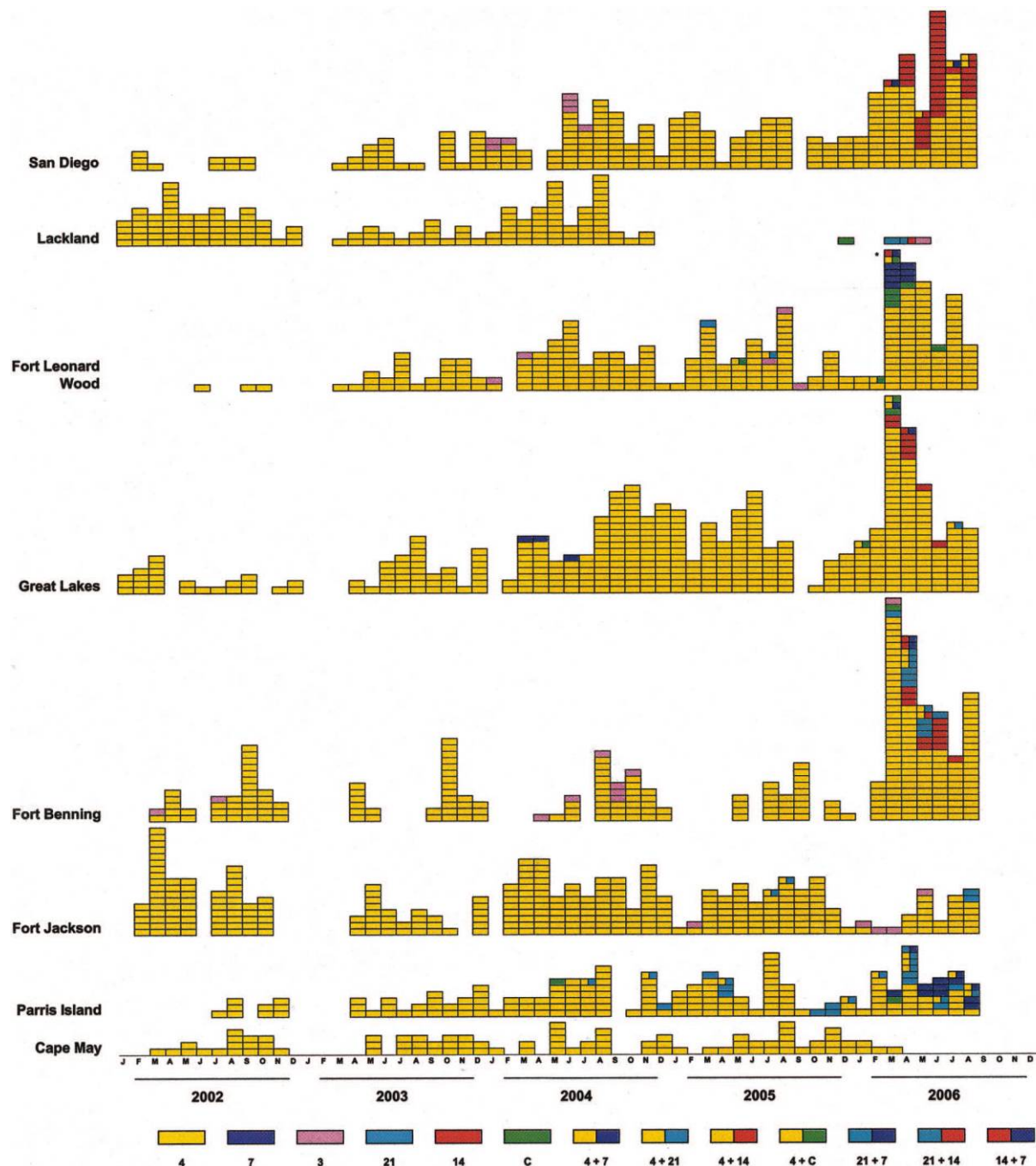


Figure 2. Serotype identification of 1867 adenovirus (Ad) isolates from military recruits at 8 US training centers, 2002–2006. Each block represents 1 sample from a recruit with febrile acute respiratory disease. Split blocks represent coinfections with 2 serotypes. The sample to the right of the asterisk was a triple infection with Ad4, Ad7, and Ad14. The decrease in sampling density at Lackland after 2004 resulted from a decrease in trainee density that essentially ended Ad outbreaks at that site. Otherwise, changes in sample volume are not representative of changes in disease rate but rather of changes in sampling effort. The relative proportions of different serotypes are representative of the relative frequency of isolation: Ad-positive samples were chosen randomly for serotyping. Results before April 2004 are from traditional serum neutralization; later results are from type-specific polymerase chain reaction.

through 2005, following the trend seen in the preceding surveillance period [20]. Ad3, Ad7, and Ad21 (all subspecies B1) were occasionally seen during this time period, as were 3 scattered cases of species C Ads. Ad3 cases were concentrated at San Diego and Fort

Benning in 2004. Ad7 cases were clustered at Great Lakes in 2004, and Ad21 was primarily identified at Parris Island in 2005.

In March and April of 2006, Ad14 (subspecies B2) simultaneously emerged at 5 training centers, including San Diego,

MCRD San Diego ARD rate status

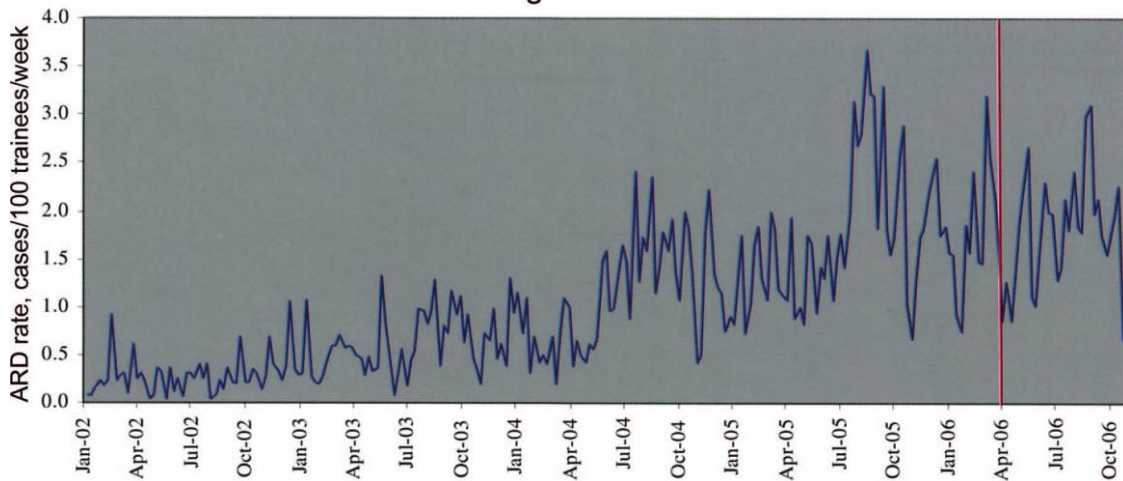


Figure 3. Acute respiratory disease (ARD) rate at the Marine Corps Recruit Depot (MCRD) San Diego, 2002–2006. The red line indicates the approximate time at which adenovirus (Ad) 14 emerged as a significant contributor to the Ad burden at this site. The figure suggests that the emergence of Ad14 did not cause a greatly increased level of ARD but rather that Ad14 partially replaced Ad4 as a causative agent, with little impact on the overall rate of ARD.

Lackland, Fort Leonard Wood, Great Lakes, and Fort Benning, thereby affecting all services except the Coast Guard (Cape May). At 3 of these centers, Ad14 first appeared in coinfections with AdB1 serotypes and in 1 case as part of a triple infection with Ad4 and Ad7. Ad14 maintained a consistent presence at Fort Benning, San Diego, and Great Lakes during summer 2006. At the same time, a variety of AdB1 serotypes emerged at all sites except Cape May. Ad3 cases were clustered at Fort Jackson, Ad7 at Fort Leonard Wood and Parris Island, and Ad21 at Parris Island and Fort Benning. All 6 identified serotypes were seen at Fort Benning during one 2-month period. Many coinfections were identified, the majority of which were coinfections with Ad4 and one of the species B serotypes.

All whole-genome *Bam*HI restriction patterns (genome types) were concordant with PCR-inferred serotypes (or subspecies type, in the case of many of the AdB serotypes—*Bam*HI patterns can readily distinguish B1 from B2 subspecies, but many serotype determinations require analysis with additional enzymes). Likewise, all identifications based on hexon sequencing were concordant with the original PCR-inferred identifications. ARD rates at MCRD San Diego, the site with the most complete turnover between Ad4 and Ad14 (i.e., between species E and subspecies B2), did not shift noticeably during the most distinct period of Ad14 dominance (figure 3). Note that the general increase in the isolation rate over time is not a function of increased disease burden but rather of increased sampling effort.

DISCUSSION

All AdB1 serotypes previously seen in recruit training centers in the United States (Ad3, Ad7, and Ad21) have simultaneously

reemerged against the background of Ad4 (figure 2). For the first time, an AdB2 serotype (Ad14) has emerged as a significant contributor to ARD in the Western Hemisphere. The simultaneous emergence of many serotypes has been broadly spread across many geographically isolated facilities, although Ad4 remains common at all sites. Population-based ARD rate data do not suggest that the emergence and subsequent dominance of Ad14 at San Diego in early 2006 had any effect on the overall rate of ARD (figure 3).

Soon after the original identification of Ads as agents of adult ARD in the 1950s [16], 4 scattered ARD outbreaks associated with the AdB2 serotype 14 were reported in military and civilian adult populations. Ad14 was initially discovered in an outbreak of ARD in Dutch recruits in 1955 [21]. Civilian outbreaks were reported in Great Britain in 1955 [26], in Uzbekistan in 1962 [27], and in Czechoslovakia in 1963 (the only sustained outbreak of Ad14 ever previously recorded) [28]. An apparent Ad11/Ad14 intermediate strain (denominated Ad14–11) was isolated after a large outbreak during a military training exercise in Spain in 1969 [29]. In general, AdB2 serotypes are more commonly associated with sporadic illness, most often kidney and urinary tract infections [3, 6].

Although both military and civilian surveys of Ad-associated ARD have commonly included assays capable of detecting and identifying Ad14 [30, 31], this serotype was not reported in large outbreaks of ARD between the 1960s and the turn of the century. Such surveys reported, for example, no Ad14 at all (0 of 1792 Ad isolates from children with respiratory illness in the United States) [30] or very little (<1% of AdB serotypes other than Ad3 or Ad7 among 3313 Ad isolates from children and adults with respiratory illness in the United Kingdom) [31].

A few recent studies yielded greater numbers of AdB2 identifications. A survey of adults and children with ARD in Egypt yielded a surprising proportion of Ad11 (4% of isolated Ads) [6], and recent studies in Taiwan yielded similar proportions of Ad14 (2%–11% of isolates) [32, 33]. Ad11 was recently identified in 2 German military troops returning from an exercise in Turkey [22]. The affected troops had severe ARD symptoms and were strictly quarantined while the agent was identified. The exercise itself was subject to a widespread outbreak of ARD, which may well have been associated with the identified strain of Ad11.

These data point to a recent emergence of AdB2 serotypes as agents of ARD in healthy adults and children in Eurasia. This is similar to the current emergence of AdB2 in US military recruits, as described here. However, given that the serotypes involved are diverse, it seems unlikely that the simultaneous emergence of AdB2 serotypes as respiratory pathogens in multiple continents is resulting from the spread of recently evolved and adaptively superior strains.

The temporal distribution of Ads causing ARD in recruits can be highly varied. Sometimes single serotypes will dominate in a specific region, being very sharply replaced by other serotypes at intervals of a few years (see van der Veen et al. [34], for example). Sometimes 2 or 3 types will share dominance in specific outbreaks; this is commonly seen with Ad3 and Ad7 (see Ryan et al. [15], for example). Before the institution of vaccination protocols, subspecies B1 (Ad3 and/or Ad7) and species E (Ad4) were often seen in significant numbers in the same year at the same site in US recruit facilities [35], and many vaccinated recruits with Ad-associated ARD were found to harbor species E and B serotypes simultaneously [36]. However, since the gradual withdrawal of vaccination measures in the United States between 1996 and 1999, Ad4 has dominated recruit training facilities. Ad4 has been associated with >98% of Ad-associated ARD cases from 1999 through 2005 ([20] and figure 2), and AdE/AdB coinfections have been rare ([36] and figure 2). The data presented here, which show many AdE/AdB coinfections associated with the general AdB reemergence in 2006, suggest that coinfections are the result of cocirculation and that neither coinfection nor cocirculation is directly related to vaccination status.

Coinfections are shown as split boxes in figure 2. Coinfections are important because they provide the opportunity for Ad strains to recombine and form new variants [6, 36]. Strains responsible for recent outbreak waves, including Ad7h and the genome type of Ad4 that has dominated most of the recruit training facilities for the last 10 years (Ad4a) [24], appear to be interserotypic recombinants [37, 38]. Coinfections with multiple Ads have only recently been recognized, owing to the advent of PCR typing [36]. Immunological methods such as neutralization are often done with low challenge doses (~ 100 TCID₅₀) using single antisera and, as such, are unlikely to identify mixtures of multiple serotypes.

Ad4 is ubiquitous in the recruits' physical environment, and transmission appears to be independent of regular import from outside sources to training centers [17]. During the past 10 years of high Ad4 prevalence, different genome types have predominated at different sites, with a pattern of circulation that suggests that Ad4 population dynamics are driven by internal, possibly environmental, reservoirs [24]. The data presented here suggest different dynamics for AdB serotypes. The emergence of Ad14, Ad21, and Ad7 at multiple sites, all in the course of 1 year and following a decade during which these types had little or no impact at all, suggests that these emergences are driven by importation from shared outside reservoirs. This reservoir is most likely the US civilian population, from which the affected trainees are recruited.

Dozens of publications from around the world during the last 40 years have documented the consistent circulation and clinical importance of Ad3, Ad7, and Ad21 in civilian populations. US recruit camps process tens of thousands of recruits from all parts of the United States every year. It would seem reasonable to assume that the influx of so many people from so many places would generate a constant influx of these common strains to the camps. It is therefore a mystery why all of the AdB1 serotypes that have ever been implicated in major ARD outbreaks in US recruits suddenly reemerged during the same year along with a previously rare AdB2 serotype.

We hypothesize that the main factor determining the variable presence of AdB serotypes among recruits is preexisting immunity in the recruit source population. In 1993, seropositivity for Ad7 among incoming recruits was estimated at 27% [39]. A study from the postvaccination era, when Ad4 was dominant, estimated incoming recruit seropositivity for Ad7 at 61% [17]. The same 2 studies reported a relatively constant level of incoming seropositivity for Ad4 (29% and 34%, respectively). Unfortunately, comparable data from 2006 or from prevaccination periods of greater Ad diversity are not available. We hope that future work can address seropositivity on a more regular basis, because changes in source-population susceptibility may well predict the relative activity of different Ad serotypes in recruit facilities.

The previously deployed Ad4 and Ad7 vaccines, essentially identical to those currently in testing for future redeployment, do not directly target some of the currently circulating Ad serotypes. During previous periods of vaccination, Ad3 and Ad21 did continue to circulate among recruits, yet they never caused a return to prevaccination levels of disease. In fact, the vaccines appeared to prevent >95% of Ad-associated ARD and >50% of total recruit ARD despite the presence of these heterotypic Ads [18]. In studies using serum obtained from military recruits vaccinated with the Ad4 and Ad7 vaccines, Ad7 immunization was shown to generate a significant increase in levels of neutralizing antibodies against Ad3 and Ad14 [40]. The cross-protective interaction between Ad7 and Ad14 may be reflected in the data

presented here (figure 2): Ad7 and Ad14 are almost exclusively clustered at different sites. It will be important to track the response of different serotypes if and when the vaccines are redeployed. It would certainly be possible to develop vaccines against other ARD-associated Ads in the advent that currently circulating strains of Ad3, Ad14, or Ad21 are found to cause high rates of disease after reinstitution of the Ad4 and Ad7 vaccines, although previous evidence suggested that the addition of Ad21 to the Ad4/Ad7 combination decreased the effectiveness of all 3 [41].

Acknowledgments

We thank Dr. Leonard Binn for suggesting critical historical references. We acknowledge the administrative support of the Henry M. Jackson Foundation for Military Medicine and the efforts of the entire Naval Health Research Center team, especially the technicians and collection personnel whose efforts are represented in this work. We also acknowledge the clinic commanders and medical staff at Fort Benning, GA; Fort Jackson, SC; Fort Leonard Wood, MO (US Army); Lackland Air Force Base, San Antonio, TX (US Air Force); Naval Training Center, Great Lakes, IL (US Navy); Marine Corps Recruit Depot, San Diego, CA; Marine Corps Recruit Depot, Parris Island, SC (US Marine Corps); and Coast Guard Training Center, Cape May, NJ (US Coast Guard), for the permissions, access, and assistance necessary to conduct these studies.

References

- Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* **1953**; 84:570–3.
- Hierholzer JC, Stone YO, Broderick JR. Antigenic relationships among the 47 human adenoviruses determined in reference horse serum. *Arch Virol* **1991**; 121:179–97.
- Wadell G. Adenoviruses. In: *Encyclopedia of virology*. Vol. 1. New York: Academic Press, **1994**:1–7.
- Wadell G. Molecular epidemiology of human adenoviruses. *Curr Top Microbiol Immunol* **1984**; 110:191–20.
- Li QG, Wadell G. Analysis of 15 different genome types of adenovirus type 7 isolated on five continents. *J Virol* **1986**; 60:331–5.
- Metzgar D, Osuna M, Yingst S, et al. PCR analysis of Egyptian respiratory adenovirus isolates, including identification of species, serotypes, and coinfections. *J Clin Microbiol* **2005**; 43:5743–52.
- Xu W, Erdman DD. Type-specific identification of human adenovirus 3, 7, and 21 by a multiplex PCR assay. *J Med Virol* **2001**; 64:537–42.
- Sarantis H, Johnson G, Brown M, Petric M, Tellier R. Comprehensive detection and serotyping of human adenoviruses by PCR and sequencing. *J Clin Microbiol* **2004**; 42:3963–9.
- Xu W, McDonough MC, Erdman DD. Species-specific identification of human adenoviruses by a multiplex PCR assay. *J Clin Microbiol* **2000**; 38:4114–20.
- Schmitz H, Wigand R, Heinrich W. Worldwide epidemiology of adenovirus infections. *Am J Epidemiol* **1983**; 117:455–66.
- Rubin BA. Clinical picture and epidemiology of adenovirus infections. *Acta Microbiol Hung* **1993**; 40:303–23.
- Van der Veen J. The role of adenoviruses in respiratory disease. *Am Rev Respir Dis* **1963**; 88:167–80.
- Gray GC, Goswami PR, Malasig MD, et al. Adult adenovirus infections: loss of orphaned vaccines precipitates military respiratory disease epidemics. *Clin Infect Dis* **2000**; 31:663–70.
- Dudding BA, Top FH Jr, Winter PE, Buescher EL, Lamson TH, Leibovitz A. Acute respiratory disease in military trainees: the adenovirus surveillance program, 1966–1971. *Am J Epidemiol* **1973**; 97:187–97.
- Ryan MAK, Gray GC, Smith B, McKeehan JA, Hawksworth AW, Malasig MD. Large epidemic of respiratory illness due to adenovirus types 7 and 3 in healthy young adults. *Clin Infect Dis* **2002**; 34:577–82.
- Hilleman MR. Epidemiology of adenovirus respiratory infections in military recruit populations. *Ann NY Acad Sci* **1957**; 67:262–72.
- Russell KL, Broderick MP, Franklin SE, et al. Transmission dynamics and prospective environmental sampling of adenovirus in a military recruit setting. *J Infect Dis* **2006**; 194:877–85.
- Russell KL, Hawksworth AW, Ryan MAK, et al. Vaccine-preventable adenoviral respiratory illness in US military recruits, 1999–2004. *Vaccine* **2006**; 24:2835–42.
- Rose HM, Lamson TH, Buescher EL. Adenoviral infection in military recruits: emergence of type 7 and type 21 infections in recruits immunized with type 4 oral vaccine. *Arch Environ Health* **1970**; 21:356–61.
- Blasiola DA, Metzgar D, Daum LT, et al. Molecular analysis of adenovirus isolates from vaccinated and unvaccinated young adults. *J Clin Microbiol* **2004**; 42:1686–93.
- Van der Veen J, Kok G. Isolation and typing of adenoviruses recovered from military recruits with acute respiratory disease in the Netherlands. *Am J Hyg* **1957**; 65:119–29.
- Chmielewicz B, Benzler J, Pauli G, Krause G, Bergmann F, Schweiger B. Respiratory disease caused by a species B2 adenovirus in a military camp in Turkey. *J Med Virol* **2005**; 77:232–7.
- Malasig MD, Goswami PR, Crawford-Miksza LK, Schnurr DP, Gray GC. Simplified microneutralization test for serotyping adenovirus isolates. *J Clin Microbiol* **2001**; 39:2984–6.
- Kajon AE, Moseley JM, Metzgar D, et al. Molecular epidemiology of adenovirus type 4 infections in US military recruits in the postvaccination era (1997–2003). *J Infect Dis* **2007**; 196:67–75.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* **1990**; 215:403–10.
- Kendall EJC, Riddle RW, Tuck HA, Rodan KS, Andrews BE, McDonald JC. Pharyngo-conjunctival fever: school outbreaks in England during the summer of 1955 associated with adenovirus types 3, 7, and 14. *BMJ* **1957**; 2:131–6.
- Mevzos LM, Il'ina TS, Makhmudov OS, Zolotarskaia EE, Dreizin RS. [An outbreak of acute respiratory infections among adults caused by adenovirus serotype 14.] *Vopr Virusol (Russian)* **1966**; 11:426–31.
- Bruij J, Farnik J, Sedmidubsky V. [Epidemic of acute respiratory disease due to adenovirus type 14.] *Cesk Epidemiol Mikrobiol Imunol (Czech)* **1966**; 15:165–71.
- Hierholzer JC, Pumarola A. Antigenic characterization of intermediate adenovirus 14-11 strains associated with upper respiratory illness in a military camp. *Infect Immun* **1976**; 13:354–9.
- Brandt CD, Kim HW, Vargosko AJ, et al. Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome. *Am J Epidemiol* **1969**; 90:484–500.
- Cooper RJ, Hallett R, Tullo AB, Klapper PE. The epidemiology of adenovirus infections in Greater Manchester, UK 1982–96. *Epidemiol Infect* **2000**; 125:333–45.
- Lin KH, Lin YC, Chen HL, et al. A two-decade survey of respiratory adenovirus in Taiwan: the reemergence of adenovirus types 7 and 4. *J Med Virol* **2004**; 73:274–9.
- Chen HL, Chiou SS, Hsiao HP, et al. Respiratory adenoviral infections in children: a study of hospitalized cases in southern Taiwan in 2001–2002. *J Trop Pediatr* **2004**; 50:279–84.
- van der Veen J, Oei KG, Abarbanel MFW. Patterns of infections with adenovirus types 4, 7 and 21 in military recruits during a 9-year survey. *J Hyg (Lond)* **1969**; 67:255–68.
- Rosenbaum MJ, Edwards EA, Frank PF, Pierce WE, Crawford YE, Miller LF. Epidemiology and prevention of acute respiratory disease in naval recruits. I. Ten years' experience with microbial agents isolated from

- naval recruits with acute respiratory disease. *Am J Public Health Nations Health* **1965**; 55:38–46.
36. Vora GJ, Lin B, Gratwick K, et al. Coinfections of adenovirus species in previously vaccinated patients. *Emerg Infect Dis* **2006**; 12:921–30.
37. Houngh HH, Clavio S, Graham K, et al. Emergence of a new human adenovirus type 4 (Ad4) genotype: identification of a novel inverted terminal repeated (ITR) sequence from a majority of Ad4 isolates from US military recruits. *J Clin Virol* **2006**; 35:381–7.
38. Kajon AE, Wadell G. Sequence analysis of the E3 region and fiber gene of human adenovirus genome type 7h. *Virology* **1996**; 215:190–6.
39. Ludwig SL, Brundage JF, Kelley PW, et al. Prevalence of antibodies to adenovirus serotypes 4 and 7 among unimmunized US Army trainees: results of a retrospective nationwide seroprevalence survey. *J Infect Dis* **1998**; 178:1776–8.
40. van der Veen J, Prins A. Studies of the significance of the recall phenomenon in the antibody response to adenovirus vaccine and infection. *J Immunol* **1960**; 84:562–8.
41. Takafuji ET, Gaydos JC, Allen RG, Top FH Jr. Simultaneous administration of live, enteric-coated adenovirus types 4, 7, and 21 vaccines: safety and immunogenicity. *J Infect Dis* **1979**; 140:48–53.

REPORT DOCUMENTATION PAGE

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB Control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. Report Date (DD MM YY) 19/03/07	2. Report Type New	3. DATES COVERED (from - to) 1999-2005
4. TITLE AND SUBTITLE Abrupt Emergence of Diverse Species B Adenoviruses at US Military Recruit Training Centers		5a. Contract Number: 5b. Grant Number: 5c. Program Element: 5d. Project Number: 5e. Task Number: 5f. Work Unit Number: 60701
6. AUTHORS David Metzgar, Miguel Osuna, Adriana E. Kajon, Anthony W. Hawksworth, Marina Irvine, Kevin L. Russell		9. PERFORMING ORGANIZATION REPORT NUMBER Report No. 07-12
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Naval Health Research Center P.O. Box 85122 San Diego, CA 92186-5122		
8. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commanding Officer Commander Naval Medical Research Center Navy Medical Support Command 503 Robert Grant Ave P O Box 240 Silver Spring, MD 20910-7500 Jacksonville, FL 332212 0140		10. Sponsor/Monitor's Acronyms(s) NMRC/NMSC
		11. Sponsor/Monitor's Report Number(s)

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

13. SUPPLEMENTARY NOTES
Published in: The Journal of Infectious Diseases, 2007, 196(1), 1465-73

14. ABSTRACT (maximum 200 words)

Adenoviruses (Ads) cause essentially continuous outbreaks of febrile acute respiratory disease (ARD) in military recruit training facilities. In 1996, the loss of highly effective vaccines targeting the most common recruit-associated serotypes (Ad4 and Ad7) precipitated the re-emergence of Ads in these highly susceptible populations. Between 1999 and 2004, Ad4 completely dominated training facilities, representing more than 95% of the adenoviruses isolated from ill recruits and causing over 50% of ARD in training facilities (15,000 cases a year). In this study, surveillance serotyping of Ad isolates from 8 military recruit training facilities in the United States was extended to the present time. A total of 1,867 Ad isolates from recruits with ARD were typed by serum neutralization and PCR. The results show that the previous dominance of Ad4 continued through 2005, but then a simultaneous emergence of diverse species B serotypes occurred at the majority of sites. This included the generally common AdB1 serotypes Ad3, Ad7, and Ad21, and a very unique emergence of the AdB2 serotype Ad14. Of these, Ad14 was the most common, appearing in significant numbers at three sites and becoming, at least temporarily, the dominant serotype at one site. Previously, AdB2 serotypes have only rarely been associated with respiratory disease outbreaks, and then only in Eurasia. Neither Ad21 nor Ad14 are directly covered by existing military adenovirus vaccines, which should be reintroduced in the near future pending final FDA trials.

15. SUBJECT TERMS

adenovirus type 14; adenovirus B2; military recruit; febrile respiratory illness; epidemiology; serotype

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UNCL	18. NUMBER OF PAGES 12	19a. NAME OF RESPONSIBLE PERSON Commanding Officer
a. REPORT UNCL	b. ABSTRACT UNCL	b. THIS PAGE UNCL			19b. TELEPHONE NUMBER (INCLUDING AREA CODE) COMM/DSN: (619) 553-8429