

# Broad Spectrum Respiratory Pathogen Analysis of Throat Swabs from Military Recruits Reveals Interference Between Rhinoviruses and Adenoviruses

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**Abstract** Military recruits experience a high incidence of febrile respiratory illness (FRI), leading to significant morbidity and lost training time. Adenoviruses, group A *Streptococcus pyogenes*, and influenza virus are implicated in over half of the FRI cases reported at recruit training center clinics, while the etiology of the remaining cases is unclear. In this study, we explore the carriage rates and

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disease associations of adenovirus, enterovirus, rhinovirus, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* in military recruits using high-density resequencing microarrays. The results showed that rhinoviruses, adenoviruses, *S. pneumoniae*, *H. influenzae*, and *N. meningitidis* were widely distributed in recruits. Of these five agents, only adenovirus showed significant correlation with illness. Among the samples tested, only pathogens associated with FRI, such as adenovirus 4 and enterovirus 68, revealed strong temporal and spatial clustering of specific strains, indicating that they are transmitted primarily within sites. The results showed a strong negative association between adenoviral FRI and the presence of rhinoviruses in recruits, suggesting some form of viral interference.

## Introduction

The unique susceptibility of military recruits to respiratory infections, including febrile upper respiratory infections, pneumonia, pharyngitis, and bronchitis, has long been recognized [1–4]. This susceptibility is assumed to stem primarily from crowding, and is exacerbated by stress, lack of sleep, and mixing of people from diverse geographic locations [5, 6]. The primary recognized causative agents of febrile respiratory illness (FRI) in recruits include adenovirus (HAdV), influenza virus, and group A *Streptococcus pyogenes* (GAS). Influenza and GAS are well controlled by standard influenza vaccination and preventive penicillin prophylaxis. HAdV is currently associated with 15,000 of the 22,000 reported cases per year of recruit FRI requiring hospitalization or restriction to bed rest [7].

Both GAS [8] and HAdV [9] have been shown to exhibit strong site-specific strain maintenance and dynamic strain turnover patterns, with specific strains dominating at specific sites for periods of months or years. For HAdV, the patterns of dominance at different sites are essentially independent, such that one serotype or genotype will account for all identified cases at one site, while another serotype or genotype will account for all cases at another site. This offers evidence that most of the identified cases result from internal transmission: infected recruits are catching the pathogen from each other or from the training environment, rather than bringing the pathogens with them.

In addition to the above-mentioned pathogens, high rates of culture- or polymerase chain reaction (PCR)-positive results for potentially pathogenic bacteria and viruses, such as *Haemophilus influenzae* and rhinovirus (HRV), among otherwise undiagnosed FRI cases have led to suggestions that these organisms might also be transmitting and causing FRI among recruits. The role of HRV as a potential causative agent and/or facilitator of infection remains an area of ongoing research. Previous studies have indicated that diverse HRVs are present in many populations [10–12]. A few studies also have shown that HRVs appear to persist in the respiratory tract in all ages without illness for relatively long periods of time [13–15]. Some viral infections of the upper respiratory tract are known to predispose a patient to bacterial superinfection by enhancing bacterial adherence capacity [16].

Certain strains of *H. influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* are known to asymptotically colonize the nasopharynx of substantial fractions of the human population, and thereby are considered commensals [17–20]. However, other strains of these bacterial species are also responsible for significant morbidity and mortality due to the occasional invasive infection [21]. The carriage rate of each species usually stays high during childhood and diminishes in adulthood [18].

This study is an exploration of the distribution patterns of several bacterial and viral species among healthy recruits and recruits with FRI (both with and without confirmed HAdV infections). To properly address carriage rates and disease associations in a controlled manner, advanced technologies that can provide comprehensive information are needed. The Respiratory Pathogen Microarray (RPM-Flu v.3.0/3.1 arrays, Tesserae, Potomac Falls, VA, USA), a high-density resequencing microarray, can provide de novo sequence data covering hundreds of thousands of base pairs (bp) from multiple targeted bacteria and viruses, as documented in previous studies [22–27]. RPM-Flu v.3.0/3.1 arrays are designed to return data from multiple genes of 86 common respiratory organisms and high human health risk zoonotic pathogens likely to be found in the upper respiratory tract, an ideal tool for addressing the

distribution and association of multiple components of the respiratory microflora. The technology has been successfully demonstrated to identify a broad range of pathogens (including bacteria and DNA and RNA viruses) in a single test at sensitivities and specificities that are similar to or improved over those of other technologies [24, 25]

In this study, RPM technology was used to explore the distribution of, and associations between, HAdV, picornaviruses (HRV and human enterovirus [HEV]), *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* among US military recruits with and without FRI. Within the limits of the sample size, we analyzed the strain distributions of the targeted organisms. The results show that pathogens associated with recruit FRI tend to be characterized by independently site-specific strain circulation, indicating that they are transmitted primarily within sites. Common commensal organisms without clear disease associations appear to be essentially randomly distributed. Results revealed a negative association between HRV and HAdV, as well as between HRV and FRI (most of which was associated with HAdV), suggesting viral interference between HRV and HAdV. Results also showed a positive association among the three most common commensal bacteria in upper respiratory tracts, supporting observations previously made in other papers [28].

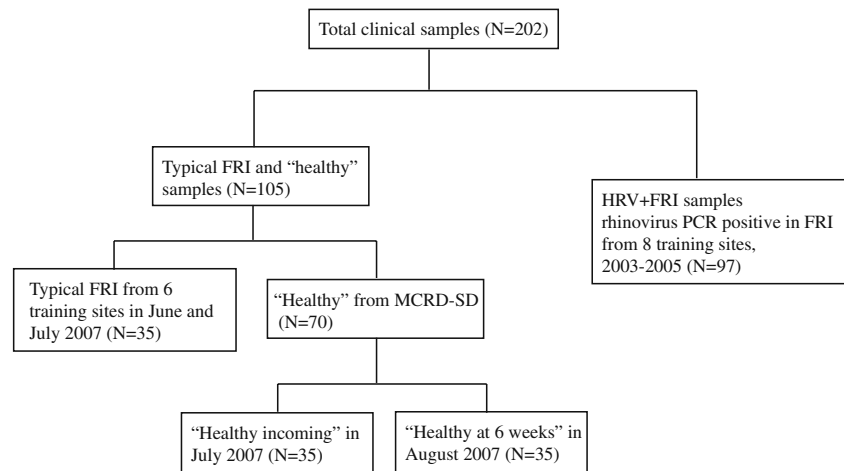
## Materials and Methods

### Specimen Collection and Sample Processing

This research has been conducted in compliance with all applicable federal and international regulations governing the protection of human subjects in research. The Naval Health Research Center (NHRC, San Diego, CA, USA) routinely collects, analyzes, and archives throat-swab specimens from consented recruits as part of respiratory infection surveillance at eight US military training camps. If available, approximately 20 recruits meeting the FRI case definition (oral temperature of  $\geq 38^{\circ}\text{C}$  and cough or a sore throat, or provider-diagnosed pneumonia) are sampled per week at clinics at each site, on a walk-in basis. Throat swabs are suspended in stabilizing transport media and archived frozen at  $-80^{\circ}\text{C}$ . Total nucleic acids were extracted from 125- $\mu\text{l}$  throat swabs using the MasterPure DNA and RNA Complete Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) and dissolved in 20  $\mu\text{l}$  of nuclease-free water.

Clinical specimens ( $N=202$ ) collected from recruits at eight US military training sites were used for this study (Fig. 1, Supplemental Table 1 and 2). These were divided into two sets for microarray studies. In the first set of 105 samples, 70 were collected during a period of 2 months (July and August) in 2007 at Marine Corps Recruit Depot,

**Figure 1** Origin and clinical correlates of samples used in this study



San Diego (MCRD San Diego). These 70 are split into two categories by disease presentation and exposure, 35 from incoming recruits ("Healthy Incoming"), and 35 from recruits in the sixth week of training who had not reported for medical treatment ("Healthy at 6 Weeks"). These were compared with 35 samples from recruits in training with FRI symptoms ("Typical FRI"), collected between June and July of 2007 at six different sites, including 16 samples from MCRD San Diego. These 105 samples were collected from the same 3-month period and 82% of them were from the same site. Demographic data from this sample set are shown in Supplemental Table 1. The second set, consisting of 97 samples, was collected from recruits at eight training sites who reported with FRI over several years, and were chosen to maximize temporal and geographic diversity for strain distribution analyses (Supplemental Table 2). Because HRV was significantly less common than any of the bacteria, we chose the second sample set on the basis of previous positive HRV PCR results. Hereafter, these will be referred to as "HRV+ FRI" specimens, though the label is not meant to indicate any etiological connection. This sample set allowed us to investigate association of HRV with FRI and other viral and bacterial species and their geographic and temporal distributions in a broad range of samples.

#### Reverse Transcriptase and Polymerase Chain Reaction

HRV was screened in clinical samples using reverse transcriptase (RT)-PCR with the following primers modified from [29]: F: 5' GGTCCCATCCCGGAATT 3' and R: 5' TCCTCCGGCCCTGAAT 3', which amplify an approximately 100-bp sequence in the conserved 5' untranslated region (5'UTR). Reactions were performed with the OneStep RT-PCR Kit (QIAGEN Inc., Valencia, CA, USA) with an initial reverse transcription at 50°C for 30 min, then preliminary denaturation at 95°C for 15 min, followed by 40

cycles of 94°C for 15 s, 52°C for 30 s, and 72°C for 30 s. Standard PCR for *S. pneumoniae*, *H. influenzae* [30, 31], and HAdV [9] was performed as previously described.

Quantitative real-time PCR assays were conducted on MyiQ instruments (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to determine the number of viral genomes in each sample. The findings for the samples were compared with those for tenfold serial dilution of prototype HRV2 templates of known copy number ( $10^1$  to  $10^6$  copies) by using specific primers (HRV-UTR-F1: TACGGTAACTTTG TACGCC and HRV-UTR-R1: ACACGGACACCCAAAG TAGT) and RT-PCR/PCR conditions as previously described [23]. Real-time PCR reactions were carried out with iQ SYBR Green supermix (Bio-Rad) in 25- $\mu$ l reaction volumes containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 200  $\mu$ M each of dATP, dTTP, dGTP, dCTP, SYBR Green I, 10 nM fluorescein, 200 nM primers, 0.625 U iTaq, and 4  $\mu$ l of extracted nucleic acid.

#### Microarray Processing and Analysis

The RPM-Flu v.3.0/3.1 design and specimen process protocols for microarray analysis have been described in detail in previous publications [22, 27]. Each sample was subjected to one microarray assay. Microarray processing, hybridization, and imaging scanning were performed according to the manufacturer's recommended protocol (Affymetrix, Inc., Santa Clara, CA, USA) using a GeneChip Resequencing Assay Kit (Affymetrix) with modification as previously described [23]. Pathogen identification for the RPM-Flu v.3.0/3.1 assay was performed using Computer-Implemented Biological Sequence Identifier Version 2.0 software [32], an automatic pathogen identification algorithm based on nucleic acid sequence alignment, which was developed and tested in detail in previous studies [24, 27]. The best match for each consensus sequence, as determined through a BLAST search (word size = 7 and E value  $<10^{-4}$ )

of the sequence against GenBank records, was regarded as the most closely related serotype of HRV.

Base call rate was calculated as number of base calls/probe length in each tiled sequence. Heat-map and clustering dendrograms were made with dChip Software 2005 (DNA-Chip Analyzer, [www.dchip.org](http://www.dchip.org)) as previously described [27]. The rows of the imported data (base call rates) were standardized and clustered. Clustering distance was set at 1 (correlation with average linkage), and gene ordering set by cluster tightness.

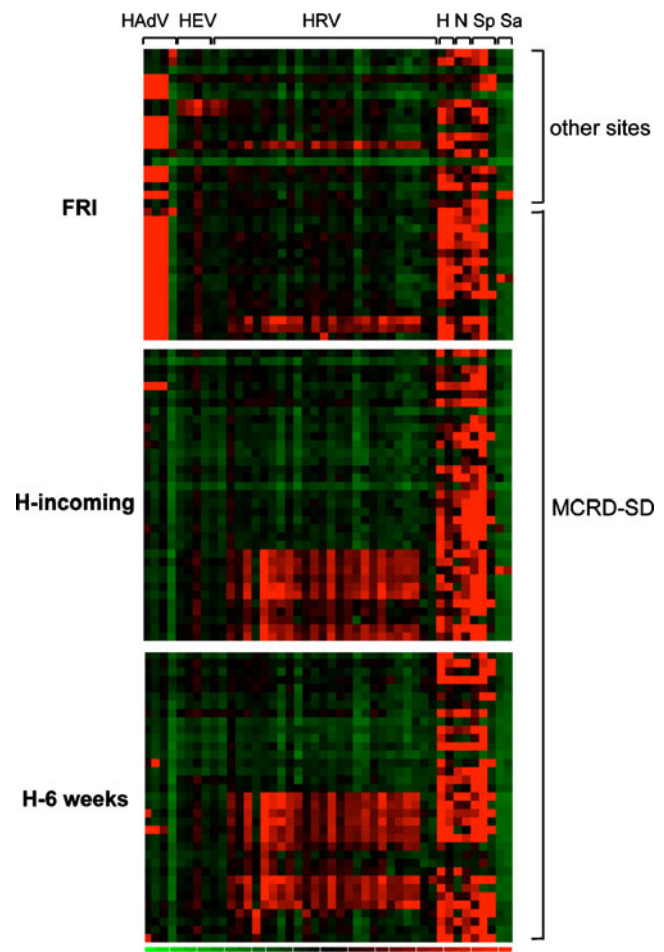
Statistical analysis of base call rates and titer for HAdV and HRV was conducted with the Microsoft Excel 2008 (Microsoft Corporation, Redmond, WA, USA) *T* test function. *p* values for differences in observed vs. expected frequencies among pathogen species were calculated using Fisher's Exact binomial test [33]. Calculations were performed using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

## Results

### Microbial Distribution in Typical FRI and Healthy Samples

The first phase of this study focuses on results from 35 Healthy Incoming recruits, 35 Healthy at 6 Weeks recruits, and 35 Typical FRI recruits. The first two categories in combination are referred to as "Healthy". This portion of samples allowed us to generate disease association data for HRV, *S. pneumoniae*, *H. influenzae*, and *N. meningitidis*, plus HAdV as, essentially, a positive control for etiology. As mild respiratory disease is ubiquitous in recruit training facilities, some unreported subclinical FRI may have been present in the Healthy at 6 Weeks population. The microarray hybridization profile of these 105 samples (Fig. 2 and Table 1) showed three major patterns of infection or colonization: HAdV concentrated in Typical FRI samples, HRV concentrated in Healthy samples, and commensal bacteria (*S. pneumoniae*, *H. influenzae*, and *N. meningitidis*) common across all samples.

The majority of Typical FRI samples [71% (25/35)] were infected with HAdV serotype 4 (HAdV-4), the primary recognized cause of acute respiratory disease in basic trainees (Table 1) [34]. Sequences generated from three HAdV-4 tiles (E1A, *fiber*, and *hexon* genes) were all identified as the currently circulating genome type, HAdV-4a [34], as represented by GenBank entry AY599837. Further analysis revealed three correlated single nucleotide polymorphisms (SNPs), including synonymous nucleotide substitutions in E1A and *hexon*, and a nonsynonymous substitution in *fiber* that clearly separate the identified HAdV-4a viruses into two distinct genotypes (genotypes 1 and 2; Table 2). Genotype 2 HAdV-4 viruses were



**Figure 2** The microarray hybridization profile of 105 samples collected from recruits in the categories Typical FRI (*FRI*), Healthy Incoming (*H-incoming*), and Healthy at 6 weeks (*H-6 weeks*). Of 35 Typical FRI samples, 16 were collected from MCRD San Diego and the remaining 19 were collected at five other training sites. All Healthy samples were collected at MCRD San Diego. *HAdV* human adenovirus, *HEV* human enterovirus, *HRV* human rhinovirus, *H* *Haemophilus influenzae*, *N* *Neisseria meningitidis*, *Sp* *Streptococcus pneumoniae*, *Sa* *Staphylococcus aureus*

exclusively present in 15 typical FRI samples collected from MCRD San Diego. Genotype 1 HAdV-4a was detected in seven samples from Fort Benning, Georgia and three samples from Fort Jackson, South Carolina. Four other samples generated hybridization signals from the HAdV-7 *fiber* gene tiles. Resequencing data from these tiles identified these as HAdV-14, an emerging serotype of HAdV recently reported at several recruit facilities [9]. HAdV-14 is not specifically tiled on the RPM-Flu arrays, but can be detected and identified from the resequencing data yielded by tiles targeted towards related serotypes. Besides HAdV, two samples from Fort Benning were positive for human coxsackievirus A21 (CAV21), an important cause of mild respiratory disease in military personnel worldwide [35]. Only four HRVs (one from Fort

**Table 1** Identification of respiratory pathogens from trainees using RPM-Flu v.3.0/3.1

Category	Samples	HAdV-4a		HAdV-14	HRVA	HRVB	HEV	Hi	Nm	Sp	Sa	
		Type 1	Type 2									
FRI	Other <sup>a</sup>	19	10	0	3	1	0	2	13 (11)	11	15 (13)	1
	MCRD	16	0	15	1	2	1	0	15 (15)	14	14 (14)	1
H-0 wk MRC	Paired <sup>b</sup>	19	0	1	0	6	0	0	14 (14)	16	15 (15)	0
	Paired <sup>b</sup>	16	0	0	0	5	0	0	13 (11)	15	16 (16)	2
H-6 wk MRC	Paired <sup>b</sup>	19	0	1	0	6	2	0	18 (18)	16	15 (14)	0
	Paired <sup>b</sup>	16	0	3	0	8	2	0	13 (13)	8	16 (15)	0
Total		105	10	20	4	29	5	2	86 (82)	80	90 (87)	4

Numbers in parentheses are samples identified positive by species-specific PCR

HAdV adenovirus, HRV human rhinovirus, HEV human enterovirus, Hi *Haemophilus influenzae*, Nm *Neisseria meningitidis*, Sp *Streptococcus pneumoniae*, Sa *Staphylococcus aureus*, FRI febrile respiratory illness

<sup>a</sup> Samples collected from six training sites other than MCRD (Marine Corps Recruit Depot San Diego). All other samples were collected at MCRD

<sup>b</sup> Paired “Healthy” samples were collected from the same 16 individuals at 0 and 6th week, respectively

Benning and three from MCRD San Diego) were detected in Typical FRI samples and all were in samples co-infected with HAdV-4a. They were identified as four different serotypes: HRV8, HRV9, HRV24, and HRV84.

Of the Healthy samples, 41% (29 of 70) were HRV+ (11 from Healthy Incoming and 18 from Healthy at 6 Weeks), in contrast to the 11% HRV+ rate in Typical FRI samples. The sequence results identified more than half of them (18) as most similar to the HRV1B serotype. The remaining had highest homologies to the 5'UTR sequences of serotypes HRV1A (one), HRV9 (two), HRV30 (two), HRV32 (one), HRV45 (one), HRV100 (one), and HRV serotype B (three). Among 70 Healthy samples, 32 were paired samples that were collected from 16 individuals at two time points. In this paired sample set, HRVs were identified in 11 individuals either upon arriving at the training site or 6 weeks later (Fig. 3). In particular, three incoming individuals carrying HRV were identified as having different serotypes 6 weeks later (Fig. 3a). For example, comparison of HRV consensus sequences from samples EVR1133T1/T2 and EVR1146T1/T2 revealed considerable divergence (Fig. 3b), demonstrating turnover in colonizing HRV serotypes during the first 6 weeks of training.

About 7% (5/70) of the Healthy samples were identified as positive for genotype 2 HAdV-4a. Four of these were identified from recruits in the sixth week of training, suggesting that these, like the symptomatic cases of HAdV-

4a, were acquired during training. This rate of asymptomatic (or less symptomatic) carriage is consistent with past reports [36]. Opposite biases in the distribution patterns of HAdV and HRV in Typical FRI samples as compared to Healthy samples (Fig. 2) suggest that HAdV infection, fever, or both might exclude or interfere with the presence of HRV (or, possibly, that HRV precludes adenoviral FRI).

The results from the first 105 specimens also revealed that all three studied commensal bacteria were common in all sample sets (Fig. 2 and Table 1). *S. pneumoniae* was detected in 86% of the samples, *H. influenzae* in 82%, and *N. meningitidis* in 80%. Detection of high frequencies of these commensal bacteria among these samples was further confirmed by species-specific PCR assays for *H. influenzae* and *S. pneumoniae* (Table 1). It was not found that any of these bacteria were associated with either disease or time spent in the recruit facility.

Distributions of HAdV, HRV, and Commensal Bacteria in HRV+ FRI samples

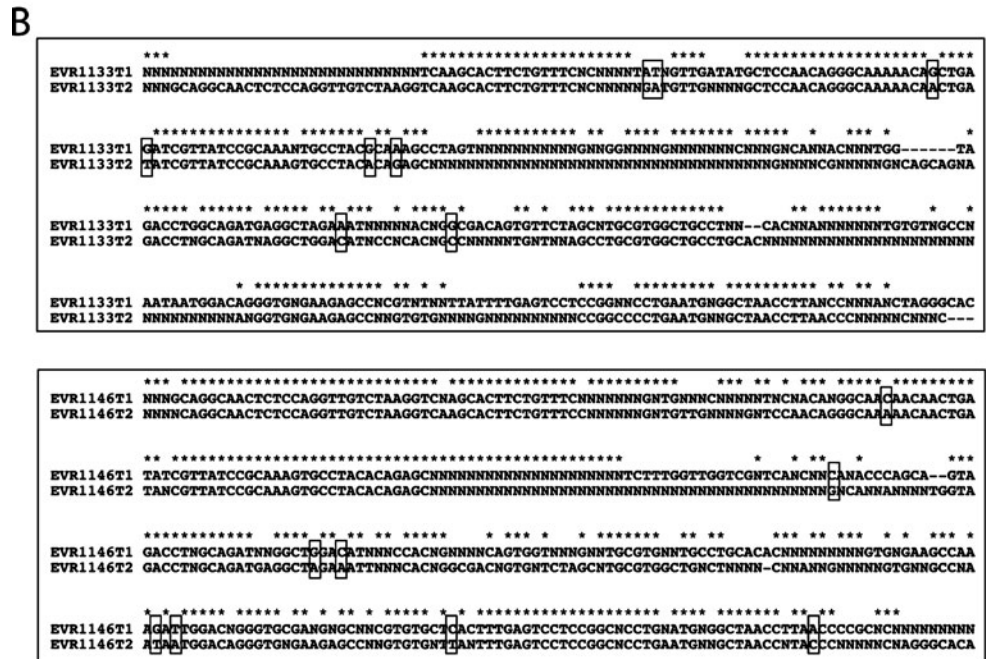
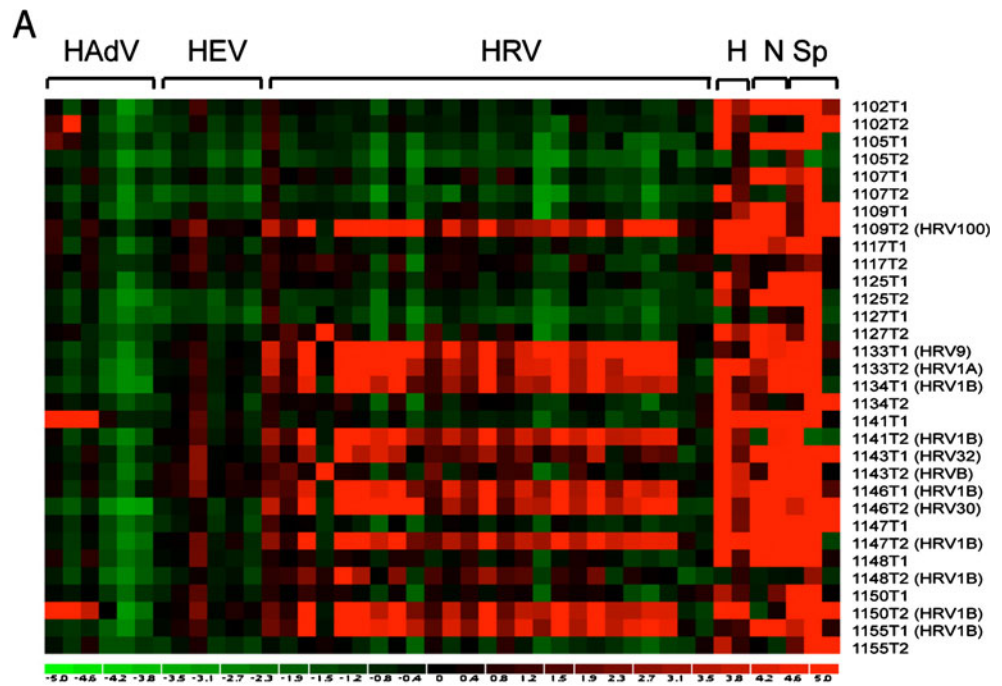
We extended the investigation of microbial flora to include more clinical samples from broader geographic sites and to further understand the associations (or lack thereof) between HRV, HAdV, and commensal bacteria. To this end, we analyzed 97 clinical samples collected from recruits with FRI symptoms at eight military bases from 2003 to 2005 (except for two, one each from 2000 and 2006), all of which were previously identified as HRV+ by PCR [29] (Supplemental Table 2). We also analyzed five cultured HAdV-14 samples and one blank used as blind negative controls for HRV.

Table 3 and supplemental Figure 1 show that 89% (86/97) of the HRV+ FRI samples were HRV+ and/or HEV+ by

**Table 2** Genotyping of adenovirus 4a

	E1A-139	Fiber-436	Hexon-192
Genotype 1	CTG/Leu	CAT/Arg	TTT/Phe
Genotype 2	TTG/Leu	CGT/His	TTC/Phe

**Figure 3 a** Microarray hybridization profile of 32 paired samples, collected from 16 Healthy individuals on arrival and again 6 weeks after arriving at the MCRD San Diego training site. In each paired sample, the upper row (T1) represents Healthy Incoming and the lower row (T2) represents Healthy at 6 Weeks. Recognized strains of human rhinovirus (HRV) most closely matching the clinical strains are designated in parenthesis. **b** Two pairwise comparisons of HRV consensus sequences from the same individual collected at different time points. Nucleotide discrepancies are framed in boxes



RPM-Flu analysis, and 51% (49/97) were HAdV-4+. The colonization rates of commensal bacteria in this sample set were *H. influenzae* (88%, 85/97), *S. pneumoniae* (81%, 79/97), and *N. meningitidis* (76%, 74/97) (Table 3), similar to the rates seen in the initial sample set. In addition, a few other common respiratory viruses and bacteria (influenza A/H3N2, parainfluenza 3, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, and *S. pyogenes*) were occasionally detected (Table 3). Overall, the majority of samples were colonized with three to six of the

respiratory viruses and/or bacteria detectable with the RPM-Flu v.3.0/3.1 assay.

**Adenovirus**

Detailed analysis of HAdV-4 resequencing information generated on the RPM-Flu v.3.0/3.1 arrays from the 49 HAdV-4+ HRV+ FRI samples resulted in identification of two different strains: HAdV-4p4 (an HAdV-4 prototype or vaccine strain, only recognized as circulating at the Coast

**Table 3** Summary of respiratory pathogen identification in eight training sites with RPM-Flu v.3.0/3.1 microarrays

Location	Sample	Date	HAdV			HRV		HEV	Hi	Spn	Nm	Mp	Pa	Kp	Spy	Mc	Flu	PIV3
			4	14	2	A	B											
MCRD-SD	37	2003–2005	22	0	1	27	3	7	34	30	31	2	5	3		2		1
MCRD-PI	19	2003–2006	12	2	1	15	2	0	18	14	14	1	3	1	4	1		
Lackland AFB	14	2000–2005	3	0		10	0	0	10	14	9		1	4		1	1	
Ft. Jackson	10	2003–2005	4	0		8	0	0	8	8	8	1			2			
Ft. Leonard Wood	7	2004–2005	4	0		4	0	0	6	4	5			1				
Ft. Benning	6	2003–2005	1	1		5	0	1	5	5	4		1			1		
CGTC	3	2003–2005	3	0		3	0	0	3	3	2		1			1		
NSTC	1	2005	0	0		1	0	0	1	1	1	0	1	0	0			
Total	97	2003–2006	49	3	2	73	5	8	85	79	74	4	12	9	6	6	1	1

*HAdV* adenovirus, *HRV* human rhinovirus, *HEV* human enterovirus, *Hi* *Haemophilus influenzae*, *Spn* *Streptococcus pneumoniae*, *Nm* *Neisseria meningitidis*, *Mp* *Mycoplasma pneumoniae*, *Pa* *Pseudomonas aeruginosa*, *Kp* *Klebsiella pneumoniae*, *Spy* *Streptococcus pyogenes*, *Mc* *Moraxella catarrhalis*, *Flu* influenza virus, *PIV* parainfluenza virus, *MCRD* Marine Corps Recruit Depot, *SD* San Diego, *PI* Parris Island, *CGTC* Coast Guard Training Center, Cape May, NJ, *NSTC* Naval Service Training Command, Great Lakes, IL

Guard Training Center Cape May, New Jersey, in the last 10 years [34]) and HAdV-4a (common at all other sites [34]). The two samples with HAdV-4p4 were, in fact, collected at Cape May, while the remaining 47 samples were collected at other sites. Further analysis of the HAdV-4a strains based on SNPs in *fiber*, *hexon*, and *E1A* genes revealed two genotypes matching those in the Typical FRI sample set: genotype 2 HAdV-4a was only present at the MCRD San Diego site while genotype 1 HAdV-4a was circulating at the remaining training sites. Hence, all three detected HAdV-4 variants were geographically and temporally segregated, with strong agreement between the sample sets and between the data generated here using the RPM-Flu v.3.0/3.1 assay and data reported elsewhere [34] generated using strain-specific PCR and whole-genome restriction enzyme analysis.

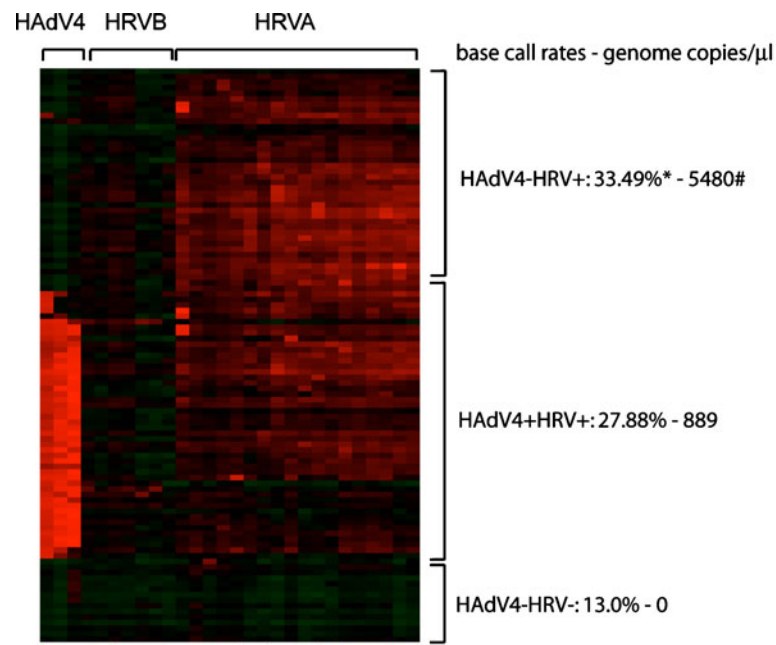
#### Rhinovirus and Enterovirus

The resequencing results from HRV and HEV tiles revealed three distinct genetic clusters of picornaviruses in the HRV+ FRI samples: HRVA (73), HRVB (five), and HEV (eight; Table 3 and Supplemental Figure 2A). Seven of eight HEVs were identified as enterovirus 68 (EV68), all of which were found in MCRD San Diego samples. These EV68 isolates shared identical sequences except for a single nucleotide polymorphism in one instance. These data suggested that clonal EV68 was endemic in MCRD San Diego during 2004–2005, showing geographic and temporal clustering reminiscent of HAdV-4 outbreaks. CAV21 was identified in one Fort Benning sample, which corresponded to the previous finding in the Typical FRI sample set, where two CAV21 were detected at the same site. This might indicate

that CAV21 was transmitted at the Fort Benning training site. In contrast, the 78 HRV isolates were identified as closely related to 41 known reference serotypes (Supplemental Table 3) and showed broad genetic diversity based on phylogenetic analysis (Supplemental Figure 2B). No dominant HRV serotype was identified from any particular training site and season. This result suggests HRV colonization in HRV+ FRI trainees was not caused by endemic transmission but instead carried over by hosts. No geographical and/or temporal dependency was observed for these samples, though the limited sample size may be insufficient to detect significant spatial or temporal patterns for a virus with this degree of diversity.

#### Association of HAdV and HRV

Microarray results showed that 46 of the 73 HRVA+ samples from the HRV+ FRI sample set were also HAdV-4+. The findings from the initial sample set (see “Microbial Distribution in Typical FRI and Healthy samples”) revealed that HAdV-4 may be negatively associated with HRV. This prompted us to further investigate the relationship between these two viruses in the HRV+ FRI data set. Visually, the hybridization strengths of HRVA+ samples co-infected with HAdV-4 (HAdV-4+/HRVA+) appear relatively weaker than those without HAdV-4 co-infection (HAdV-4-/HRVA+; Fig. 4). Base call rates were compared to see if there was a significant statistical difference. In previous studies, a correlation between stronger hybridization (i.e., more base calls) on the RPM-Flu arrays and higher concentrations of nucleic acid templates was observed, so overall base call rates indirectly reflect template titers (unpublished data). The base call rate in HRVA tiles for



\*  $p < 0.007$ , base call rate comparison between HAdV4-HRV+ and HAdV4+HRV+,  
#  $p < 0.04$  viral titer comparison between HAdV4-HRV+ and HAdV4+HRV+.

**Figure 4** HAdV-4 co-infection effect on HRV titer in 97 HRV+ FRI clinical samples. Hybridization profiling in HAdV-4 and HRVA tiling regions shows relative hybridization intensities. HRV titers calculated with quantitative real-time PCR are also shown. Average base call rates and genome copies were calculated based on three distinct clusters: HAdV-4 negative and HRV positive (HAdV-4-/HRV+), HAdV-4 positive and HRV positive (HAdV-4+/HRV+), and HAdV-4

negative and HRV negative (HAdV-4-/HRV-). Analyses of significance were generated from comparison of clusters HAdV-4-/HRV+ and HAdV-4+/HRV+. The base call rate comparison between HAdV-4-/HRV+ and HAdV-4+/HRV+ yielded  $p < 0.007$ . The viral titer comparison of HAdV-4-/HRV+ and HAdV-4+/HRV+ yielded  $p < 0.04$ . HAdV human adenovirus, HEV human enterovirus, HRV human rhinovirus

control samples and HRVA- clinical samples is 13.0%, due to background noise. Within the set of 105 samples chosen on the basis of HRV+ PCR results, the average base call rate for HRV tiles in HAdV-4- samples was 33.49%, compared with 27.88% for HAdV-4+ samples ( $p < 0.007$ ). This suggests that the titers of HRVA are lower when HAdV-4 is present.

This result was independently validated by using quantitative real-time RT-PCR. The average HRV titer in HAdV-4-/HRVA+ samples was 5,480 genome copies/ $\mu$ l, approximately six times higher than the 880 genome copies/ $\mu$ l seen in HAdV-4+/HRVA+ samples ( $p < 0.04$ ). This result may indicate that infection by HAdV interferes with the replication efficiency of HRV, or that HAdV infection is less likely in hosts with higher HRV loads.

#### Commensal Bacteria

*H. influenzae*, *S. pneumoniae*, and *N. meningitidis* were detected in more than 76% of HRV+ FRI samples (Table 3). In addition, sequences generated from microarrays were able to distinguish them at the strain level. All 85 *H. influenzae* strains belonged to nontypeable *H. influenzae*,

nonencapsulated strains that are usually harbored as normal flora in the nasopharynx [37]. The 79 *S. pneumoniae* strains were classified into two major species: *Streptococcus mitis* and *S. pneumoniae*, both of which belong to the *S. mitis* group and are recognized commensals of the upper respiratory tract [37]. The 74 *N. meningitidis* strains cluster with members of serogroup C, which usually inhabits the human nasopharynx without causing detectable diseases [38]. The three commensal bacteria were identified simultaneously in 65% (63/97) of the HRV+ FRI samples, with no statistically significant association with HRV or HAdV (Table 4). Single and double bacterial colonizations were more rarely observed. Analysis of the distributions of *H. influenzae*, *S. pneumoniae*, and *N. meningitidis* using Fisher's exact statistics showed strongly significant non-randomness (see Table 5a;  $p < 0.002$ ). Analysis of specific observed and expected frequencies of the possible combinations suggested most of the apparent significance derived from higher-than-expected rates of both triple infections (with all three co-present) and null infections (with none of the three). Removing the five null samples from the analysis on the basis that these could have resulted from poor sample quality yielded a loss of significance (see



**Table 4** Co-colonization of three major commensal bacterial in HRV+ FRI samples

	HRV/HEV positive (84)		HRV/HEV negative (13)	
	Ad+ (47)	Ad- (37)	Ad+ (5)	Ad- (8)
Hi-Spn-Nm	34	23	2	4
Hi-Spn	6	4	0	0
Hi-Nm	2	3	2	0
Spn-Nm	1	3	0	0
Hi	1	2	1	1
Spn	0	1	0	1
Nm	0	0	0	0
None	2	1	0	2

Ad adenovirus, FRI febrile respiratory illness, HEV human enterovirus, HRV human rhinovirus, Hi *Haemophilus influenzae*, Nm *Neisseria meningitidis*, Spn *Streptococcus pneumoniae*

Table 5b;  $p < 0.08$ ). Hence, the data were suggestive of a positive association between all three. There is certainly room for alternative interpretation. Only five clinical samples did not have any of the three commensal bacteria. Three of them were infected with HRVA viruses, one was infected with *M. pneumoniae*, and the remaining one had no identifiable pathogens or commensals at all.

**Discussion**

Our data clearly show that infection with HAdV, the main FRI agent in military training facilities, is associated with decreased rates and titers of HRV, suggesting some form of viral interference between these groups. The data also suggest that FRI-causing pathogens are geographically segregated and clonal, while commensals are more diverse and do not display obvious temporal or spatial clustering. Commensal bacteria are positively associated with each other in the upper respiratory tract, suggesting some synergy or common mechanism favoring colonization in specific individuals. Furthermore, while the majority of picornaviruses identified in this study were apparently randomly distributed HRVs, the MCRD San Diego site harbored an apparently site-associated strain of EV68 and Fort Benning harbored CAV21, both of which were concentrated among ill individuals, unlike other identified picornaviruses. Studies should be undertaken to determine the potential health impact of these two viruses.

As expected, our microarray results indicated that more than 50% of FRI samples (either those chosen without respect to HRV status or those chosen because they were HRV+) were infected with HAdV-4, the well-recognized dominant agent of FRI among recruits. The data supported

**Table 5** Statistic analysis of association of three commensal bacteria in HRV+FRI samples

A. with 5 “no positive”

		No. (%)	Expected frequency (%)				Observed frequency (%)					
Hi	-	11 (11.5)	None	Nm	Spn	Spn+Nm	None	Nm	Spn	Spn+Nm		
	+	85 (88.5)	Hi--	0.6	1.6	2.2	7.3	Hi-	5.2	0.0	2.1	4.2
Nm	-	22 (22.9)	Hi+	4.6	12.1	16.7	56.2	Hi+	5.2	7.3	10.4	65.6
	+	74 (77.1)										
Spn	-	17 (17.7)	Expected count (no.): 96				Observed count (No.)					
	+	79 (82.3)	None	Nm	Spn	Spn+Nm	None	Nm	Spn	Spn+Nm		
	None	5 (5.2)	Hi-	1	2	2	7	Hi-	5	0	2	4
	Something	91 (94.8)	Hi+	4	12	16	54	Hi+	5	7	10	63

Fisher’s exact test probability of the observed distribution ( $P \sim 0.0021$ )

B. with 5 “no positive” omitted

		No. (%)	Expected frequency (%)				Observed frequency (%)					
Hi	-	6 (6.6)	None	Nm	Spn	Spn+Nm	None	Nm	Spn	Spn+Nm		
	+	85 (93.4)	Hi-	0.3	0.7	1.1	4.7	Hi-	0.0	0.0	2.1	4.2
Nm	-	17 (18.7)	Hi+	4.9	10.0	15.1	65.9	Hi+	5.2	7.3	10.4	65.6
	+	74 (81.3)										
Spn	-	12 (13.2)	Expected count (No.): 91				Observed count (No.)					
	+	79 (86.8)	None	Nm	Spn	Spn+Nm	None	Nm	Spn	Spn+Nm		
	None	5 (5.2)	Hi-	0	1	1	4	Hi-	0	0	2	4
	Something	91 (94.8)	Hi+	4	9	14	60	Hi+	5	7	10	63

Fisher’s exact test probability of the observed distribution ( $P \sim 0.0759$ )

Note. FRI, febrile respiratory illness; HRV, human rhinovirus; Hi, *Haemophilus influenzae*; Nm, *Neisseria meningitidis*; Spn, *Streptococcus pneumoniae*.

previous studies showing that HAdV outbreaks in training facilities are clonal and geographically segregated. The three variants identified are consistent with the three major genome types defined by restriction enzyme analysis (REA) [34]. The identified geographic pattern of these three is also consistent with the pattern found using REA [34].

An apparent example of previously unstudied virus circulating locally in training sites is offered by EV68, one of the most rarely reported HEV serotypes. US surveillance over a 36-year period (1970–2005) only reported 26 cases (of a total of 52,812 HEV cases reported), and approximately one fourth of those reports were in adults aged 20 years or older [39]. This study identified seven EV68 cases from 37 HRV+ FRI trainees exclusively at MCRD San Diego in a 2-year period (2004–2005) (Table 3). Identical 5'UTR sequences of these isolates revealed by the microarray agrees with the monophyletic characteristics of EV68 [40]. Apparently, the outbreak of EV68 was geographically segregated, but its origin and any potential disease associations remain a mystery.

In contrast, multiple HRVs were identified both in Healthy and FRI recruits, revealing broad and scattered genetic diversity among military training sites. No geographic or temporal clustering of specific HRVs was observed. Highly diverse HRVs have also been documented in other populations in recent independent studies [10–12]. The clinical significance of HRV diversity will be an interesting and challenging topic for future investigation.

Our data show a significant negative association between HRV and FRI, though this appears to be a secondary effect of a negative association between HRV and HAdV, the primary cause of recruit FRI. There is certainly no evidence that HRV is causing FRI in this population. Multiple HRV serotypes were identified at the same training site and even from the same individual, suggesting HRV is ubiquitous, diverse, and commonly subject to turnover.

The results showed opposing distribution patterns (negative association) of HAdV and HRV, with Healthy samples showing high rates of HRV and low rates of HAdV, and Typical FRI samples showing high rates of HAdV and low rates of HRV (Fig. 2). This result suggests some form of interference between HRV and HAdV (or possibly all-cause FRI). This observation was supported by results from the HRV+ FRI samples (Fig. 4), which indicated that, even among samples chosen on the basis of HRV positivity, the presence of HAdV is associated with reduced titers of HRV. As all of these samples were from patients with FRI, this suggests that the negative association is specifically between HRV and HAdV, rather than extending to all-cause FRI. Viral interference has been recently reported in two epidemiological studies [41, 42], suggesting that HRV might have affected the spread of pandemic influenza A (H1N1) during 2009. One possible explanation for this

interference is that HRV infection causes the cellular immune response to enter an antiviral state [43] making double viral infections less common in the population [42]. Another possible explanation is that the fevers induced by influenza and HAdV are high enough to restrict replication of HRV.

The microarray data indicated that 65% of bacteria-positive samples were triple colonizations, with fewer double or single colonizations occurring than would be expected by chance (Table 5). A recent study reported that the colonization of *H. influenzae* in the upper respiratory tract is negatively associated with colonization of either *S. pneumoniae* or *M. catarrhalis*, but the combination of all three shifts the competitive interaction to positive association [28]. The association of multiple microbial organisms may be more resistant to clearance by immune response [28]. Thus, addition of one more species might alter the competitive balance between two species and allow them all to successfully colonize together. Our results are consistent with this finding; however, our sample size is too small to address this conclusively. The mechanism of this multi-organism colony needs to be further investigated.

From a clinical standpoint, possibly the most important result of this study is the failure to observe an association between colonization with *H. influenzae*, *S. pneumoniae*, *N. meningitidis*, HRV, or any combination thereof, with FRI in military recruits. The identification of EV68 with a specific site, and the similarity of the molecular epidemiology of this virus to recognized outbreak pathogens such as GAS and HAdV, suggests that this virus may warrant further study as a potential agent of significant contagious disease. The same is true for CAV21. From the standpoint of microbial ecology, the most interesting finding was the strong negative association, suggestive of interference between HRV (whether in terms of titer or absolute occurrence) and HAdV-associated FRI.

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