

Adenoviruses C in non-hospitalized Mexican children older than five years of age with acute respiratory infection

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Adenoviruses (AdV) are commonly involved in acute respiratory infections (ARI), which cause high morbidity and mortality in children. AdV are grouped in six species (A-F), which are associated with a wide range of diseases. The aim of this study was to identify the AdV species infecting non-hospitalized Mexican children with ARI symptoms, attending to the same school. For that, a PCR/RFLP assay was designed for a region of the hexon gene, which was chosen, based on the bioinformatical analysis of AdV genomes obtained from GenBank. A total of 100 children's nasopharyngeal samples were collected from January to June, 2005, and used for viral isolation in A549 cells and PCR/RFLP analysis. Only 15 samples produced cytopathic effect, and in all of them AdV C was identified. AdV C was also identified in eight additional nasopharyngeal samples which were negative for viral isolation. In summary, this outpatient population showed a rate of AdV infection of 23%, and only AdV C was detected.

Key word: PCR/RFLP - adenovirus C - ARI - non-hospitalized children

Acute respiratory infections (ARI) represent one of the main health problems in children in developing countries. Although ARI are spread worldwide, they have smaller impact in industrialized countries than in developing ones. Whereas 1 to 3% of the deaths in children under five years of age in industrialized countries are due to pneumonia, in the developing countries this disease causes 10 to 25% of the deaths in children of the same age group. (Weissenbacher & Avila 1999). ARI are also one of the main reasons for health consultations and hospitalizations, and the main basis for drug prescriptions. In most cases, these drugs are unnecessary, potentially dangerous, and can stimulate bacterial resistance. Excessive and unnecessary use of medications significantly increases health care costs without benefiting the patient's health (Benguigui et al. 1999).

The etiological agents of ARI encompass a wide variety of microorganisms, and viruses are the most frequently involved agents (Benguigui et al. 1999). Adenoviruses (AdV) are the third most common viral respiratory pathogen in children, after respiratory syncytial and parainfluenza viruses. AdV infections are common in all age groups, causing both hospital- and community-acquired epidemics (Benguigui et al. 1999). AdV constitute a large family of nonenveloped viruses containing a linear double-stranded DNA genome of approximately 36 kbp. To date, 51 different serotypes of human AdV have been classified into six species, named A, B, C, D,

E, and F (which have been associated to different clinical infections), based on erythrocyte agglutination assays and on DNA sequence homology. Respiratory infections have usually been associated with AdV species B, C, and E; whereas species F seem to be restricted to the enteric tract (Lee & Gruber 1999, Echavarría 2004, Fauquet et al. 2005, Wold & Horwitz 2007).

Diagnosis of AdV infections has typically relied on the detection of a cytopathic effect (CPE) in various cell lines (Mufson 2000). This method has been considered as the "gold standard" for AdV diagnosis. Although viral isolation provides a sensitive method for diagnosis, it can take as long as three weeks to obtain results. Immunofluorescence and other immunodiagnostic methods using direct antigen detection are faster than culture, but they lack sensitivity (Minnich & Ray 1980, Mahafzah & Landry 1989, Portillo & Cruz 2000). Recently polymerase chain reaction (PCR)-based assays have proven to be useful for AdV detection, showing higher specificity and sensitivity in comparison to the classic methods (Allard et al. 1990, Pring-Åkerblom & Adrian 1994, Alistair et al. 1996, Kajiwara et al. 1999, Xu et al. 2000, Avellón et al. 2001, Echavarría et al. 2001, Heim et al. 2003).

AdV typing is critical for epidemiological surveillance, detection of new strains, assessment of treatment efficacy and understanding of AdV pathogenesis (Casas et al. 2005, Ebner et al. 2005a, b, Metzgar et al. 2005, Morfin et al. 2005). Typing has usually been done by neutralization or hemagglutination inhibition (Rosen 1960, Hierholzer 1973, Hierholzer et al. 1991). To circumvent practical problems associated with traditional serum neutralization studies molecular typing methods have been proposed (Kidd et al. 1996, Li et al. 1999, Elnifro et al. 2000), such as, restriction endonuclease analysis of full-length adenovirus DNA or by PCR using type-specific primers (Garnet et al. 2002). Other approaches have been the PCR amplification of the hypervariable

Financial support: IPN, SIP (2004628, 20051208), INER, SSA
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Received 20 October 2007
Accepted 12 March 2008

region of the hexon gene followed by DNA sequencing (Takeuchi et al. 1999, Sarantis et al. 2004), PCR amplification followed by restriction endonuclease analysis, and multiplex PCR-enzyme hybridization methods with species-specific-oligonucleotide primers and probes (Gu et al. 2003, Pehler-Harrington et al. 2004, Echavarría et al. 2006).

There have been few studies about AdV infections in non-hospitalized children with mild respiratory infection (Freymuth et al. 1997), and in Mexico there is a lack of published data about the etiological agents causing ARI (Manjarrez et al. 2003, Cabello et al. 2006). For that reason, we investigated the presence of AdV involved in respiratory infections of non-hospitalized children, and in order to have an easy method for AdV screening in clinical samples and at the same time determine which AdV species were involved, a PCR followed by an enzymatic digestion using only one restriction enzyme was designed.

PATIENTS, MATERIALS AND METHODS

Viral propagation - Viral isolation and propagation were carried out according to the procedures described by Mufson (2000). Briefly, AdV reference strains: serotype 5 and 6 (species C), serotype 4 (species E) used in this study were obtained from American Type Culture Collection. AdV serotype 41 (species F) isolated from a patient with gastrointestinal infection, was identified and serotyped as AdV 41 at the Instituto Nacional de Diagnóstico y Referencia, SSA, México. Clinical samples: children older than five years of age (6 to 12 years old), attending to the same Elementary School in Mexico city were enrolled after the informed consent was obtained from their parents. A total of 100 nasopharyngeal aspirates were taken from January to June 2005, when children exhibited signs or symptoms of respiratory infection, within three days of the onset of the illness. Specimens were collected in transport medium (Lebovitz L-15, In Vitro, México D.F.) added with 0.5% of bovine albumin (Sigma-Aldrich) and antibiotics (penicillin/streptomycin 100 UI/ml and 100 µg/ml, Sigma), and sent to the laboratory. Samples were clarified by centrifugation for 5 min at 6,000g and transferred to cryovials and immediately frozen at -70°C.

AdV reference strains were propagated in A549 human lung carcinoma cells grown in Eagle's minimal essential medium added with 10% fetal bovine serum.

Clinical samples were inoculated by duplicate in A549 human lung carcinoma cells grown in four well Nunclon multidishes. Two wells were used as mock infected cell control. The cultures were incubated at 37°C and daily scored for CPE. When CPE was observed, the monolayer was scraped and used for AdV detection by PCR. If the cultures did not show CPE after 21 days they were discarded.

DNA extraction - Viral genomic DNA was extracted from 500 µl of reference viral lysate, viral isolates and clinical samples using the QIAamp® UltraSens™ Virus Kit (QIAGEN, cat 53704), following the manufacturer's instructions.

Primers and PCR amplification - A set of primers was designed based on the in silico analysis of the AdV hexon gene. For that, AdV sequences obtained from GenBank (Table) were aligned to identify conserved regions by using ClustalW (www.ebi.ac.uk/tools/clustalw). Forward and reverse primers were designed using the Primer3 software (Rozen & Skaletsky 2000). The specificity of each one of the primers was assessed using the BLAST algorithm available at the National Center for Biotechnology Information website (Maglott et al. 2000). The sequences of the designed primers are shown below: Forward 5'TCCTTCTTCAGAAACTTCCGCCATGAG3', and reverse 5'TCGATGACGCCGCGGTGCGGCTG3'. The PCR was carried out using the Taq PCR core kit (QIAGEN, cat 201203), according to the manufacturer's instructions. Amplification was performed in a thermocycler (TECHNE Genius) and PCR conditions were: an initial denaturing at 94°C for 10 min and 35 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 40 s, with a final elongation at 72°C for 7 min. PCR products were analyzed on a 1% agarose gel stained with ethidium bromide.

Amplicon restriction - The designed primers predicted a 434 bp amplicon for all the AdV tested. Each amplicon sequences was analyzed using the Webcutter program version 2 (www.webcutter.com), in order to obtain a different restriction fragment length pattern (RFLP) for each AdV species, using only one restriction enzyme. It was found that the digestion of the predicted amplicons with HaeIII produced a different RFLP for each AdV species (Table). A sample of 10 µl of PCR-amplified DNA product was incubated with 1U of HaeIII

TABLE
Expected and obtained RFLP for the adenoviruses (AdV) 434 bp amplicon digested with HaeIII

GenBank Accession numbers	AdV Species	Analyzed serotype ^a	Expected RFLP ^a (Size fragments, bp)	Tested serotype	Obtained RFLP (Size fragments, bp)
NC_001460	A	12	57, 122, 255	NT	
NC_004001	B	11	70, 160, 204	NT	
NC_001405	C	2	144, 290	5, 6	144, 290
NC_002067	D	17	70, 109, 118, 137	NT	
NC_003266	E	4	12, 58, 70, 144, 150	4	12, 58, 70, 144, 150
NC_001454	F	40	87, 160, 187	41	70, 118, 246

a: by bioinformatical analysis; NT: non tested.

restriction endonuclease (Invitrogen) for 3 h. After digestion, the products were analyzed by 12 % acrylamide gel electrophoresis, using the standard acrylamide monomer: N,N'-methylenebisacrylamide concentration (30:1), stained with ethidium bromide.

RESULTS

Adenovirus isolation in cell culture - Fifteen nasopharyngeal samples out of 100 samples of children with ARI showed AdV-like CPE, characterized by rounding and grapelike clustering of the swollen infected cells. These viral isolates were obtained from children in all age groups, and the number of viral isolates did not showed significative differences among the analyzed children age's groups.

Adenovirus detection by PCR - The expected 434 bp amplicon from the hexon gene was obtained in all the AdV reference strains tested (Fig. 1). A similar amplicon was observed in the 15 viral isolates and in eight nasopharyngeal samples which did not produce CPE, although all the negative CPE nasopharyngeal samples were tested for AdV presence by using the designed PCR.

Typing of Adenovirus species by RFLP - The digestion of the amplicons from the AdV reference strains with HaeIII showed the expected RFLP for the serotypes 5 and 6 (species C), and for the serotype 4 (species E). The serotype 41 (species F) did not produce the expected RFLP, instead of that, three fragments of different size were obtained (70, 118, 246 bp), but this RFLP was clearly different from the RFLP of AdV C or E (Fig. 2A, Table).

Typing of Adenovirus from children with ARI - After restriction with HaeIII, all the 23 adenovirus amplicons (8 from the children's samples and 15 viral isolates) showed the characteristic RFLP of AdV C (2 fragments of 144 and 290 bp) as shown in Fig. 2B.

Distribution of adenovirus according to children's age and time of sampling - The higher number of students with symptoms of ARI was found on January, February and March, and the lowest number was found on June. AdV were only detected in children suffering ARI from January to April; being January the month with the highest number of cases of ARI positive for adenovirus. The nasopharyngeal samples taken on May and

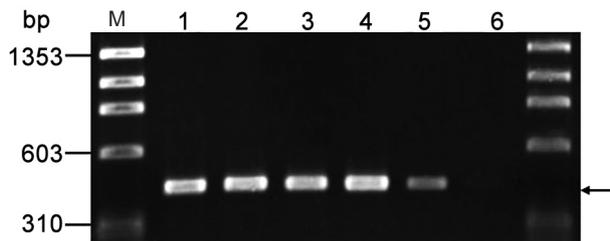


Fig. 1: adenoviruses (AdV) 434 bp amplicon obtained by polymerase chain reaction (PCR) using AdV reference strains. Agarose gel electrophoresis. Lanes - M: molecular size markers (ΦX174 HaeIII digest); 1: serotype 4 (species E); 2: serotype 5 (species C); 3: serotype 6 (species C); 4: serotype 41 (species F); 5: a nasopharyngeal sample; 6: negative control. The arrow indicates the 434 bp amplicon.

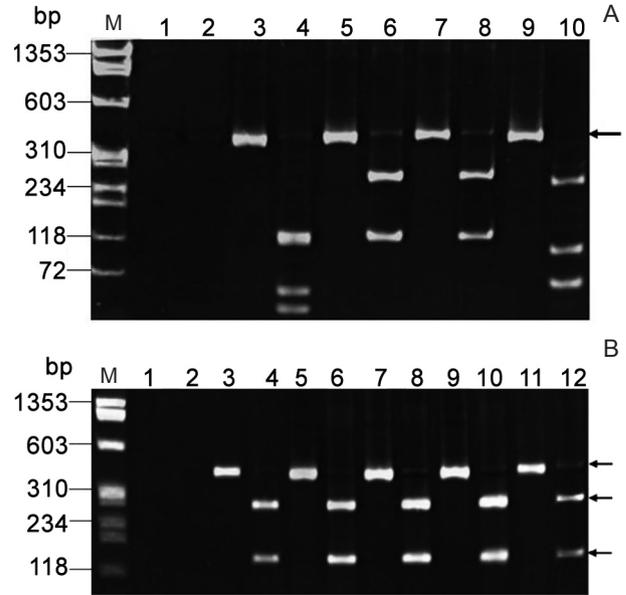


Fig. 2A: restriction fragment length polymorphism (RFLP) of the reference adenoviruses (AdV) strains amplicon digested with HaeIII. 12% polyacrylamide gel. Lanes - M: molecular size markers: ΦX174 HaeIII digested; 3, 5, 7, and 9: undigested; 4, 6, 8, and 10: digested with HaeIII. The arrow indicates the 434 bp amplicon; 1-2: negative controls; 3-4: serotype 4 (species E); 5-6: serotype 5 (species C); 7-8: serotype 6 (species C); 9-10: serotype 41 (species F). B: RFLP of the AdV amplicon obtained from representative clinical isolates. Lanes - M: molecular size markers (ΦX174 HaeIII digest); 3, 5, 7, 9, 11: undigested amplicons; 4, 6, 8, 10, 12: amplicons digested with HaeIII; 1-2: negative controls; 3-4: positive control (serotype 5, AdV C); 5-6: viral isolate; 7-8: viral isolate; 9-10: viral isolate; 11-12: viral isolate. The arrows indicate the 434 bp amplicon; and the 144 and 290 bp fragments.

June were negative for adenovirus either by PCR or by isolation in cell culture (Fig. 3). There was no difference on the rate of Adenovirus detection and the age of the children. The symptoms presented by children with acute respiratory infection were similar in both patients positive for adenovirus and in those negative.

DISCUSSION

Adenovirus infections occur worldwide in humans and are common in all age groups, causing both hospital- and community-acquired epidemics. AdV probably account for 3% of the infections in civilian population. In children younger than five years old, they cause about 5% of upper tract respiratory infections, and are probably responsible for about 10% of the pneumonias in childhood. Approximately by age 6, 95% of children are seropositive for AdV (D'Ambrosio et al. 1982).

In Mexican children suffering acute respiratory infections there have been very few studies analyzing the viral etiology for these infections. Furthermore, most of the studies have investigated respiratory syncytial viruses and recently metapneumovirus, especially in children during the first two years of life (Golubjatnikov et al. 1975, Tirado et al. 1995, Bustamante-Calvillo et al. 2001, Manjarrez et al. 2003, Noyola et al. 2005, Ca-

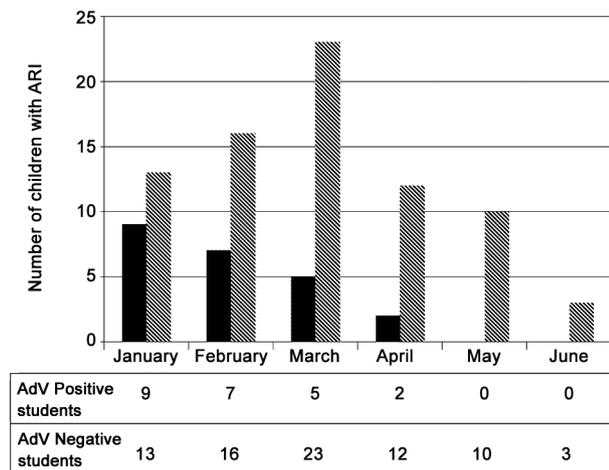


Fig. 3: AdV detection in children with acute respiratory infections (ARI) according to the date of sampling. The dark bars corresponded to the number of children with ARI and positive for AdV C, and the dashed bars show the number of children with ARI but without AdV.

bello et al. 2006). Therefore the aim of this paper was to investigate the presence of AdV associated to ARI in non-hospitalized children older than five years old, as an example of adenovirus infection in a community, and at the same time to identify the AdV species involved in these infections. For that reason, children attending to the same Elementary School in Mexico city were enrolled in this study, and nasopharyngeal samples were taken from each child when he or she had symptoms of acute respiratory infection. The period of sampling was during the winter-spring seasons (January-June, 2005). AdV were isolated from 15 nasopharyngeal samples out of 100 samples inoculated in A549 cells. These viral isolates were identified as AdV by PCR, since a fragment of 434 bp from the hexon gene was amplified. This amplicon was also obtained with the reference strains tested and had the expected size according to the bioinformatical analysis performed with the GenBank AdV sequences. Besides the 15 viral isolates, other eight nasopharyngeal samples negative for viral isolation in cell culture were positive for AdV by PCR detection. This finding agrees with previous studies which have shown that even though viral culture has been considered as the "gold standard" for AdV diagnosis, the PCR-based assays are more sensitive for this purpose (Allard et al. 1990, Pring-Åkerblom & Adrian 1994, Alistair et al. 1996, Kajiwara et al. 1999, Xu et al. 2000, Avellón et al. 2001, Echavarría et al. 2001, Heim et al. 2003).

The 23 AdV amplicons obtained from the children's samples analyzed by restriction using only one restriction enzyme such as HaeIII produced the RFLP for AdV C. This RFLP showed the number and the size fragments expected according to the *in silico* analysis carried out with AdV C serotype 2, and was similar to the one obtained by digestion of the two AdV C reference strains tested (serotypes 5 and 6). These results suggested that the PCR/RFLP method described in this paper could be a useful screening approach for AdV detection and at the

same time for AdV species typing, using a single restriction enzyme, HaeIII, in contrast to others RFLP strategies which have been used to identify adenoviruses serotypes or species by using several restriction enzymes (Li et al. 1999, Elnifro et al. 2000, Allard et al. 2001, Ebner et al. 2006). However, to evaluate the capability of the PCR/RFLP described in this paper to detect the different AdV species is necessary to test more AdV reference strains and isolates, since sequencing of the AdV hexon gene has revealed a divergence at protein level of 0.5 to 25.4% among the 51 serotypes (Ebner et al. 2005a), and this variability may explain the unexpected RFLP found with the AdV serotype 41 (species F).

The finding of AdV C in all the children's samples agrees with previous studies which have reported that AdV C is mostly associated to upper respiratory infections in children (Echavarría et al. 2006). It is known that AdV C (serotypes Ad1, Ad2, Ad5, and Ad6) infect more than 80% of the human population early in life, causing roughly 5% of symptomatic upper respiratory infections and 15% of lower respiratory tract infections in children younger than five years. Besides that, AdV C can establish an asymptomatic infection in immunocompetent hosts characterized by intermittent viral excretion in nasopharyngeal secretions, and also display prolonged fecal excretion for months, and even years (Garnett et al. 2002, Wold & Horwitz 2007). However, there is not information if persistent infections also occur in children like the population that was studied in this paper; therefore it would be important to investigate that, since persistent adenovirus DNA in the lungs has been postulated to be a cofactor in chronic obstructive pulmonary disease in adults (Matsuse et al. 1992).

AdV infections are usually more frequent in winter and spring, as it was found in this paper. The infections were distributed among the different age groups (6-12 years old) and no differences were found in the respiratory symptoms between the students' positive for adenovirus and the students with ARI without AdV, implying that other pathogens may be involved in these respiratory infections. In summary the set of primers used and the restriction of the amplicon with one enzyme allowed the detection and typing of the AdV C infecting children with ARI and attending to the same Elementary School, suggesting that AdV C is probably the most frequent adenovirus associated to upper respiratory infections in non-hospitalized patients as has been reported in hospitalized children.

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