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Detection of Adenoviruses (AdV) in Culture-Negative Environmental Samples by PCR during an AdV-Associated Respiratory Disease Outbreak

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Since 1954, adenoviruses (AdV) have been recognized as an important cause of acute respiratory disease (ARD) among U.S. military recruits. Until recently, routine oral vaccination for AdV serotypes 4 and 7 eliminated epidemic AdV-associated ARD in this population. Now that the manufacturer has ceased production, vaccination has ended and AdV epidemics have reappeared. As part of a prospective epidemiological study during the high-risk ARD season, serial samples were obtained from ventilation system filters and tested for AdV by culture and PCR. An outbreak occurred during this surveillance. Of 59 air filters, 26 (44%) were AdV positive only by PCR. Sequence analysis confirmed the presence of AdV serotype 4, the implicated outbreak serotype. The number of AdV-related hospitalizations was directly correlated with the proportion of filters containing AdV; correlation coefficients were 0.86 (Pearson) and 0.90 (Spearman's rho). This is the first report describing a PCR method to detect airborne AdV during an ARD outbreak. It suggests that this technique can detect and quantify AdV-associated ARD exposure and may enable further definition of environmental effects on AdV-associated ARD spread.

Since 1954, adenovirus (AdV) types 4 and 7 have been recognized as major pathogens in epidemics of acute respiratory disease (ARD) among U.S. military recruits (9, 10). To prevent these infections, live oral AdV type 4 and 7 vaccines were developed in the 1960s (8, 12). These vaccines were licensed in 1980, and routine administration to recruits was initiated. Until recently, these vaccines were highly successful in preventing AdV-associated ARD; however, with interruptions of the vaccine supply due to the manufacturer's decision to terminate production, AdV-associated ARD has reemerged at basic training facilities (5).

In the fall and winter of 1997 and 1998, outbreaks of AdV type 4 respiratory illness occurred among recruits at Fort Jackson, S.C. (L. Binn, J. Sanchez, F. Mitchell-Raymundo, S. Kolavic, C. Polyak, S. Cersovsky, and B. Innis, Clin. Infect. Dis. **29:**1086 [abstr. 707], 1999; J. L. Sanchez, T. Lee, R. N. Nang, J. P. Marquez, S. C. Craig, L. N. Binn, F. D. Mitchell, B. L. Innis, R. Reynolds, J. Conolly, R. M. Hendrix, and D. A. Carroll, Program Abstr. 47th Annu. Meet. Am. Soc. Trop. Med. Hyg., abstr. 310, 1998 [Am. J. Trop. Med. Hyg. **50**(Suppl. 3)**:**216]). In 1998, a prospective study of a vaccine-free cohort of Army trainees was initiated in part to identify host and environmental risk factors for AdV infection (Binn et al., abstract). Previous studies by Artenstein et al. demonstrated the detection of viable AdV in the room air of patients with ARD (3, 4).

The development of a sensitive PCR method (7) offered an additional means for AdV detection. Accordingly, studies were performed to evaluate the ability of the PCR method and conventional cell culture isolation tests to detect AdV in environmental specimens. This report describes the first study demonstrating the successful use of a PCR method to detect AdV from environmental sources. This approach may enable future evaluation of the environmental factors that may contribute to AdV-associated respiratory disease epidemics.

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MATERIALS AND METHODS

Samples. Serial environmental specimens were obtained by swabbing the surfaces of high-efficiency filters from eight air handling systems (ventilators). These ventilators provided the air circulation to the barracks at Fort Jackson, S.C. The design of the ventilation system uses 90% of the recirculated air of the barracks. Recruits were divided into three groups, companies A, C, and D, for training and sleeping during the 8-week basic training cycle. The three companies were located in the same building. Recruit sleeping areas were located in the east and west wings on the second and third floors of the building. Company A had an older ventilation system of four separate units, one for each floor (second and third) and wing (east and west). Companies C and D had newer ventilation systems located only on the second floor, one unit on each wing supplying air to both floors. Filters from all the ventilation units were swabbed on the side where the air entered into the circulation unit (dirty side). An area of 1 ft^2 in the center of each filter was swabbed every 2 weeks with a cotton swab premoistened in the cell culture medium. Specifically, in company A one sample was obtained from each filter (total $n = 4$) at each time. In companies C and D, samples from the top and bottom filters of each ventilation unit were sampled (total $n = 4$ per company) at each time. In companies A and C, filters were changed twice, between weeks 2 and 4 and between weeks 6 and 8. In company D, filters were changed only once between weeks 6 and 8. A total of 59 samples were collected at 2-week intervals from 25 September through 21 November 1998, which corresponded to training weeks 0, 2, 4, 6, and 8. Samples were inoculated into 5 ml of cell culture medium with antibiotics and frozen at −70°C until tested. In addition, swab specimens from two telephones in each of the three company areas (A, C, and D) were obtained on 21 November (week 8).

These three recruit companies comprised a cohort under observation for ARD. During the period when environmental samples were obtained, a respiratory disease outbreak occurred (Binn et al., abstract). Illness and hospitalization rates were recorded weekly, and subsequent analysis indicated that 72% of ARD cases were the result of infection with AdV type 4 (Binn et al., abstract).

Culture. The collected frozen samples were thawed, vortexed, centrifuged, and inoculated in A549 cells in tube cultures, and the remaining aliquot was refrozen

TABLE 1. Distribution of AdV PCR results from nonextracted air filter samples per ventilator

Ventilator no.	Company (location)	Result for training wk:				
		0	2	4	6	8
1	A (west, 2nd floor)	ND[b]	+	+	+	−
2	A (east, 2nd floor)	−	+	+	+	+
3	A (west, 3rd floor)	−	−	−	+	−
4	A (east, 3rd floor)	−	+[a]	−	−	−
5	C (west, top filter)	−	+	+	+	−
	C (west, bottom filter)	−	+	+	+	−
6	C (east, top filter)	−	+	+	−	−
	C (east, bottom filter)	−	−	+	+	−
7	D (west, top filter)	−	−	+[a]	+	−
	D (west, bottom filter)	+[a]	−	+[a]	+	−
8	D (east, top filter)	+	−	−	−	−
	D (east, bottom filter)	−	−	+	−	−

[a] Negative result when tested after extraction.
[b] ND, not done.

until tested by PCR. The cultures were incubated for 21 days at 35°C and observed twice a week for AdV cytopathic effects. The susceptibility of the cells was verified by titration with AdV types 4 and 7, as positive controls. Uninoculated cell culture tubes were included as negative controls.

PCR. PCR was performed as described by Echavarria et al. (7). Detection of the amplified products was done on agarose gels stained with ethidium bromide using a digital microscopy documentation system (Kodak Digital Science DC 120; Kodak, Rochester, N.Y.). DNA extraction from the samples was performed using the QIAamp blood kit (Qiagen Inc., Valencia, Calif.). From the eluted DNA, 10 μl of template was used for the PCR. Both extracted and nonextracted environmental samples were tested. Each test included a negative control (water) and a positive control (AdV type 4); assays were accepted only when all controls gave the expected result. Investigators were blinded in regard to specimen source and culture result.

Sequencing. DNA products from three air filters that produced the 139-bp fragment consistent with AdV were sequenced. DNA templates were purified with a PCR purification kit (QIAquick; Qiagen Inc.). Sequencing reactions were performed with the Applied Biosystems International (Foster City, Calif.) Prism dRhodamine Terminator Cycle Sequencing kit according to the manufacturer's specifications. Primers used for sequencing were the same as those for the generic PCR performed for AdV (7). Nucleic acid sequences were determined on an Applied Biosystems Model 377 DNA Sequencher. Editing of derived sequences was performed with Sequencher software (Gene Codes Corporation, Ann Arbor, Mich.). Sequences were compared with the current GenBank database using the BLAST algorithm (2). Probability values (*E* values) for random matches of these sequences were obtained from the BLAST search.

Definition of case. AdV-associated ARD was defined as a febrile illness (oral temperature of 100.5°F or greater) with a throat swab culture positive for AdV and the presence of one or more of the following signs and/or symptoms: sore throat, cough, rhinorrhea, nasal congestion, sinus tenderness, rales, rhonchi, or wheezing. For disease control purposes, Army recruits who meet the ARD case definition are routinely admitted to the local military hospital. The number of hospitalized patients reported only during weeks 0, 2, 4, 6, and 8 was analyzed.

Statistical analysis. A chi-square test for difference between proportions was calculated using the STATA statistical program (STATA Release 6; College Station, Tex.). Correlation coefficients were calculated using Pearson's and Spearman's rho methods with SPSS version 8.0.1 (SPSS Inc., Chicago, Ill.).

RESULTS

A total of 65 environmental samples (59 from air filters and 6 from telephones) were tested by culture and PCR. A 139-bp fragment indicating the presence of AdV was amplified from 26 of 59 nonextracted air filter samples (44%) and 22 of 59 (37%) extracted ones (Table 1). The results from extracted and nonextracted samples were concordant for 55 of 59 air filter samples (93%). None of the telephone specimens obtained at week 8 were positive for AdV by PCR. Attempts to recover AdV in cell cultures from air filters or phone surfaces were unsuccessful; all 65 samples were culture negative.

The distribution of the PCR results from air filter samples (for all three companies together) and the number of AdV-infected hospitalized patients reported during weeks 0, 2, 4, 6, and 8 of basic training are shown in Fig. 1. Based on the results from nonextracted samples, detection of AdV DNA in air filters increased from week 0 to week 4 (18 to 75%; $P = 0.004$) and declined to 8% at week 8 ($P < 0.001$). The proportion of air filters AdV positive by PCR was found to be greatest during the epidemic peak of AdV cases, at week 4. A positive correlation was seen to occur for the 8-week period, and correlation coefficients were estimated at 0.86 ($P = 0.06$, Pearson's method) and 0.90 ($P = 0.04$, Spearman's rho method).

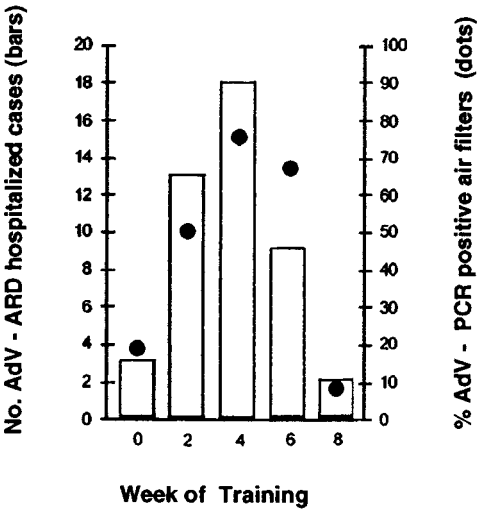

FIG. 1. Distribution of AdV-infected hospitalized patients and AdV PCR-positive air filters per week of training. Bars represent total numbers of AdV cases, and dots represent percentages of air filters positive for AdV by PCR.

To identify the amplified fragments, we analyzed the nucleotide sequence of amplified fragments from three samples selected randomly and confirmed to be PCR positive with and without extraction. The sequences had highly significant homology with AdV serotype 4; the probability values (*E* values) for a random match were 4×10^{-29}, 8×10^{-30}, and 9×10^{-36}. Eighty-one percent (78 of 96) of hospitalized ARD patients were found to be infected with AdV by throat culture; 72% (69 of 96) were culture positive for AdV serotype 4 (Binn et al., abstract).

DISCUSSION

This study clearly demonstrated that PCR is effective in detecting AdV on air filters during an outbreak of AdV-associated respiratory disease. The nucleotide sequence of three samples was identified as AdV type 4, the same AdV serotype causing 72% of the ARD cases during the outbreak (Binn et al., abstract). This work represents an advance in sensitivity, as attempts to isolate AdV from environmental samples by culture were negative. The use of controls in all assays, previous assessment of specificity (7), and correlation with clinical findings (rate of hospitalization) support the validity of these results.

Environmental factors associated with viral particle inactivation may explain the failure to detect the virus in cell culture (11). Several publications describe the relation between survival of viruses and relative humidity and temperature and the type of surface contaminated (1, 6, 11).

Moreover, the amount of virus present in the air filters may not have been sufficient to be detected by culture. Artenstein et al. reported that large air volume sampling is necessary to

detect one viable particle (3, 4). Molecular techniques such as PCR methods can be more sensitive than cell culture. The PCR method used in this study is capable of detecting concentrations as low as 0.2 PFU of AdV type 11 per ml (7).

PCR results from air filters for all three companies were correlated with the number of hospitalizations due to AdV-associated ARD during the same period. The peak of disease occurred at the same time that the greatest proportion of samples was AdV positive by PCR. We hypothesize that as the virus concentration in barracks air increases (as reflected in the proportion of positive filters) more residents are exposed and are infected. ARD cases were seen to occur in all platoons in all three companies during this study. Nevertheless, company A, which had the older ventilation system (one ventilator per floor and per wing), showed the lowest ARD attack rate (11%) in comparison to ARD attack rates for recruits sleeping in companies C (21%) and D (18%), with the newer ventilation system (one ventilator supplying air for both floors). The filter recovery rates at 2-week intervals did correlate with the total number of hospitalized ARD patients. These results suggest that testing of environmental samples for AdV by PCR could yield epidemiologically relevant data.

The outbreak waned after week 6; thus, it is not surprising that the filters (which were replaced between weeks 6 and 8) were mostly negative when sampled at week 8. On the other hand, 75% of the replaced filters were AdV positive at week 4, which correlated with the highest hospitalization rate (the peak of the outbreak).

Swab samples from telephone surfaces were negative for AdV by PCR and culture. Although these samples were limited to the end of the study period (i.e., week 8), when AdV-associated illness was at its lowest level, we considered these negative results valuable since they provided further evidence that the PCR testing was accurate and free of cross-contamination. Further studies are required to evaluate telephones as potential fomites that may play a role in AdV transmission.

The presence of inhibitory substances in samples interferes with polymerase enzymes used for the PCR method, decreasing the sensitivity of detection and producing false-negative results (7). When inhibitors are present, DNA extraction can improve test sensitivity, although loss of DNA during the process is always possible. In this study, similar results were obtained from extracted and nonextracted environmental samples. Therefore, the environmental samples that we collected probably contained no or few inhibitors. The fact that four of the samples were positive only when nonextracted samples were tested may be explained by a dilution effect introduced by the extraction procedure. A definitive evaluation of the need for extraction with air filter samples should be done.

This initial study strongly supports the utility of environmental sampling to detect AdV. As AdV tends to survive better at high relative humidity (6), prospective studies incorporating manipulation of the environment (i.e., humidity, temperature, etc.) may serve to further reveal the reasons for the high rate of AdV-associated ARD among recruits in basic training. Environmental control of airborne spread of AdV may help reduce the risk of AdV-associated outbreaks during periods of vaccine unavailability. Additional studies at Fort Jackson and/or other recruit training facilities should be conducted to confirm the value of PCR in detecting AdV from environmental samples and to determine the effect of environmental manipulations on AdV infection rates.

In summary, this is the first study utilizing a PCR method for detection of AdV from environmental samples during an AdV-associated outbreak. AdV detection by PCR represents an advance in the study of environmental factors in respiratory disease. Such a tool enables the rapid study of various environmental interventions, which may be effective in reducing or possibly eradicating important respiratory pathogens.

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