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The role of human adenoviruses type 41 in acute diarrheal disease in Minas Gerais after rotavirus vaccination

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ABSTRACT

Human adenovirus species F (HAdV-F) type 40 and 41 are commonly associated with acute diarrheal disease (ADD) across the world. Despite being the largest state in southeastern Brazil and having the second largest number of inhabitants, there is no information in the State of Minas Gerais regarding the role of HAdV-F in the etiology of ADD. This study was performed to determine the prevalence, to verify the epidemiological aspects of infection, and to characterize the strains of human adenoviruses (HAdV) detected. A total of 377 diarrheal fecal samples were obtained between January 2007 and August 2011 from inpatient and outpatient children of age ranging from 0 to 12 years. All samples were previously tested for rotavirus, norovirus, and astrovirus, and 314 of 377 were negative. The viral DNA was extracted, amplified using the polymerase chain reaction and the HAdV-positive samples were sequenced and phylogenetically analyzed. Statistical analyses were performed using the Chi-square test ($p < 0.05$), considering two conditions: the total of samples tested (377) and the total of negative samples for the remaining viruses tested (314). The overall prevalence of HAdV was 12.47% (47/377); and in 76.60% (36/47) of the positive samples, this virus was the only infectious agent detected. The phylogenetic analysis of partial sequences of 32 positive samples revealed that they all clustered with the HAdV-F type 41. The statistical analysis showed that there was no correlation between the onset of the HAdV infection and the origin of the samples (inpatients or outpatients) in the two conditions tested: the total of

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samples tested ($p = 0.598$) and the total of negative samples for the remaining viruses tested ($p = 0.614$). There was a significant association in the occurrence of infection in children aged 0–12 months for the condition 1 ($p = 0.030$) as well as condition 2 ($p = 0.019$). The occurrence of infections due to HAdV did not coincide with a pattern of seasonal distribution. These data indicate the significant involvement of HAdV-F type 41 in the etiology of ADD in Minas Gerais, which demonstrates the importance of other viral agents in the development of the disease after the introduction of rotavirus vaccine immunization.

Introduction

Acute diarrheal disease (ADD) has a major impact on global health due to the high cost of hospitalizations and outpatient visits associated with diarrhea. In developed countries, ADD is still associated with high morbidity, especially among children as well as elderly. Whereas, in developing countries, ADD is the second leading cause of morbidity and mortality in children under 5 years of age.[1,2]

Over the past few years, the Brazilian government has made considerable efforts in reducing extreme poverty and child mortality through the implementation of income transfer programs, such as the Family Support Program,[3] and the expansion of healthcare services, such as the Family Health Program.[4] Despite of the progress made, data from the Brazilian Epidemiological Information System (DATASUS) reveal that in 2009, ADD was the cause of 1,258 (2.51%) out of 50,033 deaths in the children under 5 years of age, rendering this disease still as a threat to public health.[5,6]

Different agents are involved in the etiology of ADD, and among them, the viruses play an important role, especially the group A rotavirus (RVA), the human calicivirus (norovirus [NoVs] and sapovirus [SaVs]), human astrovirus (HAstVs), and human adenovirus species F (HAdV-F) type 40 and 41.[1] The introduction of rotavirus vaccine (Rotarix®) in the Brazilian National Immunization Program in March 2006 has considerably declined the prevalence of RVA, in the number of hospital admissions, and in the mortality due to diarrhea, especially, in children under 2 years of age.[7–10] However, the role of other enteric viruses in the etiology of the ADD must be taken into account as well as the impact of RVA vaccine, which may favor the circulation of other viruses.

Human adenoviruses (HAdV) are non-enveloped particles with icosahedral symmetry, which have a linear double-stranded DNA. These viruses belong to *Mastadenovirus* genus of *Adenoviridae* family. They have been characterized and classified into species and serotypes, and correlated with the genotypes according to the characteristics of their penton, hexon, and fiber proteins.[11] So far, over 60 types of HAdV have been identified and grouped into seven species: A to G.[12] They differ in terms of tropism and pathogenicity, and are commonly associated with respiratory, ocular, urinary tract, and gastrointestinal infections.[11] The genotypes, 40, 41, and rarely 38, have been found to be associated with acute gastroenteritis.[13]

The HAdV-F type 40 and 41 account for up to 20% of the worldwide cases of ADD. This confirms the important epidemiological role of these pathogens in the etiology of the disease. Molecular characterization is a crucial step to define the HAdV species that causes ADD, since some non-enteric types can be intermittently excreted in the feces after a previous infection.[11]

In developed and developing countries, HAdV-F type 40 and 41 have been described in sporadic cases and outbreaks of the disease in inpatients and outpatients causing illness and even death, especially in children under 5 years old.[14–17] In Brazil, HAdV-F was first described by Leite et al.,[18] and since then other studies have shown the epidemiological importance of these viruses in different states.[14,19–22] However, despite being the largest state in the southeastern region and the second most populous in the country, no information is available about the types of HAdV circulating in Minas Gerais. Thus, considering the important contribution of ADD to morbidity and mortality, this study was carried out to provide epidemiological data on infection due to the HAdV-F and to determine, for the first time, the role of these viruses in the etiology of ADD in Minas Gerais, Brazil.

Materials and methods

Study design and collection of fecal specimens

This was a cross-sectional study, conducted in Juiz de Fora, Minas Gerais, southeastern Brazil. This city has about 520,000 inhabitants and highland tropical climate, with two well-defined periods: the dry season (May to September), characterized by lower temperatures and rainfall, and the rainy season (October to April), with higher temperatures and rainfall. The average temperature and rainfall, obtained from the Laboratory of Climatology and Environmental Analysis of the Federal University of Juiz de Fora,[23] were used to confirm the characteristics of dry and wet periods and to identify possible climate change throughout the study.

The fecal specimens of children of 0–12 years old with diarrhea as the main symptom at the time of clinical care were analyzed. The samples were collected within 48 h of ambulatory care or hospital admission. Diarrhea was defined as the occurrence of ≥3 liquid stools or reduction of stool consistency over a 24-h period. It was not possible to obtain information about other symptoms or whether the fecal specimens were derived from outbreaks or sporadic cases.

From January 2007 to August 2011, a total of 377 fecal specimens were obtained through passive surveillance; 341 from outpatients and 36 from inpatients. All samples were previously tested for RVA, NoV, and HAstV; and 314 of them were negative for these three viral agents (unpublished data, Rosa and Silva, 2015).

The fecal specimens, while at 4 °C, were sent to a virology laboratory and then stored at −20 °C, constituting to a sample bank at the Federal University of Juiz de Fora. This study was approved by the Ethics Committee on Human Research of the Federal University of Juiz de Fora (Protocols: CEPH/UFJF 049/2007 and CEPH/UFJF 058/2010) and written consents were obtained from the caregiver of each patient.

DNA extraction and HAdV detection

A 10% (w/v) suspension of each fecal sample was prepared and centrifuged at 1,500 × *g* for 20 min. The DNA was extracted from 400 μL of fecal suspension by the glass powder method[24] and stored at −70 °C until further use.

The presence of HAdV in the fecal sample was detected by amplifying hexon gene using generic primers Hex 1 (5′-GCC SCA RTG GKC WTA CAT GCA CAT C-3′) and Hex 2 (5′-CAG CAC SCC ICG RAT GTC AAA-3′), as described previously.[25] The PCR reaction comprised 2.5 μL of DNA and 22.5 μL PCR mixture containing 10 mM Tris HCl (pH 8.0), 50 mM KCl, 3 mM $MgCl_2$, 1 μM of each deoxynucleoside triphosphate (Promega®, Madison, USA), 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Invitrogen®, CA, USA). The amplifying conditions were: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. The PCR products were resolved by electrophoresis on 1.5% agarose gel (Promega®, Madison, USA) stained with ethidium bromide (0.5 μg/mL, Sigma Aldrich, Brazil). The stained gels were visualized with the use of a UV-transilluminator to detect the expected band of 301 bp.

HAdV sequencing

To determine the types of HAdV present in positive fecal samples, the amplicons obtained in the PCR assay (described above) were subsequently sequenced.[25,26] The PCR amplicons of the expected size were purified using the QIAquick® PCR purification kit (Qiagen, Netherlands). Only purified amplicons containing adequate amounts of DNA were submitted to sequencing. Both strands were sequenced using the ABI Prism® BigDye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). The products were sequenced in an automated sequencer ABI Prism 3130 (Applied Biosystems, Foster City, CA), following the manufacturer's recommendations.

Data and sequence analysis

The nucleotide sequences of the hexon gene were edited and aligned using the Clustal W method in the BioEdit 7.2.0 program.[27] The nucleotide sequences were compared with the prototypes and other reported strains of HAdV-F deposited in the GenBank database. Phylogenetic relationships among strains were analyzed with the MEGA software, version 5.2.2,[28] using the neighbor-joining method with 1000 bootstrap replicates. The genetic distance was calculated using the Kimura 2-parameter model.

The prototype strains used were HAdV41/Tak (X51783) and HAdV40/Dugan (X51782). The following reference HAdV strains (GenBank accession number) were selected: HAdV41/RJ-2010-544/BRA (JN654711), HAdV41/RJ-2009-527/BRA (JN654713), HAdV41/RJ-2010-547/BRA (JN654712), HAdV41/AM46-BR-06/BRA (DQ464893), HAdV41/AM16-BR-06/BRA (DQ464891), HAdV41/D30/CHN (AB610526), HAdV41/IT-703-2006/ITA (AM920721), HAdV41/IT-704-2006/ITA (AM920722), HAdV41/TAK/JPN (AB330122), HAdV41/D27/JPN (AB610523), HAdV41/D29/JPN (AB610525), HAdV41/500746-KOL-2008/IND (HQ005286), HAdV41/D31/THA (AB610527), and HAdV41/D28/THA (AB610524). The HAdV strains used as the out-group were: HAdV2 (J01917), HAdV3 (X76549), HAdV4 (AF161569), HAdV6 (AF161560), HAdV7 (X76551), HAdV8 (AF161561), HAdV12 (X73487), HAdV14 (AF161571), HAdV18 (AF161575), HAdV19 (AF161565), HAdV31 (AF161576), HAdV34 (AF161573), HAdV37 (AF161567), HAdV40 (L19443; AB330121; FJ905452), and HAdV65 (AP012285).

The HAdV nucleotide sequences obtained in this study were deposited in the GenBank/NCBI database under the accession numbers KF840481–KF840499, KF840501–KF840504 and KF840506–KF840514.

Statistical analysis

Statistical analysis was performed using the SPSS software, version 13.0 (SPSS® Inc., Chicago, USA). The Chi-square test was performed to evaluate the influence of origin of the sample, age, and the dry and rainy periods regarding the occurrence of HAdV infection. Two conditions were considered in these analyses: the total samples tested (377) and the total number of negative samples for the other previously tested (314) enteric viruses. The statistical significance was established at 5%.

Results

Table 1 shows the total and annual prevalence of HAdV observed during the period of this study, and the distribution of positive fecal samples from inpatients and outpatients. An increase in incidences of these viral infections was observed

Table 1 – Prevalence of human adenovirus in diarrheal fecal samples obtained from outpatients and inpatients in Juiz de Fora, Minas Gerais, Brazil, from 2007 to 2011.

Year	Samples' origin		
	Outpatients Positive/ tested (%)	Inpatients Positive/ tested (%)	Total Positive/ tested (%)
2007	06/83 (7.23)	0/12 (–)	06/95 (6.32)
2008	09/125 (7.20)	02/19 (10.53)	11/144 (7.64)
2009	15/79 (18.99)	0/02 (–)	15/81 (18.52)
2010	07/30 (23.34)	01/03 (33.34)	08/33 (24.25)
2011	07/24 (29.17)	–	07/24 (29.17)
Total	44/341 (12.91)	03/36 (8.34)	47/377 (12.47)

Table 2 – Prevalence of human adenoviruses by age group in all the samples tested and all the negative samples for the other enteric viruses in Juiz de Fora, Minas Gerais, Brazil, from 2007 to 2011.

Age group (months)	Positive HAdV/total tested (%)	Positive HAdV/total negative other viruses[a] (%)
0–12	23/114 (20.18)	20/102 (19.61)
13–24	15/124 (12.10)	10/97 (10.31)
25–36	04/43 (9.30)	04/35 (11.43)
37–48	02/20 (10.00)	0/16 (–)
49–60	0/26 (–)	0/22 (–)
61–144	03/50 (6.00)	02/42 (4.76)
Total	47/377 (12.47)	36/314 (11.47)

[a] Rotavirus, norovirus and astrovirus.

from 2009 to 2011 period and the statistical analysis showed that there was no significant association between the occurrence of infection due to the HAdV and the origin of the sample ($p = 0.598$).

Of the 47 samples positive for HAdV, 36 (76.60%) were positive for only enteric viral pathogen, and 34 of these 36 samples were from non-hospitalized children. Statistical analysis of these data confirmed the absence of a correlation between the incidence of infection and the origin of the sample ($p = 0.614$).

The partial nucleotide sequences of the hexon gene (301 bp) were obtained for 32 of the 47 HAdV positive strains; 6 of them were detected in 2007, 6 in 2008, 9 in 2009, 7 in 2010, and 4 in 2011. These sequences were compared with the sequences available in the GenBank database for all the 7 species of HAdV (A–G). All 32 strains were characterized as HAdV type 41 (F species). The nucleotide sequence and amino acid identities in the 32 strains were found to be 96.2–100%, and 90.5–100%, respectively. For prototype HAdV type 41 (X51783), the nucleotide and amino acid sequence ranged from 96.2% to 98.6% and 88.8% to 95.3%, respectively. Among the Brazilian sequences assessed, the HAdV type 41 strains in this study showed the highest identity with those detected in sewage samples at Rio de Janeiro, Brazil (nucleotide 97.6–100%, and amino acid 93.7–100%) in 2009 and 2010. Similar results were found after while comparing the strains in this study with the HAdV type 41 strains detected in clinical samples from China, India, Japan, and Thailand (Fig. 1).

Twenty-six (81.25%) out of the 32 strains characterized as HAdV-F type 41 were detected in fecal samples from non-hospitalized children without any other enteric viruses (RVA, NoV, and HAstV). The remaining 18.75% (06/32) were found in co-infected fecal samples with at least one of the above viruses tested, and one was from a hospitalized patient.

Table 2 shows the prevalence of HAdV by age groups in the total of samples tested (condition 1) and in the total of negative samples for the remaining viruses tested (condition 2). In both the conditions, the HAdV was found mainly within first 3 years of life, with the highest frequency of detection among children aged ≤12 months ($p = 0.030$ [condition 1]; $p = 0.019$ [condition 2]).

Majority of the 32 strains, characterized as HAdV-F type 41, were observed in fecal samples of children aged ≤12 months (40.62%; 13/32) and of 13–24 months old (37.50%; 12/32).

Fig. 2 shows the monthly distribution of HAdV positive samples and the variations of temperature and rainfall throughout the study period.

The HAdV infections were recorded in different months of the year during the study, without particular profile of occurrence. The incidence of infections of HAdV was not influenced by changes in climatic conditions (dry and rainy periods).

Discussion

The rate of mortality associated with ADD has been reduced worldwide, from 1.3 million in 2000 to about 0.6 million in 2012, due to improvements in sanitation and increased access to clean water, oral rehydration therapy, and the implementation of immunization programs (particularly for RVA) and healthcare.[2] However, the role of the ADD in morbidity and mortality in children under 5 years of age is still very relevant, especially in low-income populations inhabited in areas with little or no basic infrastructure.[29]

In Brazil, few epidemiological surveys on HAdV associated with diarrhea have been performed[14,18,21,30]; however, only one study has been conducted in Minas Gerais so far.[19] A recent survey conducted in city of Juiz de Fora from 2005 to 2008 demonstrated that ADD was the third leading cause of infant hospitalization.[31] Besides, considering the decrease in ADD cases related to RVA, verified after the introduction of the anti-RVA vaccine,[8] it is important to investigate the role of other viruses in the etiology of ADD, mainly the HAdV, due to the paucity of information available on association of this agent with ADD.

In this study, the prevalence of HAdV infections associated with ADD cases from 2007 to 2011 was 12.47%, similar to the previous Brazilian reports.[19,20,22,30] An analysis of the annual prevalence suggested an increase between 2009 and 2011, which should be interpreted with caution due to the lower number of diarrheal fecal samples collected during this period. However, it should be noted that a higher number of HAdV positive samples was detected, even taking into account the gradual reduction of the fecal specimens. This decrease primarily reflects a lower demand for clinical care reported by health professionals and the partners of this study, possibly associated with a lower occurrence of severe cases of ADD.[8] In fact, the studies conducted in Brazil after the implementation of the Rotarix® vaccine have shown a decreased association of RVA with ADD since 2007.[7,9,10,32]

The 32 strains sequenced here were characterized as HAdV-F type 41, a strictly enteric type. The involvement of HAdV-F in the etiology of ADD has been shown worldwide, with the predominance of type 41.[15–17,33,34] In a recent study conducted in sub-Saharan Africa and South Asian countries to assess the impact of ADD on children in developing countries, HAdV-F type 40 and 41 were listed among the viruses causing moderate to severe diarrhea.[35]

Despite the limited information available on the characterization of HAdV types in Brazil, previous studies have reported a greater frequency of detection of HAdV-F type 40.[14,19] In these studies, the characterization was performed using enzyme immunoassay with monoclonal antibodies[19] and using PCR followed by enzymatic restriction digestion[14]

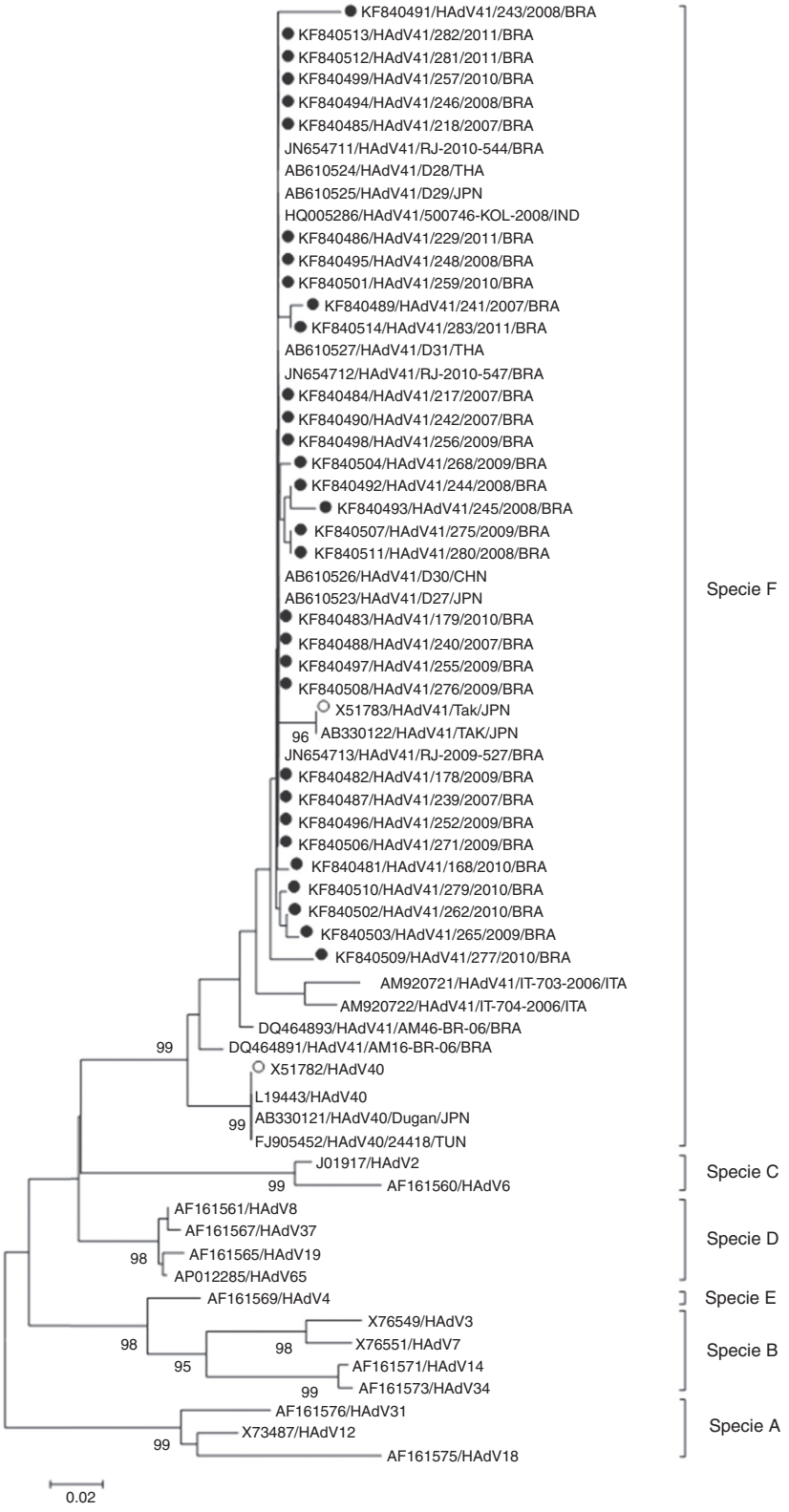

Fig. 1 – Phylogenetic dendrogram based on partial hexon nucleotide sequences of human adenovirus (HAdV) strains detected in Juiz de Fora, Minas Gerais, Brazil, from 2007 to 2011. The nucleotide sequences for the strains obtained in this study are marked with a filled circle, and the prototype strains are marked with an empty circle. Bootstrap values higher than 80% are given for each node.

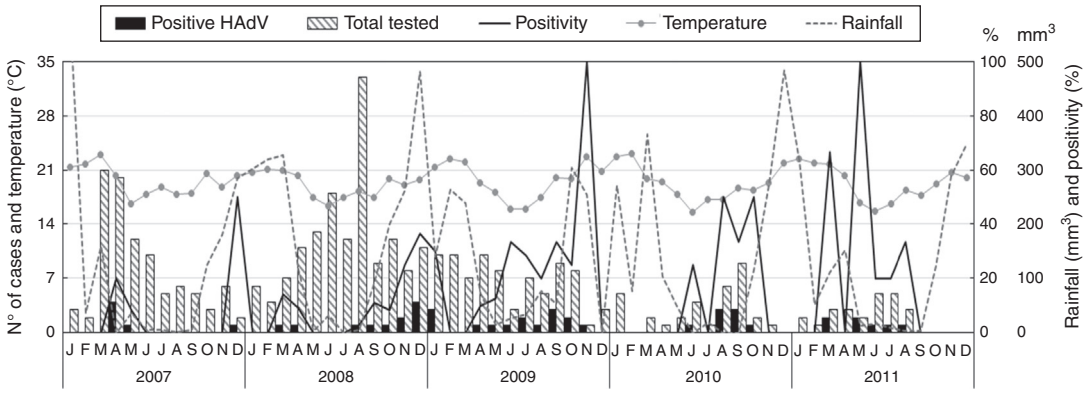

Fig. 2 – Monthly distribution of human adenovirus positive samples and average values of temperature and rainfall in Juiz de Fora, Minas Gerais, Brazil, from 2007 to 2011.
***Source of climate data*: UFJF.[23]**

in fecal specimens obtained from 1998 to 2000 and 1996 and 2003, respectively. From 2004 till date, there have been no reports dealing with the detection of type 40 and 41 in clinical samples.

The sequences obtained in this study formed a monophyletic group with other strains of HAdV-F type 41, including the prototype strain (X51783), and the strains detected in clinical samples analyzed during 2000–2008 in India,[17] China, Thailand, and Japan,[36] as well as the strains detected in Brazil (2009 and 2010) in sewage samples in Rio de Janeiro.[37] Although of environmental origin, sewage samples contain enteric virus excreted in the feces of the population of Rio de Janeiro, a neighboring state of Minas Gerais, since both the cities have heavy traffic of people traveling between them. In this study, the partial analysis of a more preserved region of the hexon gene showed a high similarity between the strains of HAdV-F type 41 obtained in distant countries, suggesting a global distribution of this type.[38] However, while evaluating hyper-variable regions of the hexon gene, some genetic characterization studies have pointed out minor changes between type 41 strains, classifying them into two genomic-type clusters.[17,38]

A comparison of the data regarding the detection of HAdV with the data on the detection of other enteric viruses in these fecal samples (unpublished data – Rosa e Silva, 2015) showed the occurrence of 10 cases of double coinfection, HAdV/RVA (5) and HAdV/NoV (5), and a case of triple coinfection HAdV/HAstV/RVA. However, in 76.60% (36/47) of the positive samples, the HAdV types were the only agents detected among the enteric viruses studied. In this group, all sequenced strains were characterized as HAdV-F type 41, pointing to the relevant role of these viruses as causative agents of ADD, a fact confirmed in studies in both Japan and in India.[39,40] Since the presence of other non-viral pathogens has not been investigated, the possibility of their association with the disease cannot be excluded.

HAdV was detected both in moderate cases (outpatients) and in severe cases (inpatients) of ADD, and a significant association between the occurrence of this infection and the origin of the fecal specimens was not observed. This result supports previous findings, in which enteric HAdV was detected in both types of the patients.[15,16,22] The information on other symptoms of ADD, besides diarrhea, could have enriched this work and contributed to a better understanding of the severity of the disease. However, these data were not available due to inconsistencies in the records, which can be considered a limitation of this study.

HAdV was detected mainly in children aged ≤12 months old, as noted earlier.[14,16,17,22] A significant association between age and the occurrence of infection was observed. In general, the demand for clinical care in ADD cases is more common for younger children, who are more susceptible to serious complications. Moreover, the cases of mild to moderate ADD that occur in older children and adults require no clinical care.

HAdV infections were distributed over the years of the study, occurring both in the dry as well as rainy season. This suggests that there is no influence of climatic conditions on the occurrence of these infections, which concords with earlier studies.[30,33,34]

Thus, this pioneer study on the molecular epidemiology of enteric HAdV conducted in Minas Gerais has proven significant involvement of HAdV-F type 41 in the etiology of ADD, occurring mainly in children during their first year of life at different periods of year. It is important to understand the contribution of other enteric viruses for which there is no vaccine and which are still causing the disease and impacting health services. These data can contribute to the knowledge about these viruses in Brazil, providing key information for the development of possible control strategies and the prevention of these infections.

Conflicts of interest

The authors declare no conflicts of interest.

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