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Detection of adenovirus in children less than 5 years old with acute gastroenteritis (AGE) from Bogota, Colombia

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ABSTRACT

Acute gastroenteritis (AGE) has a great impact on children less than five years of age, producing high levels of morbidity and mortality. Worldwide, AGE is mainly related to viruses, mainly rotavirus but human adenovirus (HAdV) in particular as an important etiologic agent. Among HAdV species F, genotypes 40 and 41 are responsible for a high number of diarrhea cases, mainly in children under 2 years of age. Two hundred and fifty four fecal samples were analyzed using conventional PCR to detect and characterize enteric HAdV in children less than five years diagnosed with AGE and recruited between 2012 and 2013 through the Sentinel Surveillance Program from Bogota DC, Colombia. Fourteen samples (5.51%) were positive for HAdV. Species-specific detection showed one HAdV-A sample, four HAdV-B samples, two HAdV-D samples, two HAdV-E samples, and five HAdV-F samples. HAdV-F samples were subjected to conventional PCR in order to identify genotypes 40 and 41. All HAdV-F samples were genotype 40, except for one sample coinfected with both genotypes (40 and 41). Our results show that enteric HAdV are important agents in the etiology of AGE in children less than 5 years old. Moreover, the species-specific PCR analysis demonstrated that species other than HAdV- F which are tightly associated with respiratory tract disease, could infect and produce gastrointestinal tract disorders.

1. Introduction

Acute gastroenteritis (AGE) is a global health problem, representing the second cause of mortality in children under five years of age (Gonzalez et al., 2011). According to the World Health Organization (WHO), every year 760,000 children die from this disease. Moreover, diarrhea exhibits the highest morbidity rate, being the major cause of death during the first year of life, after respiratory infections (Gómez-Duarte et al., 2014). Worldwide, AGE is associated with several etiologic agents, such as bacteria, viruses and parasites. Nowadays, these agents are responsible of one out of four children deaths (Gómez-Duarte et al., 2014). Thus, AGE imposes a heavy economic burden on healthcare, sustained not only by direct costs such as consultation, hospitalization and medication, but also by indirect ones, such as parent´s workdays lost and childcare.

The gastrointestinal viruses commonly involved in AGE are Rotaviruses, Adenoviruses, Noroviruses, Astroviruses and Caliciviruses. In

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AGE, rotaviruses are the main etiologic agent. Therefore, the WHO has recommended the use of rotavirus vaccines in routine immunization programs worldwide (Plata & Quevedo, 2002), since their safety and effectiveness could substantially reduce disease burden. After vaccination, surveillance programs provide unique opportunities to examine the role of other pathogens and novel viruses with unknown pathogenicity in children with AGE.

It is well established that each child presents from seven to thirty episodes of acute diarrhea in the first five years of life, where the etiologic agent is rarely identified (Plata & Quevedo, 2002). Considering that there are no effective treatments for viral gastroenteritis, the early notification of AGE cases to the Health Services is very important not only to determine the frequency of these pathogens in the general population, but also to establish control measures to overcome this health problem. Regional and local epidemiological information on viral circulation is important for healthcare practitioners and health system authorities to develop suitable vaccines and implement infection control measuresSolomon et al., 2010.

Human adenovirus (HAdV) belongs to the Mastadenovirus genus of the family Adenoviridae, implicated in acute respiratory, gastrointestinal, and urinary tract infections. Although HAdV has been mainly associated to respiratory disease, in the last few years it has consistently been involved in AGE, becoming an important cause of this disease in children less than five years of age (Godoy et al., 2012). To date, 52 HAdV serotypes have been characterized and classified according to their nucleic acid characteristics and homologies, hexon and fiber protein characteristics and biological properties, and organized into seven species, HAdV-A to -G (Wadell, 1984; Jones et al., 2007). In fact, HAdV species demarcation is based on evolutionary distance, as reflected by phylogenetic distances and genome organizational differences. From a biological point of view, species F (serotypes 40 and 41) is mainly associated with AGE, being responsible for 1 to 20% of AGE diseases in hospitalized patients as well as in outpatients (Rakhi et al., 2011). Moreover, serotypes 40 and 41 are the primarily responsible of AGE cases in children younger than two years old (Jex et al., 2012). A particular feature of HAdV-40 and -41 infections is the lengthened diarrhea, extending the disease period almost to 12 days, raising the associated costs (Rakhi et al., 2011). Worldwide, HAdVs reach prevalences of 1-8% for AGE cases in developed countries and 2-31% for AGE in developing ones (Gonzalez et al., 2011), making HAdVs a considerable public health problem.

Considering the worldwide mortality rate for AGE is quite important to find the responsible agents, characterize them, and make the appropriate epidemiologic follow-up. In Colombia, the epidemiologic surveillance is performed analyzing fecal samples from children less than five years presenting evidence of AGE. Samples are first analyzed through an immunochromatographic rapid test for the presence of rotavirus. Enteric viruses other than rotavirus are then analyzed using ELISA approaches. Traditionally, viral culture, electron microscopy, and rapid latex agglutination tests were used to detect viral pathogens, mainly rotavirus, adenovirus and other enteroviruses. However, studies performed in the last decade have shown an explosive increase in detection rates by using molecular PCR-based assays, mostly in false negative samples, due to low viral loads (Wolffs et al., 2011; Jayoung et al., 2014).

Nowadays, Colombia lacks information regarding HAdV prevalence in AGE children identified by the Sentinel Surveillance System. Thus, the development and implementation of molecular diagnostic tests is of great importance to determine such prevalence at national level. In view of this situation, the objective of the present study was to determine the frequency of enteric HAdV, particularly species F (serotypes 40 and 41) in children with AGE identified by the Sentinel Surveillance System of the city of Bogota (Colombia) during 2012 and 2013, using a validated in-house PCR-based technique.

2. Materials and Methods

2.1. Study population

Two hundred and fifty four fecal samples collected in Bogota during 2012 and 2013 were included in this study. All samples belonged to children less than five years of age (78.74% children less than 1 year and 21.26% children aged 1 year or older) with acute diarrhea, identified by the Sentinel Surveillance System.

All cases were clinically compatible with the definition of an AGE case, established by the Secretary of Health from Bogotá, Colombia. The Ethics Committee of the Secretary of Health from Bogotá DC, Colombia approved the study protocol.

2.2. DNA extraction

Viral DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (QIAGEN, Germany), according to the manufacturer´s recommendations. Isolated DNA was quantified using a Nanodrop 2000 UV/Vis spectrophotometer (Thermo Scientific, USA). DNA purity was determined by the absorbance measured at 260/280 nm. DNA concentration was finally adjusted at 10 ng/µl in TE buffer (Tris 10mM, EDTA 1mM) and kept frozen until used.

2.3. PCR analysis

Generic HAdV detection was performed by PCR amplification using the primers described by Xu and colleagues (2000) in a final volume of 25 µl, using 1.25 units of Platinum Taq DNA polymerase (Thermo Scientific, USA), 2.5 mM MgCl2, 200 µM of each dNTP in PCR buffer (Platinum Blue PCR Supermix, Invitrogen, USA), and 10 µM of each primer. The reactions were cycled as follows: 1 cycle of 94^{o} C for 5 min, 30 cycles of 1 min at 94^{o} C, 45 s at 54^{o} C, 1 min at 72^{o} C, and finally 1 cycle of 5 min at 72^{o} C for chain elongation. HAdV previously isolated from a fecal sample was used as a positive control. Briefly, HAdV was identified in stool samples by means of a conventional ELISA test. A positive stool sample was subjected to conventional PCR using the primers described by Xu and colleagues (2000). The amplified fragment was then sequenced in order to confirm the HAdV identity. Positive and negative controls were always included in the performed experiments to assure the effectiveness of the PCR reactions (Figure 1).

Species-specific HAdV identification (including serotypes 40 and 41) was also performed according to Xu et al. (2000) with some minor modifications, and using the same oligonucleotide primers complementary to the fiber gene of the respective HAdV species. Briefly, PCR reactions were done in a final volume of 25 µl, using 1.25 units of Platinum Taq DNA polymerase (Thermo Scientific, USA), 2.5 mM MgCl2, 200 µM of each dNTP in PCR buffer (Platinum Blue PCR Supermix, Invitrogen, USA), and 200 µM of each primer. The reactions were cycled as follows: 1 cycle of 94^{o} C for 5 min, 30 cycles of 1 min at 94^{o} C, 45 s at 54^{o} C (species A-D) or 45 s at 52^{o} C (species E-F), 1 min at 72^{o} C, and a final cycle of 5 min at 72^{o} C for chain elongation. Finally, HAdV serotypes 40 and 41 were identified by PCR with the same mixes as for HAdV species-specific identification, using the primers described by Samarbaf-Zadeh and colleagues (2010). These reactions were cycled as follows: 1 cycle of 94^{o} C for 5 min, 30 cycles of 30 s at 94^{o} C, 30 s at 55^{o} C, 30 s at 72^{o} C, and a final cycle of 5 min at 72^{o} C for chain elongation.

The obtained generic, species-specific and serotype 40-41 HAdV amplicons were subjected to cycle sequencing and a BLAST search (https://www.ncbi.nlm.nih.gov/BLAST/) in order to confirm the identity of the amplification products. Also, a sensitivity test was performed for generic HAdV detection, using serial dilutions of the HAdV DNAs. Finally, specificity tests were done, using the described set of primers with DNA isolated from *Escherichia coli* (ATCC 95922) and *Giardia duodenalis* as PCR targets.

2.4. Statistical analysis

The association of HAdV positivity with children age and seasonality was tested for statistical significance using the Fisher's Exact test, with a basic significance level fixed at $p < 0.05$.

3. Results

In the present work, all samples were investigated for HAdV presence using generic PCR. Samples positive for generic HAdV were further investigated using species-specific and serotype-specific PCR amplification by means of a set of primers previously reported. The DNA from all the samples was adequately amplified by the β-globin control primers. Under our experimental conditions, the PCR sensitivity for generic HAdV PCR was extremely high, reaching a detection limit of 9.77 x 10^{-4} ng/µl (Figure 2). Furthermore, PCR reactions were

highly specific, showing negative results when DNA different from HAdV was used as PCR target. DNA sequence analysis showed that all the PCR products specifically amplified the HAdV genome, in accordance with data previously reported (Xu et al., 2000).

Among the 254 AGE cases, 14 (5.51%) were positive for generic HAdV, and 78.6% of positive cases were found in children less than one year. Fisher's exact test showed no significant differences in HAdV prevalence between children less than one year and older children ($F= 1.00$; $p > 0.05$). The species-specific analysis of samples positive for generic HAdV showed that HAdV-F and HAdV-B were the most prevalent species in the analyzed population (35.7% and 28.6%, respectively). One sample was positive for HAdV-A (7.1%), two samples were positive for HAdV-D (14.3%), and other two samples were positive for HAdV-E (14.3%). None of the samples was positive for the HAdV-C species. HAdV-F was more prevalent in the rainy season, from November to May (Table 1).

HAdV-F positive samples were further analyzed for genotypes 40 and 41 by conventional PCR. All samples were infected by genotype 40 and one sample was coinfected with genotypes 40 and 41.

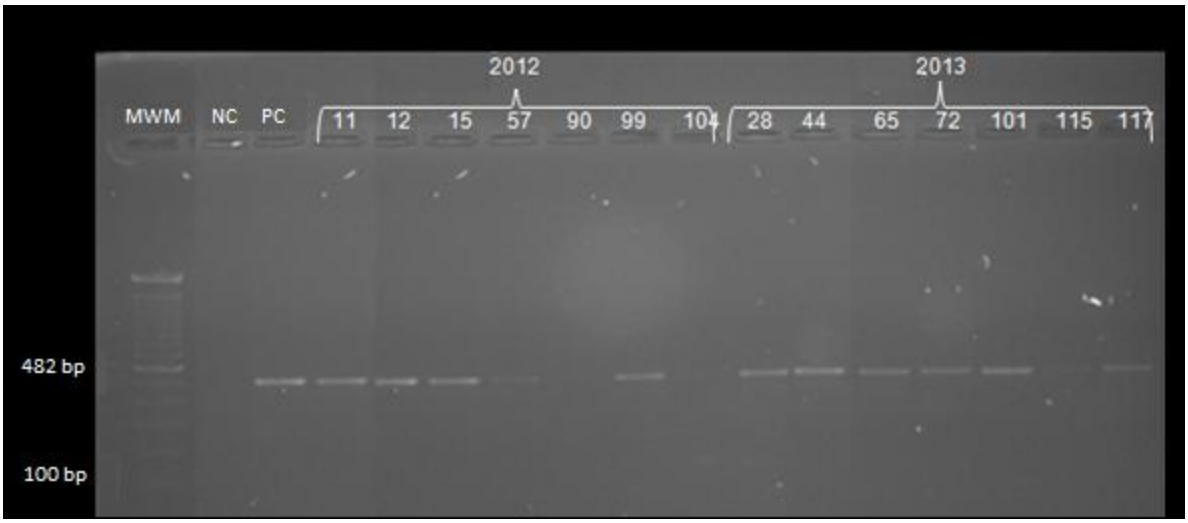

Figure 1. PCR amplification with generic primers for HAdV detection. MWM: Molecular Weight Marker. NC: Negative Control. PC: Positive Control

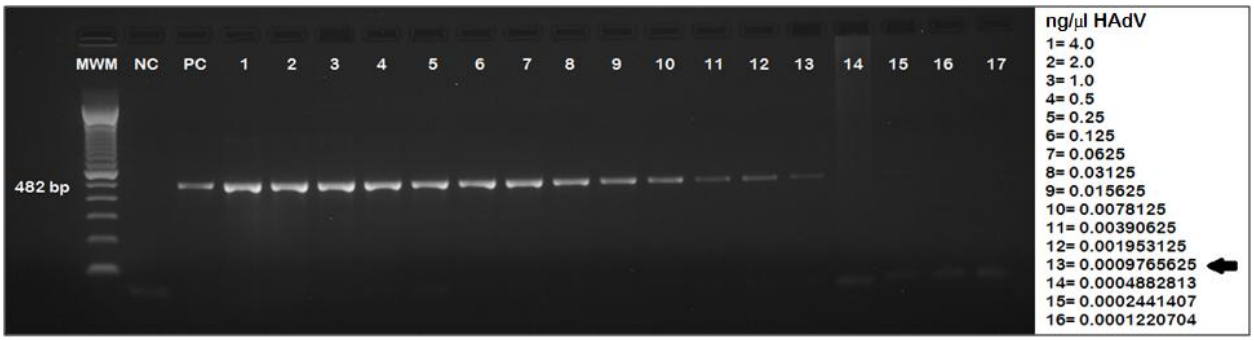

Figure 2. Detection limit reached after performing sensitivity assay for HAdV generic primers using serial dilutions of HAdV DNA. The arrow indicates the maximum dilution showing amplification.

Table 2. Seasonal distribution of the HAdV species detected in 14 patients

Sample #	Period	HAdV Species	Season
11444	May 2012	E	Rainy
11838	May 2012	B	Rainy
13377	May 2012	D	Rainy
27590	Aug 2012	F	Dry
41183	Nov 2012	B	Rainy
43309	Dec 2012	F	Rainy
45156	Dec 2012	F	Rainy
6862	Mar 2013	E	Dry
14380	Apr 2013	F	Rainy
21818	May 2013	F	Rainy
23906	May 2013	D	Rainy
35826	Aug 2013	B	Dry
43700	Sept 2013	A	Dry
43970	Sept 2013	B	Dry

4. Discussion

In this study, we evaluated 254 fecal samples obtained from children less than five years of age presenting AGE and recruited by the Sentinel Surveillance System from the Secretary of Health, Bogotá, Colombia. Contrarily to that reported for other Latin American countries (Alves et al., 2010; Luna et al., 2013), in Bogota the etiologic role of the HAdV in enteric disease is not fully understood. Our results showed that HAdV tested positive in 5.51% of the fecal samples analyzed. This HAdV prevalence is concordant with the one obtained in other studies, using the same molecular techniques. In this sense, Verma et al. (2013) and Rambha et al. (2016) found almost the same prevalence of HAdV positivity (7.74% and 5.6%, respectively) in children less than 5 years from India. González et al. (2011), in a population from Venezuela, and Sanaei et al. (2016), in a population of children with AGE from Iran, found HAdV infection rates in the order of 5%. However, other studies have reported higher or lower frequencies of HAdV in comparable populations, such as the study performed by Chhabra et al. (2013) detecting HAdV in 11.8% of AGE cases from the United States. On the other hand, Gonzalez et al. (2011) in a prospective study performed in Spain, and Wafa et al. (2013), analyzing AGE cases in children and adults from Sudan, found HAdV prevalences of 1.7% and 2.25%, respectively. All these results demonstrate that HAdV enteric infection is an inconsistent event, with variable frequencies in different populations and world regions, independently of socio-economic status.

Our results also showed that HAdV is more prevalent in males than females (64.3% vs. 35.7%). Although the proportion of infected males was larger, the statistical analysis showed no significant differences when comparing HAdV positivity with gender ($p > 0.05$; OR: 2.5424 IC: 0.7757 – 8.3325). However, reports are again contradictory. González et al. (2011) found that enteric viral infections are more common in boys (27%) than girls (16%), whereas Paleologou et al. (2008) found exactly the opposite, with prevalences showing a 1:2 male/female ratio.

Worldwide, HAdV appeared as one of the major causes of AGE (Wafa et al., 2013), mainly associated with children less than 5 years. Moreover, several studies from Asia, Europe and America showed that HAdV mainly affected children under 2 years (Motamedifar et al., 2013; Zengzhi et al., 2013; Atencio et al., 2015; Dona et al., 2016). In this study, although 85.7% of HAdV infection occurred in children less than 1 year, viral infection was apparently independent of the age of patients ($p > 0.05$; OR: 1.6556, IC: 0.5623 – 4.8743).

In the present work, HAdV species genotyping was one of our key objectives.

Worldwide, HAdV-F is the adenovirus species frequently related to gastroenteritis (Jones et al., 2007; Jayoung et al., 2014). However, recent studies have shown that species other than HAdV-F could be involved in AGE (La Rosa et al., 2015; Rambha et al., 2016). In this sense, our results evidenced that HAdV-F was the main species present in this series of AGE, reaching near 36% of cases. However, other HAdV species appeared related to gastroenteric diseases with lower frequencies. For instance, HAdV-B, uncommon in fecal samples, appeared in almost 29% of AGE cases. Meanwhile, HAdV-D (frequently present in corneal samples) and HAdV-E also appeared related to AGE, with a frequency of 14% each. Finally, HAdV-A was the less represented non-enteric adenovirus species, with a frequency of 7% in these AGE cases. This situation raises the possibility that HAdV species tightly related to acute febrile pharyngitis, pneumonia and keratoconjunctivitis (Jones et al., 2007) act as etiologic agents in gastroenteric diseases.

There is some evidence relating HAdV infection to seasonality. In the context of HAdV infection seasonal distribution, our results showed a higher prevalence of HAdV-F in the rainy season, while the non-enteric HAdV species were predominantly distributed along the dry months. A study conducted in India showed concordant results, with HAdV predominantly distributed in the rainy season (Rambha et al., 2016). Contrarily, a study from Bangladesh (Dey et al., 2009) reported that HAdV-F (serotype 40) appeared distributed from October to January (the dry winter season in that world region), being absent the rest of the year. Thus, HAdV species responsible for AGE appeared differentially distributed along seasons in different regions of the world.

Gastroenteric diseases are classified according to their etiolgy in parasitic, bacterial and viral gastroenteritis, even though many of the etiological agents have been recognized only in the last decade (Wafa et al., 2013). In the present study, the 14 HAdV-positive samples tested negative for parasites and bacteria, demonstrating that they all had a viral origin. In this regard, Wolffs et al. (2011) and Borrows et al. (2014) reported that rotavirus and adenovirus are the most prevalent pathogens, responsible for 10% to 20% of the AGE cases.

The HAdV-F species is composed of genotypes 40 and 41. Our results showed that all HAdV-F infected samples belonged to genotype 40, including a single case coinfected with both genotypes (40 and 41). Overall, 1.96% of the AGE cases were positive for HAdV-F 40 and 41. Epidemiological data show that HAdV-F is responsible for 1-20% of AGE cases, depending on the analyzed world region. For example, Rezaei et al. (2012) reported 8% HAdV-F (genotypes 40 and 41) in Iran, a higher value than that reported in this work. In addition, the data obtained by Zengzhi et al. (2013) showed that genotype 40 is the most prevalent HAdV-F in China, exactly the opposite to our results. The absence of data concerning HAdV genotype identification precludes a wide comparison of the patterns of infection in different world areas as well as the association of such data with the clinical outcome.

In conclusion, the analysis of children less than five years with AGE, identified by the Sentinel Surveillance System from Bogota DC, showed that enteric and non-enteric adenoviruses are etiological agents of great importance in AGE. These findings highlight the need for developing and employing molecular diagnostic methods in order to effectively characterize the disease and clearly identify the HAdV genotypes associated with the gastrointestinal disease. Finally, this is the first study of HAdV infection in AGE performed in Bogota DC, Colombia.

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Refereces

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