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Use of PCR To Demonstrate Presence of Adenovirus Species B, C, or F as Well as Coinfection with Two Adenovirus Species in Children with Flu-Like Symptoms

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Adenovirus (AdV) respiratory infections have usually been associated with species B, C, and E. In this study, we detected 9.4% of AdVs by PCR in 500 nasal swabs from 319 children with influenza-like symptoms. AdV typing by PCR with specific probes showed species C, B, and F as well as coinfection with two species. Coinfection with two AdV species and the presence of species F in respiratory samples are novel findings that should be further investigated.

Adenovirus (AdV) respiratory infections are common, especially during childhood and among military recruits (4, 10, 12). In children less than 4 years old, 2 to 8% of acute respiratory infections are caused by AdVs (3, 10, 15, 18). Although these infections are generally self-limited, AdVs have also been associated with severe lower respiratory infection with significant morbidity and mortality in immunocompetent children (13, 14, 17). To date, 51 AdV serotypes have been described, grouped in six species, named A, B, C, D, E, and F, associated with different spectra of diseases (2, 5). Respiratory infections with AdVs have usually been associated with species B, C, and E, whereas species F viruses seem to be restricted to the enteric tract (9). Within species B, serotype 7 h in particular has been associated with high morbidity and mortality as well as lung sequelae in children with lower respiratory infection in the south cone of America (14).

The use of molecular methods for AdV diagnosis has significantly increased in the last years although there are few studies evaluating AdV in nasal swab samples by PCR in outpatient children with mild respiratory disease (1, 6, 7, 8, 11).

The purpose of this study was to determine the AdV frequency in upper respiratory samples by PCR and to classify AdV into species in outpatient children with flu-like symptoms that were negative for influenza virus. In addition, a longitudinal study was performed for patients with sequentially obtained samples.

Patients and specimens. Specimens originated from a clinical trial of the efficacy of a live attenuated influenza vaccine in Argentina. Healthy children between 6 and 48 months of age were enrolled after informed consent was obtained from their parents. Children received either influenza vaccine or placebo and were followed up for respiratory symptoms during 10 months. Nasal swab specimens from both nostrils were taken when children exhibited any signs or symptoms of respiratory infection within 4 days of the onset of the illness. Specimens were obtained by trained physicians, placed in virus transport medium, and kept at 4°C until shipped to the Centro de Educación Médica e Investigaciones Clínicas Clinical Virology Laboratory in Buenos Aires. Each sample was cultured for influenza virus, and the remaining aliquot was kept at −70°C. From this collection of 6,000 nasal swabs, we randomly selected 500 influenza virus culture-negative samples obtained from 319 patients in August, September, October, or November 2002, corresponding to the end of winter and the spring in Argentina. The patients were seen at 10 outpatient clinics in two provinces from Argentina (Córdoba and Neuquén).

Generic hexon PCR. DNA extraction was performed with the QIAamp DNA blood minikit (QIAGEN Inc., Valencia, CA). DNA preparations were stored at −70°C until the second PCR was performed. The generic PCR amplifying a region of the hexon gene was performed as previously described (6). The bands from amplified products were categorized according to their intensities in the agarose gel. Weakly positive bands had intensity equal to or lower than the 10^2-PFU/ml positive control. Strongly positive bands showed intensities higher than this control. The analytical sensitivity of the AdV PCR was 10 PFU/ml for AdV 7. To determine the stability of the extracted AdV DNA in response to freeze-thaw cycles, control preparations containing 10^3 and 10^2 PFU/ml were evaluated.

The stronger positive AdV control (10^3 PFU/ml) remained positive by PCR after 10 freeze-thaw cycles while the lower positive control (10^2 PFU/ml) became negative by PCR after the third freeze-thaw cycle.

Commercial PCR. A commercial PCR-hybridization-immunoenzymatic assay called "Adenovirus Consensus PCR" (Argene, Varilhes, France), which amplifies the virus-associated RNA gene, was used for retesting positive PCR results. AdV typing was performed by hybridization with specific biotinylated probes to the seven AdV subspecies, including A, B:1, B:2, C, D, E, and F (16). The analytical sensitivity of this PCR was 10 PFU/ml for AdV 7.

AdV frequency. Thirty of the 319 patients tested AdV positive by the generic PCR, indicating a prevalence rate of 9.4% (95% confidence interval: 6.3% to 13.4%). No significant dif-

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TABLE 1. Comparison of positive AdV generic hexon PCR samples with the Adenovirus Consensus PCR for nasal swab samples from children with flu-like symptoms

Generic hexon PCR result[a] (*n*)	No. (%) of samples with commercial PCR (Adenovirus Consensus) result of:	
	Positive	Negative
Strong positive (17)	17 (100)	0
Weak positive (15)	9 (60)	6 (40)
Total (32)	26 (81)	6 (19)

[a] Strong positive, band intensity higher than the 10^2-PFU/ml positive AdV control; weak positive, band intensity lower than or equal to the 10^2-PFU/ml positive AdV control.

ferences were observed between the rates in both provinces and different months.

Retesting of positive samples and typing. Of the 32 specimens positive by the generic PCR, 26 (81%) scored also positive by the commercial Adenovirus Consensus PCR performed on the corresponding frozen-DNA-extracted samples (Table 1). All the 17 strongly positive samples by the generic PCR were positive by the commercial PCR. Of the 15 weakly positive samples by the generic PCR, only 9 were positive by the commercial PCR.

Viruses from these 26 AdV-positive samples were further typed into subspecies; it was found that 17 samples (65%) were infected with species C, 5 (19%) showed coinfection with two species; 3 (11%) were infected with species B:1, and 1 (4%) was infected with species F. The five samples with dual infection included one positive for species B:1 and B:2, two positive for species B:1 and C, and two positive for species C and F.

All AdV-positive patients showed mild respiratory symptoms, and five of them presented with bronchitis. In the patients with bronchitis, AdV species C was detected in three of them and a coinfection with species B:1 and C was detected in one patient, while the AdV strain from the other patient could not be typed.

Longitudinal study. Out of 30 patients with an AdV-positive sample, 11 had several specimens available for testing since they exhibited several episodes of acute mild respiratory infections. Ten of these 11 patients had a negative PCR result between 1 and 12 weeks after a positive one. Surprisingly, in one patient a simultaneous infection with two different AdV species (B:1 and C) was detected. One and 8 weeks later, he was positive for AdV species C only, and 3 weeks later he became AdV negative.

Different prevalence rates of AdV infections in respiratory samples have been published. The differences can be due to a variety of factors, including age of the patients, severity of disease, type of sample, diagnostic method, and time of the year. Using conventional diagnostic methods, a prevalence of 2 to 8% has been observed for AdV infections of hospitalized children with lower respiratory infections (3, 10, 15, 18). In the present study, we examined for the presence of AdV in nasal swabs from nonhospitalized children with influenza-like symptoms using a sensitive PCR and observed a rate of 9.4%. Here, AdV infection was associated with mild respiratory manifestations although other respiratory viruses, except for influenza virus, could not be ruled out since they had not been evaluated. Ten patients that showed an initial positive PCR result became negative in a subsequent sample, indicating the absence of a prolonged detection of DNA in nasal swabs. This may reflect the autolimitation of these infections including the clearance of viral DNA from the nasopharynx. A previous study evaluating throat swab specimens from healthy young adults showed no AdV detection by PCR or culture, suggesting the absence of latency or asymptomatic shedding (8). Further studies with children to evaluate AdV by PCR in nasal swabs after a respiratory infection are needed to better establish the period of persistence of DNA in the nasopharyngeal tract.

In general, AdV respiratory infections have been associated with species B, C, and E. Upper respiratory illnesses of children due to AdV have been reported to be mostly associated with species C, while species B infections prevail in children with acute lower respiratory disease (3, 13). In this study, patients showed mild respiratory infections and 65% of samples belonged to species C, while 11% involved species B:1. Particularly noteworthy is the detection of AdV species F in nasal swabs from two patients, one as a single species and the other mixed with another AdV species. Species F includes AdV serotypes 40 and 41, which are called "enteric AdV" because they are associated with gastrointestinal disease. To our knowledge, detection of these types in respiratory specimens has never been published. Since AdV 40 and 41 are fastidious viruses and fail to grow efficiently in the cell lines which are commonly used for clinical diagnosis, these serotypes may be not identified if conventional virus culture is used for detection, especially because they are not expected in respiratory specimens. Recently, AdV of species F has also been detected in respiratory samples from two patients when using the Adenovirus Consensus PCR, further confirmed by sequencing analysis (F. Freymuth, personal communication). However, the significance of detecting species F in the respiratory tract is not clear and deserves further investigation. Whether the replication of the AdV F virus occurs in the respiratory tract or whether this tissue can act as the "porte d'entrée" should be carefully studied.

Coinfection with two AdV species was also an unexpected finding in this population and could have been missed if nonmolecular methods had been used. When AdV typing is performed by neutralization assays after virus amplification in cell culture, coinfection is more likely to be missed if those viruses fail to grow with the same efficiency rate.

In this study, results of generic hexon-positive PCR were further evaluated with a commercial PCR which amplifies a different AdV gene. Although the analytical sensitivities were equal for both PCR assays, the latter PCR seemed less sensitive than the former. Important to note is that the commercial PCR was performed on extracted specimens conserved at −70°C; therefore there was an additional freeze-thaw cycle. We showed that preparations with DNA concentrations equivalent to 10^2 PFU/ml are less stable after several freeze-thaw cycles compared to specimens with higher DNA concentrations. A comparison of both PCR methods showed that only preparations containing low DNA concentrations, equivalent to 10^2 PFU/ml or lower, were missed by the commercial PCR. Interpretation of results should be cautious when extracted samples with low viral load are thawed several times.

In summary, our results have shown a rate of AdV detection by PCR of 9.4% in nasal swabs from nonhospitalized children with mild respiratory symptoms, mostly due to species C. Coinfection with two species and the presence of species F were also documented. Prolonged AdV infection was not detected in nasal swabs by PCR in these children. Coinfection with two AdV species and the presence of species F in respiratory specimens are interesting novel findings that need to be further evaluated.

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