Acute gastroenteritis and enteric viruses in hospitalised children in southern Brazil: aetiology, seasonality and clinical outcomes

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Viral acute gastroenteritis (AG) is a significant cause of hospitalisation in children younger than five years. Group A rotavirus (RVA) is responsible for 30% of these cases. Following the introduction of RVA immunisation in Brazil in 2006, a decreased circulation of this virus has been observed. However, AG remains an important cause of hospitalisation of paediatric patients and only limited data are available regarding the role of other enteric viruses in these cases. We conducted a prospective study of paediatric patients hospitalised for AG. Stool samples were collected to investigate human adenovirus (HAdV), RVA, norovirus (NoV) and astrovirus (AstV). NoV typing was performed by nucleotide sequencing and phylogenetic analysis. From the 225 samples tested, 60 (26%) were positive for at least one viral agent. HAdV, NoV, RVA and AstV were detected in 16%, 8%, 6% and 0% of the samples, respectively. Mixed infections were found in nine patients: HAdV/RVA (5), HAdV/NoV (3) and HAdV/NoV/RVA (1). The frequency of fever and lymphocytosis was significantly higher in virus-infected patients. Phylogenetic analysis of NoV indicated that all of these viruses belonged to genotype GII.4. The significant frequency of these pathogens in patients with AG highlights the need to routinely implement laboratory investigations.

Key words: gastroenteritis - children - rotavirus - norovirus - astrovirus - human adenovirus

Acute gastroenteritis (AG) is the most common gastrointestinal inflammatory condition affecting people in both developed and developing countries (Andreasi et al. 2008, Domínguez et al. 2009). Worldwide, diarrhoea remains the second leading cause of death in children younger than five years. Around 1.5 million of children die annually as a result of AG, which represents 15% of all deaths that occur in this population group (Boschi-Pinto et al. 2008, Black et al. 2010, Wardlaw et al. 2010). In the United States of America (USA), 200,000 children are hospitalised each year with this disease, resulting in 300-400 deaths, and thereby generating a high economic impact (McCollough & Sharieff 2006). In Brazil, AG represents a major cause of morbidity and mortality in the first year of life and, in 2006, about 120,000 hospitalisations of children less than five years occurred due to AG (RIPSA 2008).

Over 20 different types of viruses have been identified as etiologic agents of AG, but the major viruses associated with acute diarrhoea in children can be divided into four different families: Reoviridae [rotavirus (RV)], Caliciviridae [norovirus (NoV) and sapovirus], Astroviridae [astrovirus (AstVs)] and Adenoviridae [adenovirus (AdV)] (Santos et al. 2007). Other viruses, such as aichivirus, human parechovirus and human bocavirus have been described in faecal samples from patients with diarrhoea, but their association with AG has still not been established (Chhabra et al. 2013).

Since the mid-2000s, two RV vaccines became available, a monovalent RV vaccine (Rotarix®, GlaxoSmithKline Biologicals Inc) and a pentavalent RV vaccine (RV5, RotaTeq®, Merck & Co, Inc). Both vaccines are recommended by the World Health Organization, have been used in several countries and studies have demonstrated a significant reduction of hospitalisation and mortality due to RV gastroenteritis (Justino et al. 2012, Lopman et al. 2012, Soares-Weiser et al. 2012). Brazil was one of the first countries to introduce universal vaccination against RVA, Rotarix®, which has been provided free through the public health system since March of 2006. Before the introduction of the group A RV (RVA) vaccine in Brazil, the frequency for this virus in the population with AG in our institution was 30%. The number of RVA positive cases has decreased substantially since then (Pereira et al. 2011), while other pathogens are now reported more frequently.

NoVs have been recognised as the major causes of non-bacterial AG in all age groups in industrialised countries and are frequently associated with food and water-borne outbreaks. Human NoV cannot be grown in cell culture, they are non-enveloped RNA viruses with icosahedral symmetry and are divided into five genogroup (GI-GV), which are further divided into > 30 genotypes, with genogroups GI and GII being associated with human infections (Mead et al. 1999, Hardy 2005, Patel et al. 2009, Khamrin et al. 2010, Kroneman et al. 2013).

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Human AdV (HAdV) is a non-enveloped DNA virus with icosahedral symmetry. The AdV has been associated with a wide spectrum of clinical manifestations, including respiratory, gastrointestinal, ocular, neurological and urinary tract infections. This family comprises 55 different serotypes (HAdV 1-55) grouped into seven subgenera (A-G), of which the genotypes 40, 41 and more rarely, 38 are related to acute diarrhoea (Wold & Horwitz 2007, Ramani & Kang 2009, Walsh et al. 2010).

Human AstVs (HAstVs) are non-enveloped RNA viruses with icosahedral symmetry. They are classified in eight serotypes (HAstVs 1-8), which are further divided into four subtypes (1a, 1b, 1c and 1d). Studies have shown that colonisation or infection by HAstV may be associated with necrotising enterocolitis, especially in preterm infants (Santos & Cardoso 2005, Marshall et al. 2007, Bagci et al. 2010).

To assess the impact of these enteric viruses in the aetiology of AG in hospitalised children in our institution, this study aimed to identify (i) the frequency of RVA, HAdV, HAstV and NoV in stool samples, (ii) report the clinical findings of these infections, (iii) analyse the displacement of these viruses after the decrease in RVA cases during the period between September 2010-September 2011 and (iv) and genotypically characterise the NoV detected.

SUBJECTS, MATERIALS AND METHODS

Material - This study evaluated 225 stool samples from hospitalised paediatric patients, which were sent to the Virology Laboratory of the Faculty of Medicine Clinics Hospital, Federal University of Paraná (HC-UFPR) to test for RVA between the period of September 2010-September 2011. Medical records of the patients were reviewed to evaluate clinical, laboratory and demographic data. The HC-UFPR Institutional Review Board approved the study (IRB#0221.0.208.000-10).

The HC-UFPR is a tertiary academic care hospital where patients with severe infections are referred and AG is one of the most frequent causes of paediatric hospital admissions.

RV detection - RVA antigen detection was carried out using the enzymatic immunoassay Rotascreen II® kit (Microgen Bioproducts, UK), according to the manufacturer's instructions.

Viral DNA/RNA extraction - DNA/RNA was extracted from 150 µL of clarified 10% faecal suspension with the buffer Tris-HCl (0.01 M)-$CaCl_2$ (0.0015 M) by using a commercial kit (Intron Biotechnology Inc, South Korea) according to the manufacturer's instructions. Pseudotrabies viruses (PRV) were added to the lysis buffer at a concentration of 1.74×10^{-8} ng/µL for use as an internal control for extraction.

AdV detection - Generic primers and amplification tests were carried out as described by Avellón et al. (2001) (Table I) with some modifications. Briefly, 2.5 µL of extraction product was added to 22.5 µL of a PCR mix (Bioron, Ludwigshafe, GE) containing 2.5 mM dNTPs, 1.25 U of Taq DNA Polymerase, 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 25 mM $MgCl_2$ and primary amplification primers for AdV and PRV at a concentration of 10 pmol/µL. Amplification was carried out on a Mastercycler Personal (Eppendorf Inc, Hamburg, GE) thermocycler with the following cycling conditions: one hold of 94ºC/2 min, 40 cycles of 94ºC/60 s, 50ºC/60 s, 72ºC/60 s and one extension step of 72ºC/6 min. For the second reaction, 1 µL of primary amplification product was added to 24 µL of a new PCR mix similar to that for the primary amplification, but containing secondary amplification primers. PCR products were resolved on 1% agarose gel electrophoresis stained with ethidium bromide (0.5 µg/mL).

TABLE I

Sequences of the primers used in polymerase chain reaction (PCR) and reverse transcription-PCR for the detection of adenovirus (AdV), pseudorabies viruses (PRV), norovirus (NoV) and astrovirus (AstV)

Primer	Sequence 5'-3'	Gene target	Nested-PCR	Product	Reference
ADHEX 2F	CCCMTTYAACCACCACCG	AdV Hexon	Yes	169 bp	Avellón et al. (2001)
ADHEX 1R	ACATCCTTB CKGAAGTTCCA				
ADHEX 2R	KATGGGGTARAGCATGTT				
ADHEX 1F	AACACCTAYGASTACATGAAC				
PRV 1+	CGCGTGGTCTACGGGGACACGGA	PRV DNA polymerase	Yes	140 bp	Pozo and Tenorio (1999)
PRV 1-	ATGACGCCGATGTACTTCTTCTT				
PRV 2+	GGGACACGGACTCGGTCTCC				
PRV 2-	CCGGAAGGTCTTCTCGCACTC				
JV12	ATACCACTATGATGCAGATTA	NoV RNA polymerase	No	430 bp	Vinjé and Koopmans (1996)
JV13	TCATCATCACCATAGAAAGAG				
Mon 270F	CAACTCAGGAAACAGGGTGT	AstV ORF2	No	449 bp	Noel et al. (1995)
Mon 269R	TCAGATGCATTGTCATTGGT				

ORF: open reading frame.

AstV and NoV detection - Reverse transcription-polymerase chain reaction (RT-PCR) was performed for detection of HAstV and NoV. First-strand cDNA was synthesised using random primers and a RT system (SuperScript III® Reverse Transcriptase, Invitrogen, USA). Briefly, cDNA was obtained by adding 1 µL random primer (3 µg/µL, Invitrogen™) and 1 µL of ultrapure water in 7.5 mL of RNA, followed by incubation for 5 min at 65ºC. Then, it was added 10.5 µL of a RT-master mix containing 4 µL of deoxynucleotide triphosphates (dNTPs) (2.5 mM each), 4 µL of cDNA buffer (5 x), 2 µL of 0.1M dithiotreitol (DTT) (Invitrogen™) 0.25 µL of RNase inhibitor RNase OUT™ (40U/µL, Invitrogen™) and 0.25 µL of enzyme reverse transcriptase SuperScript® III (2.000U/µL, Invitrogen™), with subsequent incubation for 5 min at 25ºC, 60 min at 50ºC and 15 min at 70°C. Generic primers and amplification tests for HAstV and NoV were carried out as reported by Noel et al. (1995) and Vinjé and Koopmans (1996), respectively (Table I). Briefly, 2.5 µL of extraction product were added to 22.5 µL of a PCR mix (Bioron, GE) containing 2.5 mM dNTPs, 1.25 U of Taq DNA polymerase, 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 25 mM $MgCl_2$ and primary amplification primer for NoV or HAstV at a concentration of 10 pmol/µL. NoV amplification was carried out on a Mastercycler Personal (Eppendorf Inc) thermocycler programmed with the cycling conditions: 94ºC for 2 min followed by 30 cycles of 94ºC/60 s, 37ºC/30 s, 72ºC/30 s and an elongation step of 72ºC/6 min. HAstV amplification was performed using the same cycling protocol described for HAdV. Both PCR products were resolved on 1% agarose gel electrophoresis stained with ethidium bromide (0.5 µg/mL). The gels were photographed under ultraviolet light and the bands were analysed using the E-Capt program.

Nucleotide sequencing - Nucleotide sequencing of positive samples was performed to genotype the NoV detected. The initial amplification was performed using three sets of primers described by Vinjé et al. (2004) (Table II), with one being GI specific, one being GII specific and the third exhibiting no genogroup specificity. Briefly, 2.5 µL of cDNA was added to 22.5 µL of a PCR mix (Bioron) containing 2.5 mM dNTPs, 1.25 U Taq DNA polymerase, 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 25 mM $MgCl_2$ and primary genotyping primers for NoV at a concentration of 10 pmol/µL. The amplification was carried out on a Mastercycler Personal (Eppendorf Inc) thermocycler programmed with the cycling conditions: 94ºC for 1 min, followed by 40 cycles of 94ºC/60 s, 44ºC/60 s, 72ºC/60 s and an elongation step of 72ºC/10 min. Afterwards, the PCR products were resolved on 1.5% agarose gel electrophoresis stained with ethidium bromide (0.5 µg/mL).

The amplicons obtained from one of the three sets of primers were subjected to sequencing reactions with the same primers. PCR products were purified using the Invisorb® Fragment Clean up (Invitek GmbH, Berlin, GE) kit, according to the manufacturer's instructions. Sequencing reactions were carried out using a Big Dye® Terminator v.3.1 kit and the ABI3130 automated sequencer (Applied Biosystems, CA, USA).

Multiple sequence alignments were carried out using CLUSTALW software from BioEdit v.7.0.9 package (Ibis Biosciences, CA, USA) and phylogenetic analysis was performed using MEGA 5.1 (Tamura et al. 2011). The sequences (VP1 protein and RNA polymerase genes) were compared to a panel of reference sequences obtained from GenBank. Phylogenetic trees were generated using the neighbour-joining algorithm with bootstrap of 1,000 replicates and evolutionary distances were calculated using the Kimura two-parameter method.

Statistical analysis - Demographic and clinical data were compiled using JMP software v.5.2.1 and analysed using GraphPad Prism® v.5.03. Fisher exact or $\chi 2$ tests were used to assess differences between groups and the Mann Whitney test was used for continuous variables, as appropriate. Results for continuous data have been expressed as median ± interquartile ranges. All p-values are two-tailed and a value of < 0.05 was considered as significant.

TABLE II

Sequences of primers used for genome amplification and nucleotide sequencing of norovirus[a]

Primer	Sequence 5'-3'	Product	Gene target	Genogroup
Cap A	GGCWGTTCCCACAGGCTT	177 bp	VP1	GI
Cap B1	TATGTTGACCCTGATAC			
Cap B2	TATGTIGAYCCWGACAC			
Cap C	CCTTYCCAKWTCCCAYGG	253 bp	VP1	GII
Cap D1	TGTCTRSTCCCCCAGGAATG			
Cap D3	TGYCTYITICCHCARGAATGG			
MJV12	TAYCAYTATGATGCHGAYTA	327 bp	RNA polymerase	GI and GII
Reg A	CTCRTCATCICCATARAAIGA			

a: Vinjé et al. (2004).

RESULTS

From 225 samples sent to the virology laboratory between September 2010-September 2011, 60 (26%) were positive for at least one viral agent. HAdV was detected in 36 (16%) samples, NoV in 19 samples (8%) and RVA in 14 samples (6%). Additionally, we observed five cases of mixed infection involving HAdV and RVA, three cases of HAdV combined with NoV and one case of triple infection involving RVA, HAdV and NoV. No cases of AstV infection were detected. Most positive samples were from patients older than two years. There were five case of RVA infection in a patient aged less than two years, who had not received the full vaccination or had underlying diseases with immunological impairment (Table III).

The mean number of stool samples sent to the laboratory was approximately 17 samples/month (± 4.8), with an average of five positive samples/month (± 2.6). The distribution of positive samples and its relation to the average monthly temperature (ºC) and precipitation (cm^3) between September 2010-September 2011 revealed the absence of a seasonal pattern for the studied viruses, as the pathogen detections occurred throughout the year (Fig. 1).

The group of patients that presented infection by one or more viral agents (infected group) was compared with the group of individuals who tested negative for any of the searched viral agents (not infected group). Significant differences were observed for the presence of fever ($p = 0.0051$) and the number of lymphocytes in the blood count ($p = 0.0224$), which were higher in the infected group. Results were also obtained for faecal occult blood, parasites, reducing substances and stool culture, but the majority of the samples tested produced negative results, with no significant differences between the groups.

Nineteen samples were positive for NoV and from these we obtained 14 sequences for VP1 gene and 10 sequences for RNA polymerase gene to perform the phylogenetic analysis. These sequences were compared to VP1 genes, RNA polymerase genes and entire genome sequences of different NoV genotypes collected from GenBank. The final sequence alignment revealed 208 informative positions for analysis of VP1 gene and a phylogenetic tree was constructed (Fig. 2). Phylogenetic analysis of RNA polymerase gene was carried out, the results were the same as that performed with VP1 region (data not shown) and all sequences belonged to the genotype GII.4.

DISCUSSION

Analysis conducted previously in this hospital to assess the impact of vaccination against RVA indicated a reduction of 54.2% and 39.4% in medical consultations for children less than 12 months old and between 12-months, respectively, and a reduction of 44% in the number of hospitalisations for gastroenteritis in children less than 12 months. However, diarrhoea still represents a frequent cause of hospitalisation and, except for RVA, the role of other enteric viruses was not determined in our patients and their incidence was likely underestimated. Previously, we have carried out RV genotypic characterisation in all positive samples and G2 P[4] was the most prevalent genotype after the vaccine implementation in 2006 (Pereira et al. 2013).

Overall, 26% of samples were positive for enteric viruses, with HAdV the most frequent virus detected, corresponding to a frequency of 16%. The prevalence of HAdV in prior studies has been variable, ranging from 0.7% to > 30% (Domínguez et al. 2009, Stroparo et al. 2010). In central Brazil, the state of Mato Grosso do Sul, AdVs were found in 3.6% of cases of AG (Andreasi et al. 2008), while other studies, such as those by Simpson et

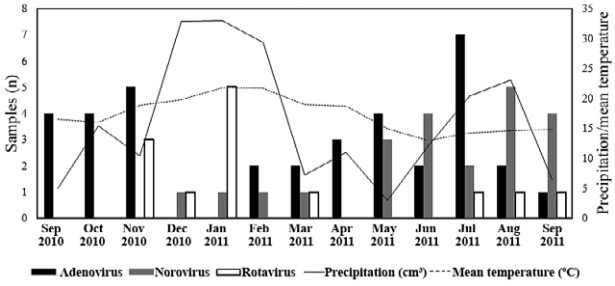

Fig. 1: distribution of positive samples for human adenovirus, norovirus, group A rotavirus and the correlation between rainfall and mean temperature from September 2010-September 2011. Source: Meteorological System of Paraná.

TABLE III

Enteric virus detected according to stratified age group

Virus	0-6 months (n)	7-12 months (n)	13-24 months (n)	> 24 months (n)
HAdV	7	3	7	19
NoV	7	5	4	3
RVA	1	2	2	9
AstVs	-	-	-	-
HAdV + RVA	-	-	1	4
HAdV + NoV	-	-	1	2
HAdV + RVA + NoV	-	-	-	1

AstVs: astrovirus; HAdV: human adenovirus; NoV: norovirus; RVA: group A rotavirus.

al. (2003) and Cunliffe et al. (2010), have reported higher frequencies of HAdV (7.9% and 15%, respectively). Previously, Pereira Filho et al. (2007) also reported a frequency of 2% of AdV antigen detection in hospitalised and community children with diarrhoea in Rio de Janeiro and Salvador, respectively. These lower frequencies might have been underestimated due to the technique used, that is, antigen detection. Subsequent introduction of molecular methods for studying this virus has led to a significant increase in the frequency of AdV detection in stools (Rohayem et al. 2004)

It is important to note that this study was carried out using a combination of generic primer for HAdVs detection, whose molecular sequencing of the products did not allow genotyping of detected virus. Then, the identified virus in the sample should not necessarily be considered the causative agent of the AG, as infections caused by some HAdV genotypes can result in an intermittent viral excretion in the stools after a previous infection. However, analysis of patient medical records did not reveal any further diseases that could be associated with other HAdV serotype infections. NoV was the second most common virus detected in the studied patients, responsible for 8% (19/225) of AG cases, with RVA cases accounting for 6% (14/225). Andreasi et al. (2008), in Brazil, reported similar findings, with NoV identified in 7.6% of the cases. Unlike the results of this study, Ferreira et al. (2012) in the states of Rio de Janeiro and Ribeiro et al. (2008) Espírito Santo identified this pathogen as the major viral agent in cases of gastrointestinal infections. Additionally, a study by Nakagomi et al. (2008) identified NoV in 15% of samples from the municipality of Recife, with clinical severity similar to that for RV, while in the municipality of São Paulo, Castilho et al. (2006) reported 33% positive cases of NoV and Siqueira et al. (2013a) found 36.5% of positive samples in the municipality of Belém.

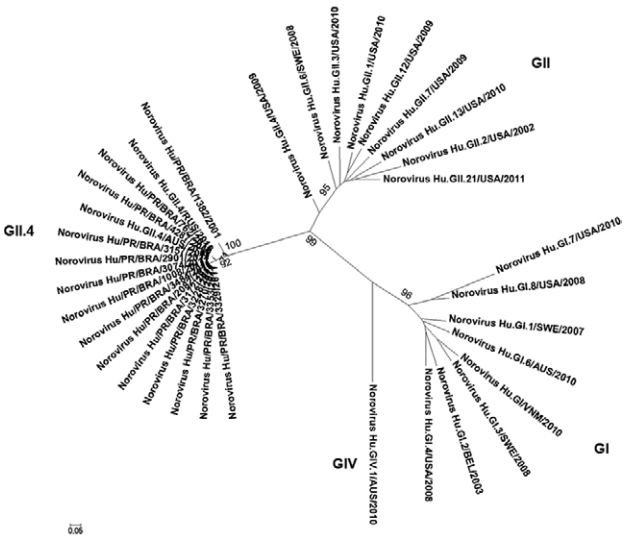

Fig. 2: neighbour-joining tree based on a 208 nucleotides sequence of the region of VP1, depicting phylogenetic relationships among norovirus from the state of Paraná, southern Brazil, and a reference panel of sequences obtained from GenBank. Sequences used as references in the analysis were as follows: JN797508.1, JQ320072.1, JN565063.1, JQ613565.1, HM596573.1, GU445325.2, JN183165.1, GU134965.1, HQ688986.1, JN899242.1, JN899245.1, JQ911594.1, FJ384783.1, FJ515294.1, JN603244.1, GQ413970.1, JQ388274.1, JN899243.1, GU299761.1 and JQ613567.1. GenBank accessions: PR_BRA_1382_2001, KF556654, PR_BRA_1008_2011, KF556655, PR_BRA_3074_2011, KF556656, PR_BRA_2082_2011, KF556657, PR_BRA_3172_2011, KF556658, PR_BRA_3228_2011, KF556659, PR_BRA_3270_2011, KF556660, PR_BRA_3329_2011, KF556661, PR_BRA_3353_2011, KF556662, PR_BRA_3484_2011, KF556663, PR_BRA_2901_2011, KF556664, PR_BRA_3157_2011, KF556665, PR_BRA_762_2011, KF556666, PR_BRA_4287_2010, KF556667. AUS: Australia; BEL: Belgium; BRA: Brazil; Hu: human; PR: Paraná; RUS: Russia; SWE: Sweden; USA: United States of America; VNM: Vietnam.

HAstV was not observed in this study. Some studies from other regions of Brazil and other countries (e.g., Japan, Greece and India) have also reported minimal circulation of this pathogen compared to other enteric viruses (Santos et al. 2007, Domínguez et al. 2009, Levidiotou et al. 2009, Chan-it et al. 2010, Verma et al. 2010). Usually, HAstV infections are not associated with severe AG, consequently, its identification in hospitalised patients might be uncommon. On contrary of our findings, a study carried out in Rio de Janeiro in 2004 shown a prevalence of 14% of AstV in the samples collected from hospitalised children with AG (Victoria et al. 2007). Interestingly, the protocol used in the present study was very similar to that reported, including the use of random primer to obtain cDNA and the sequences of specific primers employed in the PCR. Indeed, the prevalence of this virus in several studies is quite variable, epidemiological and seasonal factors should be considered in such analyses and studies that include laboratory surveillance for a longer time will be able to demonstrate the real impact of this pathogen on the children health.

HAdV and the NoV were detected during most of the months for which data were collected and although higher frequencies were observed in the fall and winter, no seasonal pattern or association with relative humidity was identified for these two viruses. Levidiotou et al. (2009) also failed to identify any seasonal pattern to the HAdV detected in Greece. However, Ozdemir et al. (2010) reported the presence of HAdV in the fall and winter months in Turkey, Kitajima et al. (2010) observed a higher frequency of NoV in winter and spring in Japan and Zeng et al. (2012) observed that the frequency of NoV peaks only during the cold months in China. Interestingly, similar to that reported by Siqueira et al. (2013b), we observed that the distribution of RVA and NoV positive cases throughout the year presented a distinct seasonal profile and a "seesaw effect".

Demographic data revealed no significant differences for age and gender between patients with or without viral infection. However, a trend was observed with infection occurring in younger children (median 30 months of age). Fever and vomiting were the most common symptoms presented and these were more frequent in the infected group than in the not infected group. Furthermore, significant lymphocytosis was observed in the infected group, although this parameter should be evaluated carefully since the quantity of lymphocytes in the blood can vary according to a child's age. Investigation of other pathogens associated

to diarrhoea, as enteric bacteria and parasites, as well as other evidence of infection, such as the presence of faecal occult blood resulted as negative.

Phylogenetic analysis identified only genotype GII.4 at the study site, including the NoV detected in a sample collected in 2001. This result confirms the dominance of the genotype GII.4 circulation, already observed in other regions of Brazil, such as São Paulo, Vitória, Rio de Janeiro, Salvador and Belém, and other countries such as Venezuela, Mexico and Thailand (Castilho et al. 2006, Ferreira et al. 2008, Xavier et al. 2009, Aragão et al. 2010, Barreira et al. 2010, Khamrin et al. 2010, González et al. 2011, Gómez-Santiago et al. 2012).

Since 1995, NoV has caused five pandemics of AG and despite it has over 30 genotypes circulating, it is only a single genotype - GII.4 - that cause mass outbreaks and pandemics (Lindesmith et al. 2008). Factors intrinsic to the host and virus are associated with the higher prevalence of this genotype, among these are cited (i) GII.4 viruses bind to all blood group antigens and the ability to use blood groups antigens may affect the viral infectivity, differently of other genotypes (Tan & Jiang 2005, Bull et al. 2010), (ii) the high capacity of the virus to alter its carbohydrate-binding targets over time, allowing to escape from protective immunity (Lindesmith et al. 2008), (iii) the higher mutation rate and rate of evolution of GII.4 genotype compared to the less frequently detected NoV (GII.b, GII.3 and GII.7 strains) and (iv) GII.4 lineage had on average a 1.7-fold higher rate of evolution within the capsid sequence allowing the viral persistence (Bull et al. 2010).

Recently, Kroneman et al. (2013) proposed for a new NoV nomenclature: the identification of new genotypes should be based on the sequences of both genomic regions, open reading frame (ORF)1 and VP1. In the present study it was performed partial sequencing of both VP1 and ORF1 regions and in the phylogenetic analysis all viruses found were identified as GII.4, without any finding suggestive of recombinant forms between these samples.

Prior to implementation of the RVA vaccine, a reduction in the number of RVA positive samples in our hospital had already been observed, likely due to a number of factors including: improved sanitary conditions, greater access to basic healthcare and conversion of the institution from primary to tertiary care, where only patients with severe disease are referred. With the introduction of the vaccine, the reduction was even more impressive. However, the reduction in RVA circulation has been followed by an increased occurrence of HAdV and NoV and it is likely that we can expect an escalation in the frequency of occurrence of other enteric viruses, implying the need for more accurate laboratory investigation and improved epidemiological surveillance to guide prevention, therapeutic measures and control of outbreaks.

To our knowledge, this work represents the first report detailing the observed frequency of enteric viruses, other than RVs, which are associated with severe conditions responsible for hospitalisation of paediatric patients in southern Brazil. It is important to note the limitations of this study. First, genetic characterisation of the AdV detected should be performed to confirm the presence of the most frequent strains associated with AG, particularly genotypes 40 and 41. Second, the investigation should be extended over a longer period to better assess the population distribution of these viruses and better evaluate seasonal patterns. Furthermore, prospective case-control studies, including the investigation AdVs in hospitalised children without diarrhoea should be implemented aiming to define the causal relation between the detection of these viruses and the presence of disease.

In conclusion, this study demonstrated that 26% of the AG identified in hospitalised children was associated with enteric viruses and that the introduction of RVA immunisation was associated with reduced frequency of this virus. However, reduction of RVA was followed by an increasing presence of other pathogens associated with severe clinical manifestations of AG in this population, highlighting the need to implement methods to routinely investigate these cases in a virology laboratory.

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