Adenoviruses associated with acute gastroenteritis in hospitalized and community children up to 5 years old in Rio de Janeiro and Salvador, Brazil

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Acute gastroenteritis is a major source of morbidity and mortality among young children in developed and developing countries. Human adenoviruses (HAdVs), and in particular species F, are related to childhood diarrhoea worldwide. This study presents the results obtained during an investigation of HAdVs causing acute gastroenteritis in children hospitalized in Rio de Janeiro, RJ, Brazil, from April 1996 to September 2003, as well as in children with diarrhoea living in the slums of Salvador, BA, Brazil, from October 2001 to September 2003. A total of 3060 stool samples was analysed by an enzyme immunoassay for rotavirus and adenovirus (EIARA) and 61 (2 %) were found to be positive. HAdV presented with low prevalence throughout the year, with a slight but not significant increase in incidence in late summer and early autumn. Children up to 2 years of age were the most frequently affected (79 % of all positive samples). All positive samples were analysed further by generic and species-specific HAdV PCR protocols, confirming 100 % specificity of this rapid and inexpensive EIARA. Species F was the most prevalent (65 %), despite the occurrence of species A (12 %), C, D and co-infection F/D (5 % each) and species B and co-infections F/A, F/C and B/D (2 % each). In order to type the species F strains as HAdV-40 or -41, generic PCR and a *Hin*fI restriction digest were performed. HAdV-40 and -41 were found to represent 62 % (23/37) and 38 % (14/37), respectively. These results demonstrated that a combination of generic and species-specific PCRs is useful and reliable for HAdV species and type identification directly from faecal specimens. The results confirmed the endemism of human adenoviruses, mainly species F, in children as aetiological agents of diarrhoea, although the limited sensitivity of EIARA as a screening method may have underestimated their prevalence.

INTRODUCTION

Human adenoviruses (HAdVs) are one the major causes of a number of different clinical syndromes including gastroenteritis, respiratory disease, conjunctivitis, haemorrhagic cystitis and exanthema (Horwitz, 2001). They comprise 51 different serotypes (HAdV-1 to -51) grouped into 6 species, A to F (de Jong *et al.*, 1999).

Abbreviations: EIARA, enzyme immunoassay for rotavirus and adenovirus; HAdV, human adenovirus.

The GenBank/EMBL/DDBJ accession numbers for the hexon gene sequences of the HAdV-41 samples determined in this work are DQ504432–DQ504434.

Acute infectious diarrhoea is a common disease in young children throughout the world. Estimated incidence rates in developing countries range from 2.1 to 3.8 episodes per child per year for children between 11 and 48 months (Kosek *et al.*, 2003). In Brazil, diarrhoea presents one of the principal morbidity indexes and causes of mortality in the first year of life (http://w3.datasus.gov.br/datasus/datasus.php).

Identification of HAdV species or serotypes can be achieved, with different degrees of efficiency, by serotype-specific neutralization tests (Hierholzer, 1995) and restriction endonuclease analysis of DNA extracted from infected cells (Wadell *et al.*, 1980; Allard *et al.*, 2001). The results of these methods, although of epidemiological value, are often of limited clinical usefulness, as up to 30 days may be required for complete characterization following the initial isolation of HAdV in cell culture. Alternative identification methods therefore have been developed and include the use of serotype-specific monoclonal antibodies (Adam *et al.*, 1996; Wood *et al.*, 1997), detection of species-specific antibodies (Akalu *et al.*, 1998) and PCR-based identification protocols (Akalu *et al.*, 1998; Xu *et al.*, 2000). Two recent advances, species-specific PCR (Pring-Åkerblom *et al.*, 1999) and generic PCR for human adenoviruses (Allard *et al.*, 2001), may facilitate the rapid and accurate diagnosis of adenovirus infection in clinical specimens.

In Brazil, a few reports have described HAdV infection in children with gastroenteritis, demonstrating the involvement of adenoviruses in cases of acute infantile gastroenteritis (Leite *et al.*, 1985; Pereira *et al.*, 1993; Harsi *et al.*, 1995; Soares *et al.*, 2002).

The main objective of the present report was to investigate and characterize HAdV species in cases of acute gastroenteritis in children hospitalized in Rio de Janeiro, RJ, Brazil, and in children living in the slums of Salvador, BA, Brazil, using two molecular protocols for the detection and characterization of these viruses directly from clinical specimens.

METHODS

Subjects, specimens and adenovirus screening. The study population consisted of children up to 5 years of age with acute diarrhoea who attended two public hospitals in Rio de Janeiro, in the south-eastern region of Brazil, from April 1996 to September 2003, and children up to 5 years of age presenting with diarrhoea and living in the slums of Salvador, in the north-eastern region of Brazil, from October 2001 to September 2003. The hospitalized children in Rio de Janeiro were more severely dehydrated compared with those living in the poor communities in Salvador, who were assisted at home.

A total of 2452 faecal samples were collected from the two public hospitals in Rio de Janeiro and 608 were collected from children living in the slums in Salvador. Only one specimen was collected per child. The faecal specimens were collected and maintained at 2–8 °C and sent to the Laboratório de Virologia Comparada, Departamento de Virologia, Instituto Oswaldo Cruz, and stored at −20 °C for viral testing.

This project was approved by the Ethical Research Committee of the Hospital Municipal Jesus and by the Instituto de Saúde Coletiva, Universidade Federal da Bahia.

Virus prototypes, virus isolation and indirect immunofluorescence assay. Lyophilized aliquots of adenovirus types 4 (species E), 6 (species C), 7 (species B), 9 (species D), 12 (species A) and HAdV-40 strain Dugan and HAdV-41 strain Tak (species F), provided by the Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London, UK, were used as prototype strains. Lyophilized aliquots were reconstituted in 150 μl Dulbecco's minimum essential medium (Gibco-BRL Life Technologies) and used as sample patterns (positive controls).

Prototype strains and negative samples for the PCR were inoculated (0.1 ml per tube) onto HEK-293 cells (kindly provided by Dr Jean Claude D'Halluin, INSERM, France) at 70 % confluence, as described previously (Leite *et al.*, 1985).

Supernatants from infected cells showing a positive cytopathic effect were tested by fluorescent antibody technique (Light Diagnostics; Chemicon International) according to the manufacturer's recommendations and by generic and species-specific PCRs, as described by Allard *et al.* (2001) and Pring-Åkerblom *et al.* (1999), respectively.

Enzyme immunoassay for rotavirus and adenovirus (EIARA). Faecal suspensions (10 %, w/v) were prepared in 0.01 M Tris/Ca^{2+} buffer (pH 7.2), as described by Leite *et al.* (1985), and clarified by centrifugation at 2000 ***g*** for 5 min. Supernatants were submitted to screening with a commercial EIARA test according to the manufacturer's recommendations (BioManguinhos-Fiocruz). The sensitivity and specificity of the EIARA test in faecal specimens has been established previously (Pereira *et al.*, 1985) and is largely used in Brazil by the Ministry of Health as the standard method for rotavirus and adenovirus detection.

HAdV detection and characterization. Virus DNA was extracted from 250 μl 10 % faecal suspension and from the supernatant of cell cultures infected with the HAdV prototype strains using the QIAmp DNA mini kit according to the manufacturer's instructions (Qiagen).

Generic and species-specific primers and PCR amplification tests for HAdV were performed as described by Allard *et al.* (2001) and Pring-Åkerblom *et al.* (1999), respectively. Amplicons were resolved on a 2 % agarose gel by electrophoresis. Restriction endonuclease digestion to discriminate between HAdV-40 and -41 was performed as described by Allard *et al.* (2001). For molecular procedures, four separate rooms were used to avoid cross-contamination of samples. Milli-Q water was used as a negative control in all procedures.

DNA purification, sequencing and phylogenetic analysis. Amplicons were purified using a QIAquick PCR purification kit (Qiagen). DNA sequencing was performed by dideoxynucleotide chain termination using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems). Sequence editing, analysis and multiple alignments were performed using BioEdit (Hall, 1999). Phylogenetic analysis was conducted using MEGA 2.1 (Kumar *et al.*, 2001).

RESULTS

Of the 3060 faecal samples from children up to 5 years of age with acute gastroenteritis, 61 (2 %) were positive for HAdV by EIARA, comprising 44 of the 2452 faecal samples from hospitalized children in Rio de Janeiro (1.8 %) and 17 of the 608 samples from the children living in the slums of Salvador (2.8 %).

From the 61 faecal samples positive by EIARA, 59 were available in sufficient amounts for testing by PCR. All 59 (100 %) were positive by the generic PCR for human adenoviruses and 57 (97 %) by the species-specific PCR. Four samples were initially negative for species-specific PCR but positive by generic PCR for human adenoviruses. These samples were inoculated onto HEK-293 cells and two were found to be positive and characterized as species C, probably due to the increased virus concentration.

The species-specific multiplex PCR showed non-specific amplicons when the assay was carried out with HAdV species prototypes (A–F) (Fig. 1a), as well as with positive samples. In order to check the origin of these non-specific amplicons, we performed nucleotide alignments between the primers and the nucleotide hexon region of species A–F (GenBank accession nos NC_001460, NC_004001, NC_001405, NC_002067, NC_003266 and NC_001454, respectively). The analysis, using CLUSTAL W software (Thompson *et al.*, 1994; www.ebi.ac.uk/clustalw), showed the possibility of cross-hybridization among the different primers, corroborating the unexpected results obtained with the HAdV prototypes (data not shown). To resolve this problem, amplification was carried out using an individual set of primers for each species (Fig. 1b). Electrophoretic analyses of species F multiplex PCR amplicons and PCR using individual sets of primers are also shown (Fig. 1c, d). Using this methodology, species F (65 %) was the most prevalent, followed by species A (12 %), C, D and co-infection F/D (5 % each) and species B and co-infections F/A, F/C and B/D (2 % each). Similar percentages for the prevalence of HAdV species F were observed in both hospitalized children and those living in slums.

To characterize the enteric HAdV species F, the amplicon of 301 bp obtained with the generic PCR for HAdVs was subjected to RFLP analysis using the restriction endonuclease *Hin*fI (Invitrogen). Using this methodology, HAdV-40 was characterized in 23/37 species F-positive samples (62 %) and 14 (38 %) were characterized as HAdV-41 (Fig. 2). The 301 bp amplicons of four samples were chosen randomly for sequencing: strains of HAdV-41 from Rio de Janeiro (samples 929 and 934, GenBank accession nos DQ504432 and DQ504433, respectively) and Salvador (sample 1139, GenBank accession no. DQ504434), and an HAdV-40 strain from Rio de Janeiro (sample 2795). The sequence analysis corresponded to the 253 nt at positions 47–299 within the hexon gene. The Brazilian strains showed 100 % nucleotide homology among the three HAdV-41 Brazilian strains and 97 % with the HAdV-41 prototype Tak (GenBank accession no. X51783) and with isolate Vaalriver (GenBank accession no. AJ608285). The HAdV-40 Brazilian strain showed 100 % nucleotide homology with the HAdV-40 prototype strain Dugan (GenBank accession no. X51782) (Fig. 3).

Case distribution according to age group showed that 45/57 (79 %) of the children aged up to 2 years presented species F

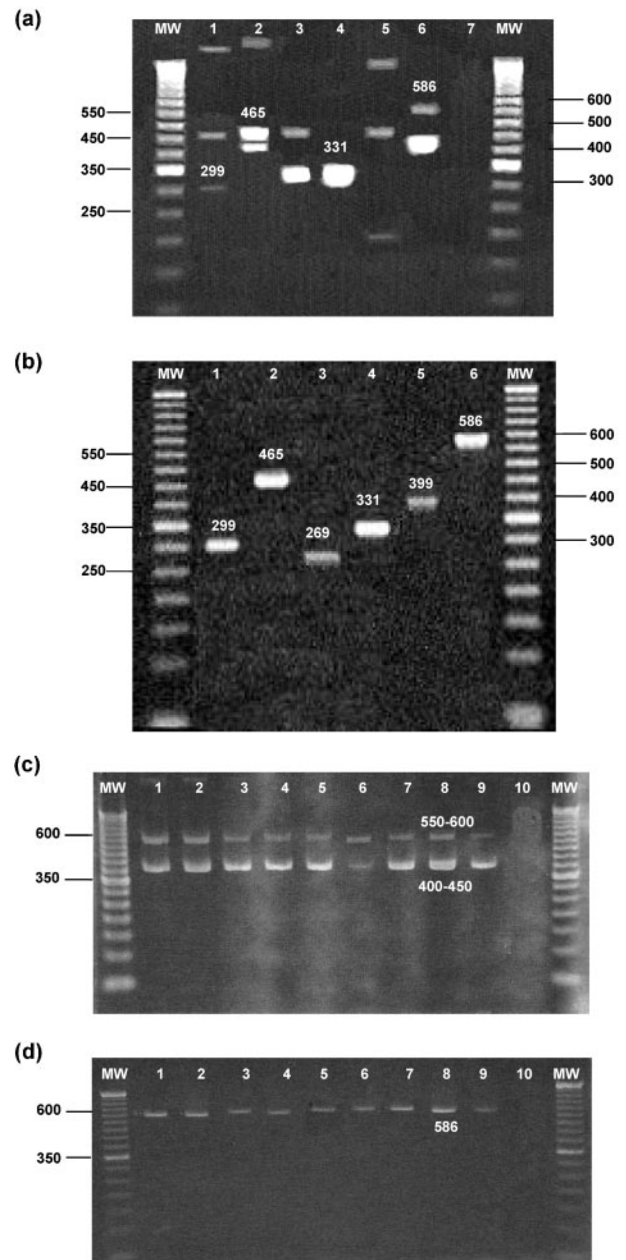

Fig. 1. Detection of adenovirus genotypes using species-specific PCRs. (a, b) Detection of prototype viruses using multiplex PCR (a) and PCR using individual primers for each species (b). Lanes: 1, HAdV-A; 2, HAdV-B; 3, HAdV-C; 4, HAdV-D; 5, HAdV-E; 6, HAdV-F; 7, negative control. (c, d) Detection of HAdV species F using species multiplex PCR (c) and PCR using individual primers for species F (d). Lanes 1–9 show sample amplicons. Lanes: 1, sample 929; 2, sample 934; 3, sample 139; 4, sample 1412; 5, sample 1595; 6, sample 2346; 7, sample 2501; 8, sample 2530; 9, sample 2790; 10, negative control. MW, 50 bp DNA ladder size marker.

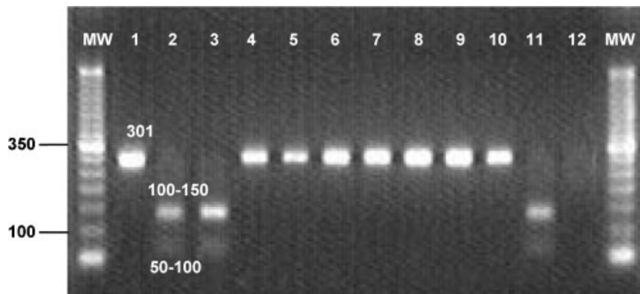

Fig. 2. Characterization of HAdV-40 and -41 by *Hin*fI endonuclease digestion. The prototype virus restriction fragments are shown in lanes 1 (HAdV-40 strain Dugan) and 2 (HAdV-41 strain Tak). HAdV-41 sample restriction fragments are shown in lanes 3 (sample 929) and 11 (sample 934). Lanes 4–10 show HAdV-40 sample restriction fragments (samples 1412, 896, 1595, 2501, 2795, 3118 and 6006, respectively) and lane 12 shows the restriction endonuclease-negative control. MW, 50 bp DNA ladder size marker.

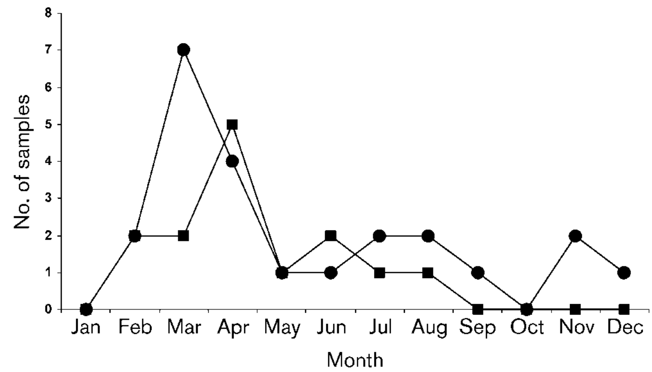

Fig. 4. Seasonal distribution of adenovirus species F during the period studied. The number of samples represents the monthly distribution of the accumulated positive cases during the study. ●, HAdV40; ■, HAdV41.

as the main species observed, corresponding to 31/45 positive cases (69 %) from the entire study.

The monthly distribution of the accumulated positive cases during the study showed that the period from February to April contributed to 36/57 (63 %) of the positive cases, and April was the only month where HAdV was observed annually (data not shown). No pattern of serotype distribution was observed, although peaks of HAdV-40 and -41 were observed in March and April, respectively (Fig. 4).

DISCUSSION

Gastroenteritis represents one of the main problems of paediatric public health in developing countries, second only to acute respiratory infection as the most important cause of infant morbidity and mortality in these countries. In Brazil, acute diarrhoea is one of the most common diseases in childhood, responsible for 15 % of deaths among children (Scaletsky *et al.*, 1999; Linhares, 2002). Faecal samples from children up to 5 years of age who had been hospitalized in Rio de Janeiro or were living in the slums of Salvador were screened by EIARA and HAdV species were characterized using two PCR methodologies. A total of 61 (2 %) of 3060 samples were positive for HAdV by EIARA screening, indicating a low prevalence of HAdV. A higher percentage of HAdV detection might have been obtained if a more sensitive methodology, such as an amplification assay, had been used as a screening test (Rohayem *et al.*, 2004). Of the EIARA-positive samples that were available in sufficient amounts to allow testing by PCR, 100 % were positive using the generic PCR for human adenoviruses. In contrast, species-specific PCR (either multiplex or singleplex) (Pring-Åkerblom *et al.*, 1999) should not be used for HAdV screening, as the sensitivity (97 %) was lower compared with the generic PCR and EIARA. Reports from other countries associating HAdV with acute gastroenteritis range from 2.3 to 38 % (Herrmann *et al.*, 1988; Lew *et al.*, 1991; McIver *et al.*, 2001; Marie-Cardine *et al.*, 2002). Studies in other Brazilian cities have found higher rates of HAdV: Leite *et al.* (1985) found 5.2 % in Rio de Janeiro, Harsi *et al.* (1995) found 10 % in São Paulo and Soares *et al.* (2002) found 4.9 % in four Brazilian cities. However, our results are similar to those described by Pereira *et al.* (1993) in a report

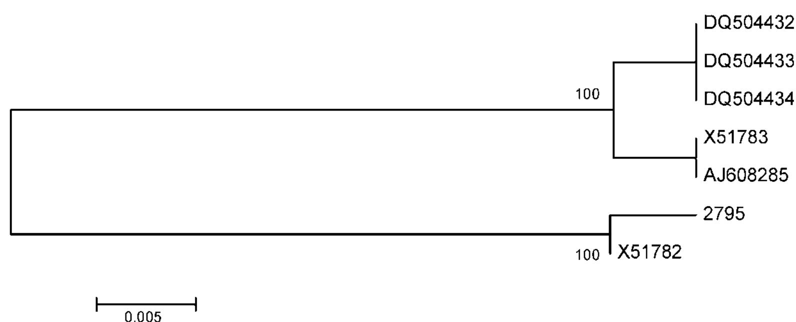

Fig. 3. Neighbour-joining phylogenetic tree based on partial nucleotide sequences of the adenovirus hexon gene of HAdV-41 [GenBank accession nos X51783 (strain Tak), AJ608285 (isolate Vaalriver), DQ504432 (sample 929), DQ504433 (sample 934) and DQ504434 (sample 1139)] and HAdV-40 [GenBank accession no. X51782 (strain Dugan) and sample 2795]. Numbers adjacent to the nodes represent the percentage bootstrap support (1000 replicates). Bar, 0.005 nucleotide substitutions per site.

on an HAdV and rotavirus surveillance programme in 14 Brazilian States from 1984 to 1989.

Species F (HAdV-40 and -41) was the most prevalent HAdV, corresponding to 37/57 positive samples (65 %). Similar percentages of HAdV species F were observed in hospitalized children and those living in slums. Hospitalization of children living in slums was probably avoided due to the oral rehydration therapy provided at the start of their symptoms.

These results are similar to those obtained by Uhnoo *et al.* (1984) and higher than previous reports where HAdV species F has been found to be responsible for 2–40 % of positive cases (Herrmann *et al.*, 1988; Lew *et al.*, 1991; Saderi *et al.*, 2002; Oh *et al.*, 2003). In countries like Sweden, Thailand, Korea, the USA, the UK, Australia, France, Iran and Argentina, prevalence of enteric adenovirus from 1.55 to 15 % has been observed, compared with the prevalence found in our study of 1.2 % (37/3060) (Uhnoo *et al.*, 1984; Herrmann *et al.*, 1988; Kim *et al.*, 1990; Lew *et al.*, 1991; Barnes *et al.*, 1998; Bon *et al.*, 1999; Saderi *et al.*, 2002; Bereciartu *et al.*, 2002; Amar *et al.*, 2004; Chen *et al.*, 2005; Liu *et al.*, 2006; Venacchio *et al.*, 2006). In Brazil, Soares *et al.* (2002) found a similar prevalence (1.55 %) of enteric HAdV in four Brazilian cities.

In a specific comparison of the results of enteric HAdV species F from studies performed in Brazil, the 65 % positive samples in this study was higher than the 36 % obtained by Leite *et al.* (1985) and the 32 % obtained by Soares *et al.* (2002). In the present study, HAdV-40 and -41 contributed to 62 % (23/37) and 38 % (14/37) of positive samples, respectively. The results obtained here used PCR followed by restriction endonuclease digestion to characterize species F; Soares *et al.* (2002) used monoclonal antibodies for HAdV-40 or -41 and detected 50 % HAdV-40 (11/22) and 27 % HAdV-41 (6/22), whilst 23 % (5/22) were recognized indistinctly as HAdV-40/41. This suggests that the higher level of species F detection is a direct result of the improvement in the techniques of viral characterization (McIver *et al.*, 2001), as it has been demonstrated previously that the monoclonal antibodies for enteric adenovirus (EAdV) are highly specific reagents that can fail to detect genomic variations of EAdV, probably due to the alteration of external neutralizing epitopes under immunological pressure (Scott-Taylor *et al.*, 1990).

Comparing the results of the present study with those of Soares *et al.* (2002) from February 1998 to July 2000, both carried out in Rio de Janeiro, we detected a lower percentage (0.86 %) of positive samples for HAdV compared with their result of 2.7 %, although we observed a greater percentage of HAdV species F (89 %) compared with their result of 47 %, possibly due to the larger number of inpatients involved in the current study.

In an analysis of the age group of HAdV infection, we observed that 79 % (45/57) of positive cases were in children under 2 years of age and species F was present in 31/45 (69 %) of the cases studied. Our data reveal a greater percentage of positive cases than previously described in the literature, which showed that species F was prevalent in approximately 50 % of adenovirus-positive samples. Taken together, these results show that EAdV plays an important role in acute diarrhoeal infection (Uhnoo *et al.*, 1984; Kim *et al.*, 1990; Dennehy *et al.*, 2001; Saderi *et al.*, 2002; Bereciartu *et al.*, 2002; Oh *et al.*, 2003).

With regard to other species of HAdV associated with cases of gastroenteritis, we observed the presence of species A (12 %), C, D and co-infection F/D (5 % each) and species B and co-infections F/A, F/C and B/D (2 % each). These results are similar to previous reports that have described other species of adenovirus that could be associated with acute gastroenteritis, particularly HAdV-12, -18 and -31 (species A), HAdV-3 and -7 (species B), and HAdV-1, -2 and -5 (species C) (Brown *et al.*, 1984; Uhnoo *et al.*, 1984; Leite *et al.*, 1985; Gomes *et al.*, 1989; Brown, 1990).

The monthly distribution of the accumulated number of positive samples studied by species-specific PCR showed a greater prevalence in the number of cases between February and April and highlighted the month of April as the only month with positive cases in 7 years of study (Fig. 4). Unfortunately, the number of samples was too small to draw any significant conclusions about the seasonality of adenovirus infection. Dennehy *et al.* (2001) described the occurrence of low rates throughout the year, Barnes *et al.* (1998) observed the existence of random peaks during the year and de Jong *et al.* (1993) observed that HAdV species F occurred more frequently at the beginning of autumn. Jarecki-Khan *et al.* (1993) found prevalence peaks of EAdV in dry months, which corresponded to winter. McIver *et al.* (2001), in Australia, observed that species F infection increased from February to April. Comparing our results with those of Soares *et al.* (2002) in Rio de Janeiro, in spite of no evidence for seasonal variation of EAdV in their study, they observed peaks of HAdV-40 and -41 in March and May, respectively. In the current study, similar results were found and peaks of HAdV-40 and -41 were observed in March and April, respectively (Fig. 4).

Mixed infections were found in the present work, which can be explained by the fact that some viruses from a particular episode continue to be excreted while another virus causes the acute disease. In any individual case, determining which aetiological agent is the cause of gastroenteritis can be difficult (Oh *et al.*, 2003). The identified HAdV in the sample should not necessarily be considered to be the causative agent of the infection, as infections caused by members of species C and HAdV-3 can result in lingering intermittent excretion in the faeces after a previous infection (Pring-Åkeblom *et al.*, 1999; Allard *et al.*, 2001).

The combination of generic PCR for human adenoviruses (Allard *et al.*, 2001) and the species-specific PCR (Pring-Åkeblom *et al.*, 1999) are tools available for use in the molecular characterization of HAdV, and have the advantage

that they can be used to test clinical specimens directly compared with other methodologies that require virus isolation in cell culture prior to identification.

Continuous studies are essential to verify the impact of HAdV in the community and compare this with hospitalization cases, as well as investigating the seasonality of these viruses compared with rotaviruses, astroviruses and human caliciviruses. In association with potential sanitary and health-care improvements, continuous investigations will become increasingly important with the introduction of the rotavirus vaccine campaign in 2006 in Brazil and probably in other South American countries.

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