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*G. J. Vora  
B. Lin  
K. Gratwick  
C. Meador  
C. Hansen  
C. Tibbetts  
D. A. Stenger  
M. Irvine  
D. Seto  
A. Purkayastha  
N. E. Freed  
K. Russell  
D. Metzgar*

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NAVAL HEALTH RESEARCH CENTER  
P. O. BOX 85122  
SAN DIEGO, CA 92186-5122



BUREAU OF MEDICINE AND SURGERY (M2)  
2300 E ST. NW  
WASHINGTON, DC 20372-5300

# Coinfections of Multiple Virulent Adenoviral Species in Previously Vaccinated Patients

Gary J. Vora<sup>1,2f</sup>  
Baochuan Lin<sup>1,2f</sup>  
Kevin Gratwick<sup>3</sup>  
Carolyn Meador<sup>4</sup>  
Christian Hansen<sup>3</sup>  
Clark Tibbetts<sup>1,5</sup>  
David A. Stenger<sup>1,2</sup>  
Marina Irvine<sup>3</sup>  
Donald Seto<sup>1,5,6</sup>  
Anjan Purkayastha<sup>1,5,6</sup>  
Nikki E. Freed<sup>3</sup>  
Kevin Russell<sup>1,3</sup>  
David Metzgar<sup>1,3</sup>

<sup>1</sup>Epidemic Outbreak Surveillance Consortium, USAF/SGR  
Falls Church, Virginia, USA

<sup>2</sup>Center for Bio/Molecular Science and Engineering, Code 6900  
Naval Research Laboratory, Washington, DC, USA

<sup>3</sup>Department of Defense Center for Deployment Health Research  
Naval Health Research Center, San Diego, California, USA

<sup>4</sup>Nova Research Inc., Alexandria, Virginia, USA

<sup>5</sup>Headquarters, USAF Surgeon General Office  
Directorate of Modernization (SGR), Falls Church, Virginia, USA

<sup>6</sup>Bioinformatics and Computational Biology Division  
School of Computational Sciences, George Mason University  
Manassas, Virginia, USA

<sup>f</sup>Both authors contributed equally to these studies.

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Nonstandard abbreviations used: acute respiratory disease (ARD); bovine serum albumin-casein solution (BSA-C); febrile respiratory illness (FRI); human adenovirus (HAdV); hemagglutination inhibition (HI); infected tissue culture fluid (ICTF); Naval Health Research Center (NHRC); Naval Research Laboratory (NRL)

## Abstract

Population-based febrile respiratory illness surveillance was used to monitor the effectiveness of the mandatory adenovirus vaccine administered to all US military trainees. Despite the general success of the vaccination program, surveillance still detected breakthrough infections — cases of acute respiratory disease associated with the adenovirus serotypes (4 and 7) specifically targeted by the vaccine. Termination of the vaccine program in 1999 allowed collection of matching samples from an unvaccinated population in the same environment. To explore the possible role of adenoviral coinfection (simultaneous infection by multiple pathogenic adenovirus species) in the facilitation of vaccine breakthrough we compared vaccinated and unvaccinated populations using three independent methods capable of simultaneously detecting multiple adenoviral species: a 70-mer DNA microarray, a commercially available PCR-ELISA, and a multiplex PCR assay. Analysis of 52 patient throat swab samples (21 vaccinated, 31 unvaccinated) collected from 1996-2000 revealed that all vaccinated samples harbored adenoviral coinfections. Most of the coinfections were composed of community-acquired, pathogenic serotypes of both species B (serotypes 3, 7, and/or 21) and species E (serotype 4). Unvaccinated samples primarily contained just serotype 4. Coinfections may be selected in vaccinated individuals because they can produce mosaics with unique antigenicity, giving them the ability to bypass vaccine-induced immunity specific to virus particles grown in isolation. This study highlights the previously undocumented phenomenon of natural adenoviral coinfections in an environment suitable for the generation of new, potentially virulent, and uniquely selectable recombinational variants, and suggests that vaccination may play a role in coinfection.

## **Introduction**

Adenovirus is an important human pathogen, estimated to cause 8% of clinically relevant viral disease globally (1). Human adenoviruses (HAdV) are divided into 51 serotypes (HAdV-1 - HAdV-51) based on type-specific antiserum-mediated neutralization of infectivity, and into six species, also referred to as subgenera or subgroups (HAdV-A, B, C, D, E, and F) based on hemagglutination inhibition (HI) (2). HAdV-B is further classified into subspecies B1 and B2. Serotype is determined primarily by the hexon coat protein, while HI is determined primarily by the receptor-binding fiber protein. In civilian populations, HAdV-B1 serotypes 3, 7, 16, 21, and 50, HAdV-E serotype 4, and one member of the otherwise gastric species HAdV-B2 (serotype 14) are known to cause outbreaks of illness ranging from mild febrile respiratory infections and conjunctivitis to potentially lethal disseminated infections in both adults and children (3). HAdV-C serotypes 1, 2, 5 and 6 cause widespread upper respiratory infections in infants and children (4), and occasional outbreaks in adults. Other HAdV species are usually not associated with respiratory disease in otherwise healthy humans.

HAdV seems to have found a particularly destructive niche in military training camps. HAdV-B1 serotypes 3, 7, and 21, HAdV-E serotype 4, and HAdV-B2 serotype 14 have been identified as the causal agents in severe outbreaks of acute respiratory disease (ARD) among military recruits in training centers (5, 6). Prior to the initiation of an HAdV vaccination program in 1971, outbreaks occurred regularly during which approximately one out of six recruits in affected camps required hospitalization (1). Systematic vaccination of recruits against the two most common agents of ARD in the military, HAdV-4 and HAdV-7, decreased HAdV-specific respiratory illness by 95-99% and overall respiratory illness rates by 50-60% (7). Despite this general efficacy, vaccine breakthrough (infection of vaccinated individuals by the targeted pathogen) was still regularly reported (2). Production of the vaccine was suspended in

1996, at which point vaccination became sporadic until the existing stocks ran out in 1999. ARD rates quickly returned to prevaccine levels, with HAdV as the apparent causal agent, and reintroduction of the vaccine is being actively pursued (2).

To explore the possibility that unique HAdV strains were causing ARD in vaccinated individuals, throat swab samples were selected from the Naval Health Research Center (NHRC) population-based febrile respiratory illness (FRI) surveillance collection from vaccinated (N = 21) and unvaccinated (N = 31) recruits who reported with ARD between 1996 and 2000. Samples were chosen that had previously tested positive for HAdV-4 or HAdV-7 by culture and serotypic antibody neutralization. The gene coding for the primary adenoviral antigen, the hexon coat protein, was sequenced from these isolates. The sequence data suggested that the detectable HAdV-4 and HAdV-7 strains apparently responsible for vaccine breakthrough were the same as those circulating in unvaccinated military and civilian populations (2). In this study we reanalyze the same set of samples, using newly emerging technologies capable of resolving complex concurrent coinfections of multiple HAdV strains, to address the possibility that breakthrough is facilitated by coinfection.

## Results

*Methods of detection.* The methods used here, including a recently developed 70-mer spotted microarray (8), a commercially available multiplex PCR-ELISA assay (Adenovirus Consensus kit; Argene Inc., North Massapequa, New York, USA), and a published species-specific multiplex PCR assay (9), used here as both a multiplex and as a parallel series of separate monoplex PCR tests, can all specifically assay for the presence of more than one adenoviral strain at once. Although they vary in the degree to which signals from multiple strains or species will interfere with each other and in the breadth and specificity of their coverage, these tests can yield measurable signals from "secondary" (less numerous) coinfectants against the background of stronger signals produced by "primary" infecting strains. Using these three methods, data profiles for each of the 52 samples were generated. Two representative profiles, from vaccinated sample #7274 and unvaccinated sample #10756, are presented in Figure 1. Many of the positive results from these tests were further verified by comparison with results from independent methods such as microneutralization, *hexon* sequence analysis, serotype-specific PCR (using primers not used in the multiplex tests — see Table 3), and sequencing of PCR amplicons. The assembled data profiles for all 52 samples are presented in Table 1.

*Validation and statistical analysis.* While most apparent coinfections could be verified by serotype-specific PCR with independent primers, some could not (Table 1). Since two of the assays (microarray and Adenovirus Consensus kit) use detection signal amplification techniques that could render them more sensitive than traditional PCR/agarose gel visualization techniques, it is likely that these PCR-unverifiable signals are accurate. In the case of the microarray, the test is self-validating: the array tests for the presence of three physically distinct serotype-specific alleles from the *E1A*, *hexon*, and *fiber* genes. In other cases, the primary techniques tested here verified each other even when traditional PCR was unable to verify particular

identifications. Numbers of independently verifiable strain identifications correlated well with total numbers of strain identifications (verifiable + unverifiable). For the sake of simplicity, we chose to count all positive results as "real" for statistical comparison of vaccinated and unvaccinated populations. Samples were analyzed in mixed batches from vaccinated and unvaccinated groups, and any potential false positive reactions would theoretically affect the vaccinated and unvaccinated data sets equally.

To address the hypothesis that vaccination selects for coinfection (which may in turn result in vaccine breakthrough), we performed the Fisher Exact Test to compare the numerical distribution of HAdV strains infecting previously vaccinated patients with the numerical distribution of strains infecting unvaccinated patients. Each of the methods designed to test for multiple species or serotypes revealed a significantly higher number of pathogenic serotypes in vaccinated individuals than in unvaccinated individuals (Table 2). Count distributions were generally normal except in the case of the Adenovirus Consensus kit, which yielded a bimodal distribution. The correlation between coinfection and previous vaccination appears to derive almost entirely from the high rate of coinfection with both of the species commonly associated with ARD (HAdV-B1 and HAdV-E) in vaccinated individuals, whereas unvaccinated individuals are primarily infected with HAdV-E (HAdV-4). The most commonly paired coinfectants in vaccinated individuals appear to be HAdV-4 and HAdV-7. These are the two most common ARD-associated serotypes, but also the two strains contained in the (live virus) oral vaccine. Sequence analysis of the *hexon* gene shows that most, if not all, of these coinfectants are modern circulating HAdV-4 and HAdV-7 species, genetically distinct from the vaccine strains [see Table 1 and reference (2)]. Hence, the isolates obtained from these patients represent community-acquired pathogens, and not the vaccine strains themselves. Forthcoming reference genome data for HAdV-4 and 7 vaccine and field strains (D. Seto, manuscripts in

preparation) will allow further and comprehensive genome-wide comparisons of the coinfecting samples containing HAdV-4 and HAdV-7.

## **Discussion**

This study unequivocally demonstrates a strong and significant association of adenoviral coinfections composed of multiple currently circulating pathogenic serotypes [as defined in (6) — HAdV-4, 7, 21, 3, and 14] with vaccine breakthrough (Tables 1 and 2). Almost all samples contained species E (HAdV-4), while the vaccinated coinfections were most often composed of at least one serotype of species B (HAdV-7, 3, and/or 21) and HAdV-4.

Adenoviruses are capable of coinfection. The concurrent and productive infection of the same cell can allow sharing of enzymatic functions. This has been demonstrated by studies in which otherwise nonproductive adenoviral mutants grow efficiently in the presence of wild-type strains that can provide missing functions *in trans*, even if the mutant and complementing strains are divergent members of different species. For example, HAdV serotypes 3, 4, and 9 (species B, E, and D) can provide the hexon protein *in trans* to serotype 5 (species C) hexonless mutants, a phenomenon termed "pseudopackaging" (10). The resulting viruses are infectious and can deliver both the parental and coinfecting mutant genomes to new host cells.

The *fiber* gene product can also be shared *in trans*. Tissue-culture coinfections of both wild-type HAdV-5 and an HAdV-5 mutant in which the *fiber* knob has been replaced with the HAdV-3 counterpart can generate mosaic viruses bearing single genomes but displaying mixtures of both fiber protein knobs (11). Since the fiber protein is responsible for tissue tropism, these mosaics are able to infect all tissue types independently targeted by both HAdV-3 and HAdV-5. This example shows that viruses resulting from coinfection can display phenotypes distinct from those of either parental strain, leading to the possibility that breakthrough coinfections could overcome vaccine-induced immunity specific to strains grown



in isolation (the HAdV-4 and 7 vaccine strains were grown and administered as separate entities). Other functional proteins, including regulators of both viral processes and host immune responses, can also act *in trans* (12).

Coinfection is further interesting because it can provide an environment suitable for recombination between serotypes. Recombination can generate new strains with unique and stable phenotypes. Intra- and inter-species recombinations have been demonstrated many times in laboratory cell-culture coinfection studies (13-16) and generate viable hybrids with intermediate or unique immunogenic and tropic properties. Deliberate (*in vitro*) recombinants are also viable, showing broad (interspecies and intergenera) complementarity of functional elements (17-20). Evidence suggests recombination can generate hybrids in immunocompromised patients (1, 21), possibly as a result of coinfection with normally isolated serotypes. Most importantly, recombination (particularly intraspecies) seems to play a major role in the evolution of new, virulent strains of HAdV (1, 16, 22).

For example, the sequence homology relationships among HAdV-11, HAdV-35, HAdV-7, and HAdV-21 show that either HAdV-11 or HAdV-35 is a recombinant strain resulting from a *fiber* gene transfer between two of the other aforementioned strains (23). HAdV-7 subtype 7h, a recently emerged and currently common pathogenic strain, has been shown to be an otherwise normal Ad7 with a recombined Ad3 *fiber* gene (22). It has been suggested that HAdV-4 appears to be a recombinant between two species, essentially an HAdV-B with an HAdV-C-derived fiber protein (24), though this result has been brought into question by recent whole-genome sequencing efforts (D. Seto, manuscript submitted). The currently dominant pathogenic HAdV, a considerably diverged variant HAdV-4 strain (2) appears to be a very recent recombinant between HAdV-4 and an HAdV-B1 serotype, probably 7 or 3 (D. Seto, manuscript in preparation). Given that these two are the most common coinfectants seen in our sample set, this

raises the possibility that the observed dominance of coinfections during the period of vaccination may have contributed to the emergence of the new variant. Given the propensity of adenovirus to evolve through recombination, conditions that might favor symbiotic coexistence are of considerable concern.

Many identified coinfectants were strains not generally associated with ARD in the military. HAdV-C, though rarely associated with pharyngitis outbreaks in recruits (6), is usually seen in children and can produce latent infections lasting into young adulthood (4). These latent infections can be revived in tissue cultures such as those used to amplify HAdV from the samples used in this study. The causal nature of the HAdV-B2 and HAdV-F species identifications with respect to ARD is also questionable. HAdV-B2 is primarily associated with urinary tract infections in immunocompromised patients (15), though it is occasionally associated with gastroenteritis, pharyngoconjunctival fever, and ARD (1). HAdV-F is almost exclusively associated with gastroenteritis. The signals from these strains were invariably weak (as were most signals from HAdV-C) in comparison with the strong signals often seen from HAdV-B1 and HAdV-E.

Coinfections with these virus species (B2, F, C) did not correlate well with vaccination in any case, but results were included because these agents are capable of genetic complementation with upper respiratory strains (11, 25), and serve to suggest the general complexity of the human adenoviral load. The question of whether these infections are latent or active parts of symbiotic coinfections (unassociated with vaccination) will have to wait for data collected from concurrent ill and healthy populations.

The HAdV vaccine (an enteric-coated live-virus tablet designed to transiently and selectively infect the gastrointestinal tract with normal respiratory HAdV strains) contained viable HAdV-4 and HAdV-7, so it must be asked whether the detected coinfectants arose from

the vaccine itself or from community acquisition of currently circulating strains. Most HAdV-4 strains seen in this study are clearly not the vaccine strain, but rather a highly divergent variant (26) that appears to have recently replaced the vaccine strain. This was shown previously through sequence analysis of 1500 bp of the *hexon* gene (2). These data were conclusive, as the variant HAdV-4 isolates consistently differ from the vaccine strain by 32 base substitutions (including 9 coding changes) in this region [see GenBank records associated with (2) and Table 1]. This variant, represented in GenBank by strain Z-G 95-873, is fourfold reduced in neutralization compared with the AV4 RI-67 prototype (essentially the vaccine strain) using anti-AV4 RI-67 serum (26), suggesting a potential advantage of this strain (compared with strains more closely related to the vaccine strain) in vaccinated populations. The three HAdV-4 vaccine-group isolates identified in this study have two conserved base substitutions in the sequenced *hexon* region relative to the vaccine strain itself, and two of them share a third difference [see GenBank records associated with (2)].

*Hexon* sequence analysis also revealed that many of the HAdV-7 coinfectants are HAdV-7d2. These are distinguished from the HAdV-7 vaccine strain (HAdV-7a) by a single coding polymorphism in the *hexon* sequence, but this polymorphism [protein:L443Q, or nucleotide:T1328A in the GenBank records associated with (2)] is specific to HAdV-7d and HAdV-7d2 and is not found in HAdV-7a, b, c, g or h, nor in the vaccine strain itself (2, 3, 27). HAdV-7d2 has recently been reported as a circulating upper respiratory pathogen among both vaccinated military populations in the United States and among unvaccinated civilian populations in Japan (3, 28), while outbreaks of the older subtypes (such as HAdV-7a) have become less common. One of the HAdV-7 coinfectant's *hexon* sequence was grouped with the highly divergent HAdV-7 prototype (HAdV-7p) (2). Three of the other HAdV-7 coinfectants were shown to be HAdV-7h by *fiber* gene sequencing. The *fiber* gene of HAdV-7h appears to

have been horizontally transferred from HAdV-3, and hence is highly diverged from the usual HAdV-7 *fiber* gene (as found in the vaccine strain) (22). Still other coinfections contain HAdV-3 or HAdV-21 instead of (or in addition to) HAdV-7, and these coinfectants clearly did not come from the vaccine. Only one HAdV-7 from this collection shared an identical *hexon* sequence with the vaccine strain. The bulk of the evidence supports the idea that the coinfectants seen in this study did not arise from direct infection by the vaccine itself.

Three lines of evidence support the idea that most of the apparent genetic complexity in these samples comes from multiple independent strains, as opposed to recombinants with mixed genetic character. The first comes from the microarray data. The microarray tests for hybridization of three independent probes. These probes were designed to match strain-specific sequences in three genes (8). Hence, hybridization of genes from one strain to the identifying probes for two strains would require the redundant presence of two different alleles in all three genes. Since both natural recombination in hosts (21, 22) and artificially encouraged recombination in cell culture (15) strongly favors homologous recombination and the generation of nonredundant hybrid strains, redundant characterization of paired, divergent alleles is inconsistent with the presence of a single recombinant genome.

The second piece of data supporting coinfection with independent genomes comes from comparisons of relative coinfectant titers before and after potentially selective events, such as growth in tissue culture. PCR amplification of *fiber* gene sequences using species B- and E-specific primers was performed on serial dilutions of vaccinated sample #7274 before and after passage of the original infected tissue culture fluid (ITCF) through two further cycles of growth in A549 cells. In this instance, the relative titers of HAdV-4 and HAdV-7 changed by two orders of magnitude (K. Gratwick, unpublished data). The rapid drift in relative concentrations of PCR

targets from paired coinfecting strains strongly suggests that the coinfectants' genomes are replicating independently and are thus likely to be physically separate entities.

The third piece of evidence supporting coinfectant independence comes from whole-genome sequencing efforts. Several molecular methods indicated that vaccinated sample #7151 harbored an HAdV-5/HAdV-21 coinfection (Table 1). The genome of the HAdV-5 coinfecting strain was completely sequenced (Commonwealth Biotechnologies, Inc., Richmond, Virginia, USA) and all of the sequence ladders assembled cleanly into a single contiguous sequence, suggesting no recombination of foreign DNA (D. Seto and C. Tibbetts, complete sequence and annotation will be accessible upon publication at GenBank under accession number AY601635). However, this effort generated several sequences that did not fit into the assembled scaffold, despite its apparent completeness and consistency with a published HAdV-5 genome, GenBank accession number AY339865 (29). These "orphan" sequences were subsequently identified as functionally redundant HAdV-21 regions. Further amplification and sequencing of several genetically distant fragments from the same sample using HAdV-21-specific primers yielded approximately 2 kb of HAdV-21 sequence, clearly distinct from the corresponding HAdV-5 sequences found with the original sequencing protocol. Based on the whole genome and partial PCR-sequencing analyses, it may be concluded that there are at least two coinfecting HAdV genomes contained in the original specimen collected, characterized and archived as NHRC strain #7151.

It is important to note that conventional clinical microbiological methods, including microneutralization and hemagglutination inhibition, are comparative and designed to identify the primary HAdV serotype (or species) present in a sample. Therefore, secondary infections are masked in these methods by nature of the tests themselves (for example, microneutralization is reported in terms of the strongest reaction, not the spectrum of reactions across all serotypes).

Likewise, the use of direct sequencing (2) and restriction enzyme analysis methods (30) are likely to restrict identification to a single strain, particularly if one coinfectant is numerically dominant. For the sake of comparison, it is satisfying that the predominant serotype or species signal generated in both the microarray and PCR-ELISA tests matched the results obtained from the original microneutralization experiments in every documented case. In terms of discerning coinfections, as opposed to primary single infections, all three molecular methods used here served their purpose. Each method revealed a similar correlation between multiplicity of infection and previous vaccination (Table 2). Furthermore, the microarray analyses revealed several coinfections of multiple serotypes from the diverse HAdV-B1 subspecies (most commonly HAdV-7 and HAdV-3), coinfections that could not have been resolved by species-specific methods. In this context, the microarray provides a distinct advantage to traditional methods, one relevant to both single and multiple infections: HAdV-7 and HAdV-3 crossreact in neutralization assays because they share very similar hexon proteins, while HAdV-7h is misidentified as HAdV-3 by HI assays because they share very similar fiber proteins (31). The microarrays used here independently test for strain-specific differences in three different genes (*hexon*, *fiber*, and *EIA*), and can therefore resolve pairs with differences in only one of the three genes. This is important due to the potential role of recombination in strain evolution, since recombination is likely to generate strains like HAdV-7h that resemble one serotype at one locus but another serotype at another locus. HAdV-7h has become an epidemiologically important pathogen in recent years (22).

In all but two vaccinated individuals testing positive for HAdV-7 by microarray (and one of the two unvaccinated HAdV-7-positive individuals), the HAdV-B strains present are either HAdV-7h [an HAdV-7/HAdV-3 recombinant (22)] or a combination of HAdV-7 with a marginally detectable HAdV-3. These two conditions may well represent different steps on the

same path, the latter representing a coinfection with functional sharing of proteins and the former representing a unique strain (HAdV-7h) formed by recombination between the two original strains. Perhaps the combination of HAdV-7 and HAdV-3, by either recombination or by complementation *in trans*, allows partial avoidance of vaccine-induced immunity while retaining aspects of the HAdV-7 phenotype that make it a much more common agent of disease than is HAdV-3.

The vaccinated samples collected here (from 1996–1998) are not perfectly concurrent with the unvaccinated samples (collected from 1998–2000). Vaccination was done systematically, if at all; hence, vaccinated individuals tended to be sampled at times when all recruits were being vaccinated, and *vice versa*. It is possible that the phenomenon of coinfection documented here is temporally correlated rather than being related to vaccination, though it would seem highly unlikely that a vaccination-independent event would be so tightly correlated with the termination of the vaccination program. Furthermore, the change from HAdV-4/HAdV-7 coinfections to HAdV-4 monoinfections (rather than a shift between strains) would be unusual in the absence of a shift in selective pressure. The reintroduction of the vaccine in the next few years will allow further examination of the relationship between vaccination and coinfection.

Whether coinfections are composed of multiple circulating pathogens, retained vaccine strains, or latent strains revived by the primary infection, they represent an optimal condition for the generation of new, potentially virulent, and uniquely selectable recombinant variants. Since these sorts of variants appear to play a major role in the evolution of new pathogenic strains of HAdV (1, 16, 22), the control of situations that enable or promote recombination should be a significant consideration. If coinfection-derived HAdV-4 and HAdV-7 are immunologically distinct from independently grown HAdV-4 and HAdV-7, then vaccine protection against coinfections might be achieved by vaccination with coinfection-derived viral mixtures.

## Methods

*Sample collection and preparation.* Samples were collected as throat swabs in viral transport medium from military recruits suffering from FRI at a variety of training camps as previously described (2). The throat swab samples were cultured on A549 cells, tested with traditional serological methods, and both the original swabs and the ITCF samples were stored at -80°C. Samples initially testing positive for HAdV-4 or HAdV-7 by culture and serotype-specific Ab neutralization were chosen for analysis and grouped by previous vaccination status. DNA extracts obtained from ITCF samples were collected and used in the molecular assays conducted in this study. Collection details and symptomological definitions were previously published (2) and sample details are listed in Table 1.

The first 13 of 52 samples sent by NHRC for testing by Naval Research Laboratory (NRL) personnel were tested in a masked fashion (i.e., the vaccination status of the individuals from which these samples came were unknown to NRL personnel). The study was expanded to include all 52 samples, as used in a previous study (2) after the initial tests and subsequent unmasking revealed a high rate of respiratory HAdV coinfection, primarily in vaccinated individuals.

To control for the introduction of contaminating or coinfecting adenoviral strains during tissue culture, five original throat swab samples (vaccinated) were directly tested for the presence of coinfectants using serotype-specific PCR and microarray analyses (see Table 1). Although sensitivity of detection, on average, was lower in original specimens (as expected, since tissue culture greatly increases titer), coinfections were still detectable.

*Microarray-based genotyping.* One  $\mu$ l of purified DNA extract from each of the 52 ITCF samples was used as the template in 50  $\mu$ l degenerate PCR amplification reactions. The primers, degenerate PCR amplification protocol, probes, and microarray fabrication techniques have been



previously documented (8). Once constructed, the spotted microarrays were blocked with a 3% bovine serum albumin-casein solution (BSA-C) for 15 min at room temperature and the slides were outfitted with MAUI Mixer DC hybridization chambers (BioMicro Systems, Salt Lake City, Utah, USA). Twenty  $\mu\text{l}$  hybridization reactions (13.6  $\mu\text{l}$  biotinylated degenerate PCR amplicons, 2  $\mu\text{l}$  3% BSA-C, 4  $\mu\text{l}$  20X SSC, and 0.4  $\mu\text{l}$  10% SDS) were denatured for 3 min at 98°C and immediately applied to the microarrays. Hybridizations were performed for 2 hours at 63°C on a MAUI Hybridization System (BioMicro Systems). Post-hybridization, slides were washed twice with 4X SSC-0.2% SDS buffer for 3 min at 63°C and twice with 2X SSC buffer for 1 min at room temperature. Hybridization events were detected by the sequential addition of Cy5-conjugated mouse anti-biotin IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) where each Ab was incubated for 15 min at room temperature. Images were captured with a ScanArray Lite confocal laser scanning system (Perkin–Elmer Inc., Torrance, California, USA) at a laser power between 60-80 and a photomultiplier tube gain of 60-80. The fluorescent signal from each microarray element was considered positive only when its quantified intensity was >3X that of known internal negative control elements. Each ITCF sample was subjected to 2-5 independent amplification and hybridization experiments.

*Detection of amplified adenovirus DNA on microwell plates.* To aid in confirming the results obtained from the microarray analyses we chose to utilize a commercially available kit capable of typing adenoviruses to the species level. Briefly, the Adenovirus Consensus kit (Argene Inc.) utilizes a PCR-ELISA protocol that amplifies a fragment from the adenovirus VA RNA gene and subsequently detects and types the amplicon with species-specific biotinylated oligonucleotide probes in a colorimetric microwell format. The Adenovirus Consensus kit was applied and interpreted according to the manufacturer's "Adenovirus typing" protocol. All colorimetric

reactions were read at 450 nm on a Biotrak Visible Plate Reader (Amersham Biosciences, Piscataway, New Jersey, USA).

*Adenovirus-specific PCR.* The species-specific PCRs were done using the primers described in (9) and the Qiagen Multiplex PCR Kit (Qiagen, Valencia, California, USA) as per the PCR kit instructions (with .5X Q solution). These PCRs were done in 25 µl reaction volumes with a 52°C annealing temperature. In general, PCR reactions were run on an iCycler (Bio-Rad Laboratories, Hercules, California, USA) and analyzed on 1.5% agarose gels. We used both a standard DNA ladder and an in-house ladder composed of a multiplex PCR reaction containing DNA from all six species as size standards. Monoplex PCR was performed under identical reaction conditions, except that primers were used in independent reactions. Sequencing reactions and microneutralizations were performed as in (2). Strain-specific PCRs for verification (see Table 1) were done, when possible, in the manner described in the publications to which they are referenced (Table 3), with occasional substitutions of polymerase type and annealing temperature adjustments.

*Statistical analysis.* For each of the four molecular methods tested (microarray, Adenovirus Consensus kit, species-specific multiplex PCR, and serotype/species-specific monoplex PCR) the total numbers of strains identified in each vaccinated (N = 21) and unvaccinated (N = 31) sample isolate were enumerated. Using these numbers, we performed the Fisher exact test using SAS software (Version 9.0, SAS Institute, Inc., Cary, North Carolina, USA) to compare the distributions of coinfectant counts in the two populations.

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Affiliations — Department of <sup>1</sup>USAF/SGR, <sup>2</sup>USAF/SGR (Ctr), <sup>3</sup>Lackland AFB, TX, <sup>4</sup>The George Washington University (IPA), <sup>5</sup>Texas A&M University San Antonio (IPA), <sup>6</sup>Virginia Tech (IPA), <sup>7</sup>George Mason University (IPA), <sup>8</sup>George Mason University, <sup>9</sup>Armed Forces Institute of Pathology, <sup>10</sup>Naval Research Laboratory, <sup>11</sup>Wilford Hall Medical Center, <sup>12</sup>Air Force Institute for Operational Health, <sup>13</sup>Naval Health Research Center.

Corresponding author: David Metzgar, Naval Health Research Center, Phone: (619)-553-0606,

Fax: (619)-553-7601, E-mail: [metzgar@nhrc.navy.mil](mailto:metzgar@nhrc.navy.mil)

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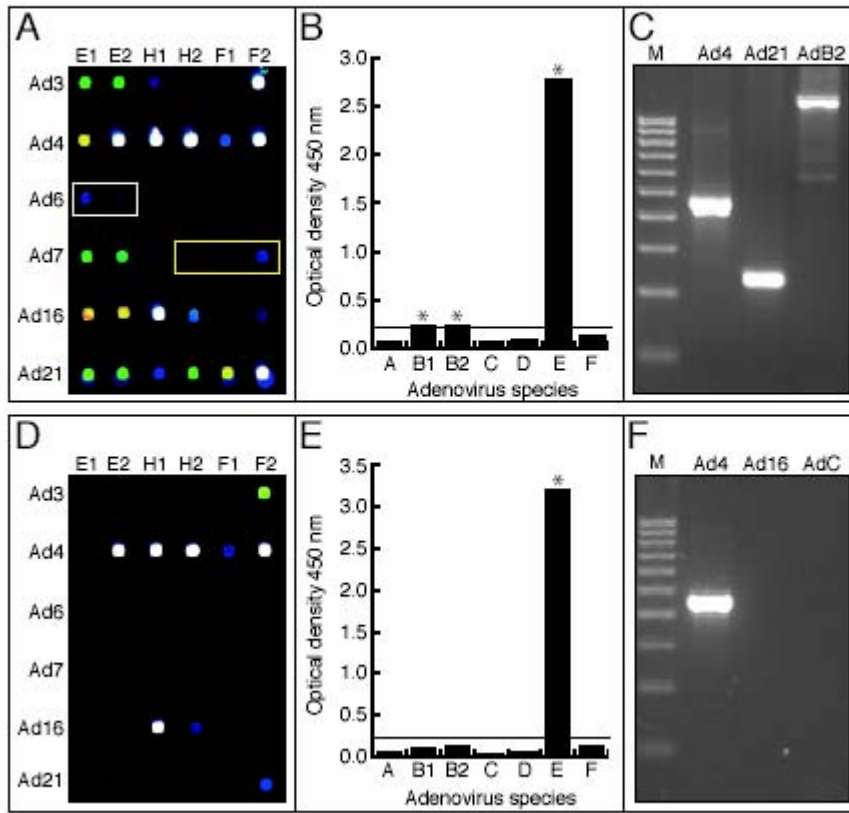
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## Figure Legends

**Figure 1** Molecular methods used for the identification of adenoviral coinfections. The data profiles for 2 of the 52 samples, vaccinated sample #7274 (A-D) and unvaccinated sample #10756 (E-H), are shown. **(A)** Microarray hybridization profile of sample #7274 indicating the presence of HAdV-4, HAdV-21, HAdV-C, and HAdV-B2. The white and yellow boxes outline the detection of low positive HAdV-C and HAdV-B2 targets, respectively. **(B)** Adenovirus Consensus kit optical density values indicating (\*) HAdV-B1, HAdV-B2, and HAdV-E amplification. The horizontal line is the manufacturer's significance threshold. **(C)** Multiplex species-specific PCR indicating HAdV-B and HAdV-E amplification. **(D)** PCR verification of HAdV-4, HAdV-21, and HAdV-B2 using independent serotype or species-specific primers (the apparent low-level HAdV-C component was not detected with this reaction). **(E)** Microarray hybridization profile of sample #10756 indicating the presence of HAdV-4 only. **(F)** Adenovirus Consensus kit optical density values indicating (\*) HAdV-E species amplification only. **(G)** Multiplex species-specific PCR indicating HAdV-E amplification only. **(H)** PCR verification of HAdV-4 using independent serotype-specific primers.



Figure 1



**Table 1 Molecular detection of adenoviral coinfections from vaccinated and unvaccinated patients**

Original Designation <sup>a</sup>	Vaccination Date	NHRC Data					NRL Data			
		Microneutralization Serotype	Multiplex PCR	Group-Specific PCR (B, C, and E)	Sequencing <sup>b,c</sup>	GenBank Accession	Microarray	Adenovirus Consensus Kit	PCR Determination	
									Positive	Negative
1649.AV7.V.98.GL	13.Jun.98	7	B	B	7d2 (Δ-2)	AF321311	7, 3, 4	B1	7, 3	4
1152.AV7.V.97.GL	08.Oct.97	7	B	B	7 Vaccine (Δ-1)	AY337253	7, 4, 3	B1	7, 4	3
141.AV7.V.96.GL	12.Nov.96	7	B	B	7d2(prototype)	AY337258	7, 4, 3	B1, B2, E	7, 4, B2	3
1251.AV7.V.97.GL	08.Oct.97	7	B	B	7d2 (Δ-2)	AF321311	7, 4, 3	B1, E, F	7	4, 3
1302.AV7.V.97.GL	08.Oct.97	7	B	B, E	7 Vaccine (Δ-2)	AY337256	7, 4, 3	B1, E, F	7, 4	3
1186.AV7.V.97.GL	08.Oct.97	7	B	B, E	7d2 (Δ-2)	AF321311	7, 4	B1, E, F	7	4
1150.AV7.V.97.GL	08.Oct.97	7	B	B, E	7 Vaccine (Δ-2)	AY337254	7, 4, 3	B1, E, F	7, 3, F	4
1212.AV7.V.97.GL	29.Sep.97	7	B	B, E	7d2 (Δ-2)	AY337255	7, 4, 3	B1, E, F	7, 4, 3, F	
79.AV4.V.96.GL	07.Oct.96	4	E	E	4 Vaccine (Δ-3)	AF065062	4, C, 7	E	4, C, B2	7
275.AV4.V.97.GL	31.Jan.97	4	E	E	4 Vaccine (Δ-3)	AY337239	4, C, 7	E, B2, F, B1	4, C, B2	7
7333.AV4.V.98.FJ	25.Mar.98	4	E	E, B	4 Variant	AY337242	4, C, B2	E	4, 1, 5, B2	
4476.AV4.V.97.FLW	24.Oct.97	4	E	B, E	4 Variant	AY337249	4, C, B2	E, B2, F, B1	4, 5, B2	
1856.AV5.V.98.GL	25.Mar.98	4	C	B, C, E	5 (7h)		C, 7	C	C	7
1275.AV7.V.97.GL	08.Oct.97	7	B	B, E	7 Vaccine (Δ-1)	AY337257	7, 4, 3	B1, E	7, 4, 3	
7307.AV5.V.98.FJ	09.Feb.98	4	C, B	B, C, E	5		C, 21	C	C	21
7137.AV4.V.97.FJ	01.Dec.97	4	E	B, E	4 Variant	AY337237	4, C, B2	E	4, 1	B2
7151.AV5.V.98.FJ	05.Nov.97	4	B, C	B, C, E	5		C, 21	C, B1	5, 21	
7274.AV4.V.98.FJ	11.Feb.98	4	E, B	E, B	4 Vaccine (Δ-2)	AF065062	4, 21, C, B2	E, B1, B2	4, 21, B2	C
1122.AV7.V.97.GL	08.Oct.97	7	B	B	7d2 (Δ-2)	AF321311	7, C, 3	B1	7, C	3
1108.AV7.V.97.GL	08.Oct.97	7	E, B	B, E	7 Vaccine (Δ-0)	AF065067	7, 4, C, 3	B1, E, F	7, 4, C	3
4185.AV4.V.97.FLW	24.Mar.97	4	E	B, E	4 Variant (7h)	AY337252	4, C, B2	E, B2, F, B1	4, B2	C
40183.AV4.98.FJ		4	E	E	4 Variant	AY337237	4	E	4	
10213.AV4.98.GL		4	E	E	4 Variant	AY337240	4, C, B2	E, B2, F	4, B2	
40098.AV4.98.FJ		4	E	E	4 Variant	AY337241	4	E, F	4, F	
10258.AV4.98.GL		4	E	E	4 Variant	AY337237	4	E	4	
20142.AV4.98.MCRD		4	E	E	4 Variant	AY337250	4	E	4	
10756.AV4.00.GL		4	E	E	4 Variant	AY337243	4	E	4	
60406.AV7.99.FB		7	B	B	7 Vaccine (Δ-2)	AY337256	7	B1	7	
20139.AV4.98.MCRD		4	E	E	4 Variant	AY337237	4	E	4	
10190.AV4.98.GL		4	E	E, B	4 Variant	AY337237	4	E	4	
40160.AV4.98.FJ		4	E	E, B	4 Variant	AY337237	4	E	4	
10257.AV4.98.GL		4	E	E	4 Variant	AY337237	4, B2	E	4, B2	
40844.AV4.99.FJ		4	E	E	4 Variant	AY337237	4, C	E, B2	4, B2	C
10060.AV4.98.GL		4	E	E	4 Variant	AY337237	4, C, B2	E, B2	4, B2	C
20145.AV4.98.MCRD		4	E	E	4 Variant	AY337245	4, C, B2	E, B2, F	4, B2	C
40781.AV4.99.FJ		4	E	E	4 Variant	AY337238	4, C	E, B2	4, B2	C
10206.AV4.98.GL		4	E	E	4 Variant	AY337244	4, C, B2	E, B2	4, B2	C
20143.AV4.98.MCRD		4	E	E, B	4 Variant	AY337237	4, C, B2	E, B2, F	4, C, B2, F	
41059.AV4.99.FJ		4	E	E	4 Variant	AY337237	4, C	E, B2, F	4, C, B2, F	
60673.AV4.00.FB		4	E	E	4 Variant	AY337237	4, C	E	4	C
60691.AV4.00.FB		4	E	E, B	4 Variant	AY337238	4, C	E	4	C
60697.AV4.00.FB		4	E	E	4 Variant	AY337246	4, C	E	4, 1	
60708.AV4.00.FB		4	E	E	4 Variant	AY337237	4, C	E	4	C
60716.AV4.00.FB		4	E	E	4 Variant	AY337247	4, C	E	4	C
CHPPM44.AV4.00.FB		4	E	E	4 Variant	AY337237	4, C	E	4	C
CHPPM2.AV4.00.FB		4	E	E, B	4 Variant	AY337237	4, C	E	4	C
CHPPM9.AV4.00.FB		4	E	E, B	4 Variant	AY337237	4, C	E	4	C
CHPPM13.AV4.00.FB		4	E	E, B	4 Variant	AY337237	4, C	E	4	C
CHPPM29.AV4.00.FB		4	E	E	4 Variant	AY337237	4, C	E	4	C
20044.AV4.98.MCRD		4	E	B, E	4 Variant	AY337248	4, C, 7, 3	E	4, 1, B2	7, 3
7372.AV5.98.FJ		4	C	B, C, E	5 (7h)		C, 7	C	C	7
50108.AV4.00.LAC		4	E	B, E	4 Variant	AY337251	4, B2	E	4, B2	

<sup>a</sup>Format: AcquisitionNumber.SerotypeIsolationYear.IsolationLocation.

<sup>b</sup>Variant/Vaccine grouping based on *hexon* gene sequence as defined in Blasile et al. (2).

<sup>c</sup>(A-#) Reflects number of base substitutions from vaccine strain in 1490 bp of *hexon* sequence (Blasile et al. (2)).

<sup>d</sup>7d2 designation based on (2).

<sup>e</sup>7h designation based on fiber gene sequence as defined in Kajon et al. (10).

Text in **Bold** indicates weak positives.

Serotype/Subgroups in order of predominance.

**Species and Serotype-Specific Primer References**

Name	Sequence	Target Gene	Authors	Reference
Primer1 Primer3	CTT GGT CTA CGA CCA GAC GG GTT TGC TCA TGA ACA TGG CCA GAT CGC AC	Species B2 E3	Basler & Horwitz	Virology. 1996 Jan 15;215(2):165-77.
F30 F969	CTT CAA CCG TGT CTA CCC TAT GAA TTC TCT AAT GTA GTA AAA GG	HAdV11 Fiber	Mei & Wadell	Virology. 1993 Jun;194(2):453-62.
HsgF1 HsgF2	ATT TCT ATT CCT TCG CG TCA GGC TTG GTA CCG CC	Species F Hexon	Pring-Akerblom et al.	J Med Virol. 1999 May;58(1):87-92.
HsgC1 HsgC2	ACC TTT GAC TCT TCT GT TCC TTG TAT TTA GTA TC	Species C Hexon	Pring-Akerblom et al.	J Med Virol. 1999 May;58(1):87-92.
Ad3F Ad3R	GGT AGA GAT GCT GTT GCA GGA CCC ATC CAT TAG TGT CAT CGG T	HAdV3 Hexon	Xu et al.	J Med Virol. 2001 Aug;64(4):537-42.
Ad7F Ad7R	GGA AAG ACA TTA CTG CAG ACA AAT TTC AGG CGA AAA AGC GTC A	HAdV7 Hexon	Xu et al.	J Med Virol. 2001 Aug;64(4):537-42.
Ad21F Ad21R	GAA ATT ACA GAC GGC GAA GCC AAC CTG CTG GTT TTG CCG TTG	HAdV21 Hexon	Xu et al.	J Med Virol. 2001 Aug;64(4):537-42.
Ad4F5 Ad4R4	GTT GCT AAC TAC GAT CCA GAT ATT G CCT GGT AAG TGT CTG TCA ATC C	HAdV4 Hexon	This Study	
Ad7F-F Ad7F-R	ACA ACT GCC TAT CCT TTC AAT G GAC CAA GTT ACA CGA ATA CAA TAT G	HAdV7 Fiber	This Study	
Ad5 E1A-F1 Ad5 E1A-R1	CCT AAA ATG GCG CCT GCT ATC CTG GCG ACG CCC ACC AAC TCT CAC	HAdV5 E1A	This Study	
Ad5 E1A-F2 Ad5 E1A-R2	GAG CCT TGG GTC CCG TTT CTA TG CCA TTT TAG GAC GGC GGG TAG	HAdV5 E1A	This Study	
Ad5 hexon-F1 Ad5 hexon-R1	GAC GGA GCC AGC ATT AAG TTT GAT GTT GGC GGG TAT AGG GTA GAG CAE	HAdV5 Hexon	This Study	
Ad5 fiber-F1 Ad5 fiber-R1	TAT TCA GCA TCA CCT CCT TTC C AAG CTA TGT GGT GGT GGG GC	HAdV5 Fiber	This Study	
AdA1 AdA2	GCT GAA GAA MCV GAA GAA AAT GA CRT TTG GTC TAG GGT AAG CAC	Species A Fiber	Xu W et al.	J Clin Microbiol. 2000 Nov;38(11):4114-20.
AdB1 AdB2	TST ACC CYT ATG AAG ATG AAA GC GGA TAA GCT GTA GTR CTK GGC AT	Species B Fiber	Xu W et al.	J Clin Microbiol. 2000 Nov;38(11):4114-20.
AdC1 AdC2	TAT TCA GCA TCA CCT CCT TTC C AAG CTA TGT GGT GGT GGG GC	Species C Fiber	Xu W et al.	J Clin Microbiol. 2000 Nov;38(11):4114-20.
AdD1 AdD2	GAT GTC AAA TTC CTG GTC CAC TAC CCG TGC TGG TGT AAA AAT C	Species D Fiber	Xu W et al.	J Clin Microbiol. 2000 Nov;38(11):4114-20.
AdE1 AdE2	TCC CTA CGA TGC AGA CAA CG AGT GCC ATC TAT GCT ATC TCC	Species E Fiber	Xu W et al.	J Clin Microbiol. 2000 Nov;38(11):4114-20.
AdF1 AdF2	ACT TAA TGC TGA CAC GGG CAC TAA GTT TGT GTT ACT CCG CTC	Species F Fiber	Xu W et al.	J Clin Microbiol. 2000 Nov;38(11):4114-20.

**Table 2**

Fisher's Exact Test analyses of coinfection data

Method	Status	Number of samples with X coinfectant strains				Fisher's Exact Test statistics	
		X=1	X=2	X=3	X=4	Sample <i>P</i>	<i>p</i> value
Microarray	Vaccinated	0	4	15	2	< 0.001	< 0.001
	Unvaccinated	9	16	5	1		
Adenovirus Consensus kit	Vaccinated	8	2	8	3	< 0.001	0.011
	Unvaccinated	22	5	4	0		
Multiplex PCR	Vaccinated	17	4	0	0	0.022	0.022
	Unvaccinated	31	0	0	0		
Monoplex PCR	Vaccinated	7	11	3	0	0.004	0.031
	Unvaccinated	21	9	1	0		

Sample *P* is sample probability (or table probability). A *p* value of < 0.05 was considered significant. All *P* values are right-handed, to address the specific hypothesis that vaccinated patients carried a higher multiplicity of infection than unvaccinated patients.

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<b>14. ABSTRACT (maximum 200 words)</b>
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Population-based febrile respiratory illness surveillance was used to monitor the effectiveness of the mandatory adenovirus vaccine administered to all US military trainees. Despite the general success of the vaccination program, surveillance still detected breakthrough infections — cases of acute respiratory disease associated with the adenovirus serotypes (4 and 7) specifically targeted by the vaccine. Termination of the vaccine program in 1999 allowed collection of matching samples from an unvaccinated population in the same environment. To explore the possible role of adenoviral coinfection (simultaneous infection by multiple pathogenic adenovirus species) in the facilitation of vaccine breakthrough we compared vaccinated and unvaccinated populations using three independent methods capable of simultaneously detecting multiple adenoviral species: a 70-mer DNA microarray, a commercially available PCR-ELISA, and a multiplex PCR assay. Analysis of 52 patient throat swab samples (21 vaccinated, 31 unvaccinated) collected from 1996-2000 revealed that all vaccinated samples harbored adenoviral coinfections. Most of the coinfections were composed of community-acquired, pathogenic serotypes of both species B (serotypes 3, 7, and/or 21) and species E (serotype 4). Unvaccinated samples primarily contained just serotype 4. Coinfections may be selected in vaccinated individuals because they can produce mosaics with unique antigenicity, giving them the ability to bypass vaccine-induced immunity specific to virus particles grown in isolation. This study highlights the previously undocumented phenomenon of natural adenoviral coinfections in an environment suitable for the generation of new, potentially virulent, and uniquely selectable recombinational variants, and suggests that vaccination may play a role in coinfection.

<b>15. SUBJECT TERMS</b> febrile respiratory illness, coinfection, adenovirus, vaccination
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