Molecular characterization of astrovirus in stool samples from children in São Paulo, Brazil

Hugo Reis Resque, Veridiana Munford, Juliana Galera Castilho*, Herika Schmich, Thabata Alessandra Ramos Caruzo***, Maria Lucia Rácz/+**

Laboratório de Gastroenterites Virais, Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1374, Cidade Universitária, 05508-900 São Paulo, SP, Brasil *Seção de Diagnóstico, Instituto Pasteur, São Paulo, SP, Brasil **CEPES, Faculdade de Medicina do ABC, Santo André, SP, Brasil ***Diagnostic Systems, Becton Dickinson Indústrias Cirúrgicas Ltda, São Paulo, SP, Brasil

The purpose of this study was to characterize astrovirus in faecal samples collected from children with and without diarrhea in São Paulo, Brazil, grouped into two sets: EPM and HU. Detection and genotyping were carried out using reverse transcription nested polymerase chain reaction (RT-PCR) with specific primers directed towards the genome open reading frame 2 (ORF2). Results for EPM set showed that 66/234 (28.2%) were positive: 28/94 (29.7%) from children with acute diarrhea, 14/45 (31.1%) with persistent diarrhea, and 9/55 (16.3%) from control individuals. No data was available for 15/40 (37.5%) of samples. Mixed infections with other viruses were found in 33 samples. In the HU, 18/187 (9.6%) were positive: 12/158 (7.6%) from individuals with acute diarrhea and 6/29 (20.7%) from control children. Four samples were mixed with other viruses. Out of 66 astrovirus positive EPM samples, 18 (27.2%) were characterized as human astrovirus type-1 (HAstV-1), two (3.0%) as HAstV-2, two (3.0%) as HAstV-3, and three (4.5%) as HAstV-8. Among 18 astrovirus positive HU samples, one (5.5%) was characterized as HAstV-1, six (33.3%) as HAstV-2, and one (5.5%) as HAstV-8. Two HAstV-8 genotyped samples were further confirmed by nucleotide sequencing. Our results shows that astroviruses are circulating in a constant manner in the population, with multiple serotypes, in higher frequency than it was described for other Brazilian regions. For the first time in Sao Paulo, Brazil, it was shown that astroviruses play an important role in children gastroenteritis, as described for most locations where they were detected.

Key words: astrovirus - gastroenteritis - molecular characterization - RT-PCR - nucleotide sequencing - São Paulo - Brazil.

Acute gastroenteritis is one of the most common illnesses of humankind and has its greatest impact on people at extremes of age: children and the elderly (Glass et al. 2001). It is one of the most common diseases affecting children under five years old (Victoria et al. 2007). Astroviruses were associated to infantile gastroenteritis for the first time in 1975, by Madeley and Cosgrove. Using electron microscopy, these researchers observed particles that, at that time, were called "small round structured viruses". Nowadays, astrovirus incidence in children with gastroenteritis in both developed and developing countries is usually 2 to 9% (Guix et al. 2002, Liu et al. 2007).

Human astroviruses (HAstV) are members of the *Astroviridae* family, genus *Mamastrovirus*, and are recognized as a common cause of gastroenteritis not only in children, but also in the elderly and immunocompromised individuals (Méndez & Arias 2007). The virion is small and spherical (28-30 nm in diameter), non-enveloped, with icosahedral symmetry and a star-like capsid. However, only about 10% of particles display the star-like appearance, which makes the morphological identification difficult (Fauquet et al. 2005). The genome is a positive sense single strand RNA (ssRNA), with approximately 6,8 to 7,2kb in length. The astrovirus genome has three open reading frames (ORFs): ORFs1a and 1b, which encode viral protease and polymerase, respectively, and ORF2, which encodes the capsid precursor protein (Bhattacharya et al. 2006). Regarding serotypes/genotypes, there are eight HAstV serotypes/genotypes described so far (HAstV-1 to HAstV-8), grouped into genogroup A (HAstV-1 to 5 and HAstV-8) and genogroup B (HAstV-6 and 7). The main astrovirus infection symptom is watery diarrhea that can occur associated with fever, vomiting, anorexia and abdominal pain (Gabbay et al. 2005).

Recently, three studies in Brazil described the detection and/or genotyping of astrovirus: one carried out in Rio de Janeiro, in the Southeast region, one in the city of Belém, in the North region, and another in the Midwest region (Victoria et al. 2007, Gabbay et al. 2007a, Santos et al. 2007). This study presents results of astrovirus characterization in faeces from children up to six years old, and it is the first of this kind carried out in São Paulo, also in the Southeast region Brazil.

SUBJECTS, MATERIALS AND METHODS

Faecal samples - The present study had two sets of samples, obtained from children with or without diarrhea. The first set (EPM), with 234 faecal samples, was collected from children up to six years old, from February 1995 to December 1999, at the ambulatories of

Financial support: FAPESP (2001/14335-7, 2000/11857-0, 2000/14461-0, 2001/07298-8), CNPq (140.167/2002-6)
+Corresponding author: mlracz@usp.br
Received 27 July 2007
Accepted 19 December 2007

Hospital São Paulo, Hospital Municipal de Jundiaí, and Hospital Darci Vargas, and at the gastro-pediatric unit of the University Federal of São Paulo, São Paulo (SP), Brazil. Ninety four (40.2%) out of the EPM samples were from children with acute diarrhea, 45 (19.2%) from children with persistent diarrhea, 55 (23.5%) from control individuals (no diarrhea) and 40 (17.1%) samples had no clinical data available. The second set (HU) was composed of 187 faecal samples from children up to five years old, being 158 samples (84.5%) from individuals with acute diarrhea and 29 (15.5%) without diarrhea (control individuals), obtained at the ambulatory of Hospital Universitário of the University of São Paulo (USP), from March 1994 to August 1996. Both sets of samples were previously tested for rotavirus and norovirus and they were kept frozen at -70°C until use.

Acute diarrhea was defined as diarrhea lasting less than 14 days at the time of admission. Persistent diarrhea was defined as diarrhea lasting more than 14 days. Control individuals had no gastrointestinal symptoms for at least 30 days prior to the inclusion into the study (Scaletsky et al. 2002). This study was approved by the ethical committee of the Institute of Biomedical Sciences, USP (Protocol 273/CEP).

Extraction of viral RNA from faecal suspensions - Viral RNA was extracted from faecal suspensions using Trizol® (Invitrogen-Carlsbad, CA, USA) according to manufacturer's instructions. The pellet was resuspended with DEPC Milli-Q water, treated with 1µl of RNAse inhibitor (Invitrogen-Carlsbad, CA, USA) and stored at -70°C until use.

Detection of astrovirus by RT-PCR - Both sets of samples were submitted to reverse transcription polymerase chain reaction (RT-PCR) for astrovirus detection, as described by Noel et al. (1995), using MON269 (forward 5'- CAACTCAGGAAACAGGGTGT-3') and MON270 (reverse 5'-TCAGATGCATTGTCATTGGT-3') primers, which amplify a 449 bp region located at ORF2. SuperScript™ One-Step RT-PCR with Platinum® Taq kit (Invitrogen-Carlsbad, CA, USA) was used in the reaction. Prior to RT-PCR itself, denaturation of viral nucleic acid was carried out at 70°C/5 min, followed by 2 min in ice. Reverse transcription for 1 h at 42°C was followed by 40 cycles of amplification (94°C/30 s, 50°C/30 s, 72°C/1 min) and a final extension at 72°C/10 min; 10 µl of the final product were submitted to electrophoresis in 1.5% agar gel and detected by ethidium bromide staining.

Genotyping by nested/RT-PCR - Genotyping by nested/RT-PCR was done for all astrovirus positive samples according to Sakamoto et al. (2000), with some modifications. RT-PCR was carried out with SuperScript™ One-Step RT-PCR with Platinum® Taq kit, and PreCAP1 and 12Gr common primers. The procedure included denaturation of viral nucleic acid at 95°C/5 min, followed by 5 min in ice. Reverse transcription for 1 h at 42°C was followed by 35 amplification cycles (94°C/1 min, 45°C/2 min, 72°C/3 min) and a final extension at 72°C/7 min. For nested-PCR, 13 µl Milli-Q H_2O, 4 µl dNTP's pool, 2.5 µl 10X PCR Buffer minus Mg (Invitrogen), 0.7 µl 50 mM $MgCl_2$ (Life Technologies-Carlsbad, CA, USA), 1 µl of primer END, 1 ml of specific primers pool (Ast-S1 to Ast-S8, 33mM each), 0.3 ml Platinum® Taq DNA Polymerase (Invitrogen-Carlsbad, CA, USA), and 2.5 µl of the cDNA resulting from RT-PCR were added to a plastic microtube. After that, 40 amplification cycles (94C/1 min, 45°C/2 min and 72°C/3 min) were carried out, followed by a final extension of 72°C/7 min. PCR product for each genotype was expected to be 212 bp (HAstV-1), 158 bp (HAstV-2), 119 bp (HAstV-3), 258 bp (HAstV-4), 388 bp (HAstV-5), 427 bp (HAstV-6), 548 bp (HAstV-7) and 599 bp (HAstV-8).

Nucleotide sequencing - Nucleotide sequencing was used in this study as a method of confirmation for two samples previously tested by nested/RT-PCR. For gene sequencing, cDNA resulting from RT-PCR was directly extracted using rapid PCR purification system (GibcoBRL-Carlsbad, CA, USA) protocol. Gene sequencing was performed using BigDye terminator cycle sequence ready kit (Applied Biosystems-Foster City, CA, USA), with some modifications. Sequencing reaction used 30 cycles (96ºC/2 min, 96ºC/10 s, 50ºC/10 s and 60ºC/4 min). The same primers used for astrovirus detection by RT-PCR were used for sequencing, resulting in a 449 nucleotides sequence, in ORF2. The sequencing reaction product was purified using the DyeEx™ Spin kit (Qiagen-Hilden, Germany) and then analyzed in an ABI-Prism 3100 DNA sequencer (Applied Biosystems-Foster City, CA, USA). The final consensus sequence for each sample was aligned with homologous sequences in GenBank using the Clustal/W method with the Bioedit program and the identities between the aligned sequences were calculated (Hall 1999). The alignments were then used to build a neighbor-joining distance-based phylogenetic tree using the Kimura two-parameter correction model with 1,000 replicates for statistical bootstrap support with MEGA version 3.1 (Kumar et al. 2004).

RESULTS

Astrovirus detection by RT-PCR - From 234 EPM faecal samples, 66 (28.2%) were positive for astrovirus, being 28/94 (29.7%) from children with acute diarrhea, 14/45 (31.1%) from children with persistent diarrhea, and 9/55 (16.3%) from control individuals. Fifteen positive samples out of 40 (37.5%) had no clinical data available (Table I). Among the 66 positive samples, mixtures of astrovirus and other viruses were found in 33 samples: seven were positive for astrovirus and rotavirus, 25 were positive for astrovirus and norovirus and one was positive for all three viruses. Monthly distribution of astrovirus positive samples is illustrated in Fig. 1a. From 187 HU faecal samples, 18 (9.6%) were positive for astrovirus, being 12/158 (7.6%) from individuals with acute diarrhea, and 6/29 (20.7%) from control children (Table I). Among these 18 positive samples, two were also positive for rotavirus, one was positive for both astrovirus and norovirus, and one was positive for all three viruses. Monthly distribution of positive samples is in Fig. 1b.

Genotyping by nested/RT-PCR - From EPM positive samples, 25 (37.8%) were genotyped: 18 were characterized as HAstV-1, two as HAstV-2, two as HAstV-3 and three as HAstV-8. From HU positive samples, eight (44.4%) were genotyped: one was characterized as HAstV-1, six as HAstV-2 and one as HAstV-8. (Table II).

Nucleotide sequencing - Samples ICB2622 (EPM) and ICB1333 (HU), which were characterized as HAstV-8, were further analyzed by nucleotide sequencing as a method of confirmation. They were also genotyped as type 8, showing identities of 98.1% (ICB2622) and 92.4% (ICB1333) with standard HAstV-8, Yuc-8 (Méndez-Toss et al. 2000) found in the GenBank. Samples genotyped by nucleotide sequencing, as well standards HAstV strains found in GenBank, are shown in the phylogenetic tree (Fig. 2).

TABLE I

Occurrence of astrovirus in diarrheic children and controls in sets of samples from São Paulo, Brazil

	EPM		HU	
Clinical condition	Pos/Total	%	Pos/Total	%
Accute diarrhea	28/94	29.7	12/158	7.6
Persistent diarrhea	14/45	31.1		
Control (no diarrhea)	9/55	16.3	6/29	20.7
No data	15/40	37.5		
Total	66/234	28.2	18/187	9.6

EPM: samples collected from four ambulatories from 1995-1999; HU: samples collected at one ambularory from 1994-1996.

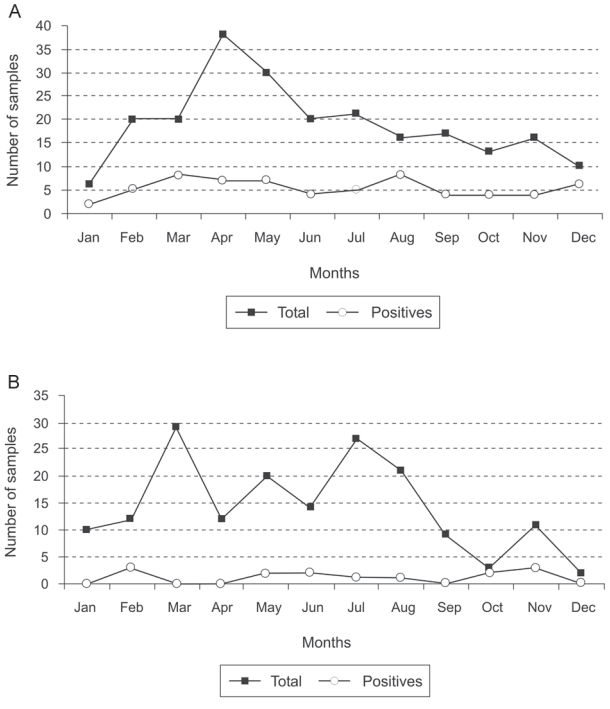

Fig. 1: monthly distribution of astrovirus in São Paulo, Brazil. A: EPM samples, 1995-1999; B: HU samples, 1994-1996.

DISCUSSION

According to literature regarding astrovirus detection in stool samples, the usual percentage of positive samples varies from 2 to 9% (Liu et al. 2007). In the present study, particularly regarding EPM set, 28.2% of the samples were found to be positive for astrovirus. This percentage is much higher than the ones commonly found in other studies, as Jakab et al. (2004) (1.6%), Fodha et al. (2006) (7.0%) and Pazdiora et al. (2006) (3.6%). However, frequencies higher than 20% of astrovirus infections have been reported, as described in the USA, where 26% of all diarrheal episodes reported were associated with astrovirus (Maldonado et al. 1998). Prevalence found for HU are similar to data on astrovirus detection usually found in literature, as described in Mexico (7.9%) (Méndez-Toss et al. 2004), Italy (7.0%) (Colomba et al. 2006), and some Brazilian cities. Thus, Victoria et al. (2007) found that 13.5% (43/318) of samples from hospitalized children in Rio de Janeiro were positive for astrovirus. In the Midwest region of Brazil, Santos et al. (2007) found a lower prevalence for astrovirus (3.7%; 46/1244 samples). Both Brazilian studies used the same pair of primers as ours for RT-PCR.

One reason for that notable difference between the results of the two sets of samples from SP maybe due to the children social economical status. The EPM set was obtained from children from a lower income population than the HU children. Another possible explanation for the high percentage of positivity is the number of individuals with mixed infections. It is not possible to state which virus is responsible for the gastroenteritis symptoms in patients infected by more than one virus. Multiple viral infections have also been described in a study with norovirus (Castilho et al. 2006).

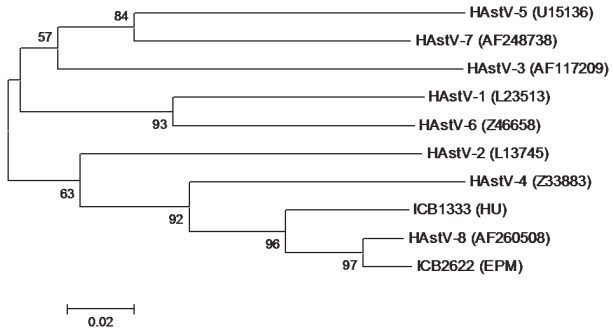

Fig. 2: neighbor-joining tree with K2P model based on partial ORF2 nucleotide sequences of astroviruses samples ICB2622 (EPM) and ICB1333 (HU), and standard human astrovirus (HAstV) samples. Each taxon is represented by its respective GenBank accession number. Number at each node is 1,000 bootstrap replicate values. The bar indicates genetic distance.

TABLE II
Genotypes of human astrovirus (HAstV) in two sets of samples from São Paulo, Brazil

		Genotyping (HAstV)									
	Tested	1	2	3	4	5	6	7	8	Total Genotyped	Non-Typeable[a]
EPM	66	18	2	2					3	25	41
HU	18	1	6						1	8	10

a: samples that show no PCR products (bands) in the agar gel. EPM: samples collected from four ambulatories from 1995-1999; HU: samples collected at one ambularory from 1994-1996.

Astrovirus was detected almost every month during the years of collection for both sets of samples and no seasonaly was observed. Mustafa et al. (2001) suggested that, although astroviruses were found to be circulating in the population through the whole year, they can be more frequently isolated during the winter in countries of temperate climate, and during the rainy season, in tropical countries. However, in Rhode Island, USA, the peak of astrovirus infection was observed in the spring, between March and June over five years of the study.

Prevalence of HAstV in children without diarrhea in both sets of samples from SP (16.3% for EPM and 20.7% for HU) was higher than those observed in the Midwest region of Brazil (0.5%) (Santos et al. 2007) and in Zaria, Nigeria (5.7%) (Pennap et al. 2002).

Regarding astrovirus genotyping, results show that, particularly for EPM, HAstV-1 still is the most frequent genotype circulating in the population, as described worldwide (Noel et al. 1995, Traoré et al. 2000, Espul et al. 2004). However, for HU, HAstV-2 was the most commonly found, which is not common in studies regarding astrovirus genotyping. Victoria et al. (2007) found HAstV-2 as the second most common genotype, accounting for 17% (6/35) of the analyzed samples, while HAstV-1 was the most prevalent (71%) (25/35).

In this study, the characterization of two samples as HAstV-8 and further confirmation by nucleotide sequencing were achieved for the first time in SP. These data suggest that the concordance observed between the two genotyping techniques was 100%, which is higher than that found by Gabbay et al. (2007a) (69.2%). HAstV-8 is not commonly found, or not found at all, in studies worldwide related to astroviruses genotyping (Sakamoto et al. 2000, Schnagl et al. 2002, Cardoso et al. 2002, Galdiero et al. 2005, Gabbay et al. 2007b). Few studies have reported the detection of HAstV-8 in Brazil, such as described by Silva et al. (2006). Thus, results from the present work increase the knowledge about HAstV circulating in the country. It is important to stress the use two genotyping techniques to confirm results have been reported elsewhere, as described in studies carried out in Mexico, Spain and Hungary (Walter et al. 2001, Dalton et al. 2002, Jakab et al. 2004).

Genotyping was not achieved in some samples in the present study. Long storage periods (around 10 years), with several freezing and thawing steps may have contributed for this result. According to Méndez & Arias, (2007), those situations may disrupt astroviral particles, making astroviruses detection and/or genotyping difficult. Primers used in the nested/RT-PCR genotyping technique in this study have not been extensively tested in samples from Brazil and elsewhere. Only a few studies have used genotyping technique worldwide (Phan et al. 2004, Nguyen et al. 2007) and in Brazil (Cardoso et al. 2002, Gabbay et al. 2007a).

Mixtures of astrovirus, rotavirus and norovirus occurred in both HU and EPM sets of samples. These findings suggests that mixtures of enteric viruses are common, as described by other authors (Jaaskelainen & Maunula 2006, Klein et al. 2006), and this could also reflect the low socioeconomic environment of these children who are living in heavily contaminated environment.

These results all together show that astroviruses are circulating in a constant manner in the population and its role in children gastroenteritis is becoming more important. Studies on astrovirus are essential for understanding its pathogenesis and epidemiology.

REFERENCES

Bhattacharya R, Sahoo GC, Nayak MK, Ghosh S, Dutta P, Bhattacharya MK, Mitra U, Gangopadhyay D, Dutta S, Niyogi SK, Saha DR, Naik TN, Bhattacharya SK, Krishnan T 2006. Molecular epidemiology of human astrovirus infections in Kolkata, India. *Infect Genet Evol 6*: 425-435.

Cardoso DDP, Fiaccadori FS, Souza MBLD, Bringel RMM, Leite JPG 2002. Detection and genotyping of astroviruses from children with acute gastroenteritis from Goiania, Goias, Brazil. *Med Sci Monit 8*: 624-628.

Castilho JG, Munford V, Resque HR, Fagundes-Neto U, Vinje J, Rácz ML 2006. Genetic diversity of noroviruses among children with gastroenteritis in São Paulo State, Brazil. *J Clin Microbiol 44*: 3947-3953.

Colomba C, De Grazia S, Giammanco GM, Saporito L, Scarlata F, Titone L, Arista S 2006. Viral gastroenteritis in children hospitalized in Sicily, Italy. *Eur J Clin Microbiol Infect Dis 25*: 570-575.

Dalton RM, Roman ER, Negredo AA, Wilhelmi ID, Glass RI, Sanchez-Fauquier A 2002. Astrovirus acute gastroenteritis among children in Madrid, Spain. *Pediatr Infect Dis J 21*: 1038-1041.

Espul A, Martínez N, Noel JS, Cuello H, Abrile C, Grucci S, Glass R, Berke T, Matson DO 2004. Prevalence and characterization of astroviruses in Argentinean children with acute gastroenteritis. *J Med Virol 72*: 75-82.

Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA 2005. Family Astroviridae. In CM Fauquet, MA Mayo, J Maniloff, U Desselberger and LA Ball, *Virus Taxonomy: Eighth Report of the International Committee on the Taxonomy of Viruses*, Elsevier Academic Press, San Diego, p. 859-864.

Fodha I, Chouikha A, Peenze I, De Beer M, Dewar J, Geyer A, Messaadi F, Trabelsi A, Boujaafar N, Taylor MB, Steele D 2006. Identification of viral agents causing diarrhea among children in the Eastern Center of Tunisia. *J Med Virol 78*: 1198-1203.

Gabbay YB, Da Luz CRNE, Costa IV, Cavalcante-Pepino EL, Sousa MS, Oliveira KK, Wanzeller ALM, Mascarenhas JDP, Leite JPG, Linhares AC 2005. Prevalence and genetic diversity of astroviruses in children with and without diarrhea in São Luís, Maranhão, Brazil. *Mem Inst Oswaldo Cruz 100*: 709-714.

Gabbay YB, Linhares AC, Cavalcante-Pepino EL, Nakamura LS, Oliveira DS, Da Silva LD, Mascarenhas JDP, Oliveira CS, Monteiro TAF, Leite JPG 2007a. Prevalence of human astrovirus genotypes associated with acute gastroenteritis among children in Belém, Brazil. *J Med Virol 79*: 530-538.

Gabbay YB, Linhares AC, Oliveira DS, Nakamura LS, Mascarenhas JDP, Gusmão RHP, Heinemann MB, Macêdo O, Leite JPG 2007b. First detection of a human astrovirus type 8 in a child with diarrhea in Belém, Brazil: comparison with other strains worldwide and identification of possible three lineages. *Mem Inst Oswaldo Cruz 102*: 531-534.

Galdiero E, Marinelli A, Pisciotta MG, Pagliara I, Di Monteforte ES, Liguori G 2005. Reverse transcriptase-PCR for the detection of astrovirus in children with nosocomial acute diarrhoea in Naples, Italy. *Med Mal Infect 35*: 213-217.

Glass RI, Bresee J, Jiang B, Gentsch J, Ando T, Fankhauser R, Noel J, Parashar U, Rosen B, Monroe SS 2001. Gastroenteritis viruses: an overview. In D Chadwick, JA Goode, *Gastroenteritis viruses*, J. Wiley & Sons, Chichester, West Sussex, England, p. 5-25.

Guix S, Caballero S, Villena C, Bartolomé R, Latorre C, Rabella N, Simó M, Bosch A, Pintó RM 2002. Molecular epidemiology of astrovirus infection in Barcelona, Spain. *J Clin Microbiol 40*: 133-139.

Hall TA 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/nt. *Nucl Acids Symp Ser 41*: 95-98.

Jaaskelainen AJ, Maunula L 2006. Applicability of microarray technique for the detection of noro- and astrovirus. *J Virol Methods 136*: 210-216.

Jakab F, Meleg E, Bányai K, Melegh B, Tímár L, Péterfai J, Szücs G 2004. One-year survey of astrovirus infection in children with gastroenteritis in a large hospital in Hungary: occurrence and genetic analysis of astroviruses. *J Med Virol 74*: 71-77.

Klein EJ, Boster DR, Stapp JR, Wells JG, Qin X, Clausen CR, Swerdlow DL, Braden CR, Tarr PI 2006. Diarrhea etiology in a Children's Hospital Emergency Department: a prospective cohort study. *Clin Infect Dis 43*: 807-813.

Kumar S, Tamura K, Nei M 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinformatics 5*:150-163.

Liu MQ, Yang BF, Peng JS, Zhou DJ, Tang L, Wang B, Liu Y, Sun SH, Ho WZ 2007. Molecular epidemiology of astrovirus infection in infants in Wuhan, China. *J Clin Microbiol 45*: 1308-1309.

Madeley CR, Cosgrove BP 1975. 28nm particles in faeces in infantile gastroenteritis. *Lancet 1*: 451-452.

Maldonado Y, Cantwell M, Old M, Hill D, Sanchez ML, Logan L, Millan-Velasco F, Valdespino JL, Sepulveda J, Matsui S 1998. Population-based prevalence of symptomatic and asymptomatic astrovirus infection in rural Mayan infants. *J Infect Dis 178*: 334-339.

Méndez E, Arias CF 2007. Astroviruses. In: DM Knipe, PM Howley, DE Griffin, RA Lamb, SE Straus, MA Martin, B Roizman (eds), *Fields Virology,* Vol.1, 5th ed., Lippincott-Williams and Wilkins, Philadelphia, p. 981-1000.

Méndez-Toss M, Griffin DD, Calva J, Contreras JF, Puerto FI, Mota F, Guiscafré H, Cedillo R, Muñoz O, Herrera I, López S, Arias CF 2004. Prevalence and genetic diversity of human astroviruses in Mexican children with symptomatic and asymptomatic infections. *J Clin Microbiol 42*: 151-157.

Méndez-Toss M, Romero-Guido P, Munguía ME, Méndez E, Arias CF 2000. Molecular analysis of a serotype 8 human astrovirus genome. *J Gen Virol 81*: 2891-2897.

Mustafa H, Bishop RF, Palombo EA 2001. The biology and epidemiology of human astroviruses. *Rev Med Microbiol 12*: 75-85.

Mustafa H, Palombo EA, Bishop RF 2000. Epidemiology of astrovirus infection in young children hospitalized with acute gastroenteritis in Melbourne, Australia, over a period of four consecutive years, 1995 to 1998. *J Clin Microbiol 38*: 1058-1062.

Nguyen TA, Yagyu F, Okame M, Phan TG, Trinh QD, Yan H, Hoang KT, Cao ATH, Hoang PL, Okitsu S, Ushijima H 2007. Diversity of viruses associated with acute gastroenteritis in children hospitalized with diarrhea in Ho Chi Minh City, Vietnam. *J Med Virol 79*: 582-590.

Noel JS, Lee TW, Kurtz JB, Glass RI, Monroe SS 1995. Typing of human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing. *J Clin Microbiol 33*: 797-801.

Pazdiora P, Jelinkova H, Svecova M, Taborska J 2006. First experience with diagnosing astroviral infections in children hospitalized in Pilsen (Czechia). *Folia Microbiol (Praha) 51*: 129-132.

Pennap G, Pager CT, Peenze I, De Beer MC, Kwaga JKP, Ogalla WN, Umoh JU, Steele AD 2002. Epidemiology of astrovirus infection in Zaria, Nigeria. *J Trop Pediatr 48*: 98-101.

Phan TG, Okame M, Nguyen TA, Maneekarn N, Nishio O, Okitsu S, Ushijima H 2004. Human astrovirus, norovirus (GI, GII), and sapovirus infections in Pakistani children with diarrhea. *J Med Virol 73*: 256-261.

Sakamoto T, Negishi H, Wang QH, Akihara S, Kim B, Nishimura S, Kaneshi K, Nakaya S, Ueda Y, Sugita K, Motohiro T, Nishimura T, Ushijima H 2000. Molecular epidemiology of astroviruses in Japan from 1995 to 1998 by reverse transcription-polymerase chain reaction with serotype-specific primers (1 to 8). *J Med Virol 61*: 326-331.

Santos RAT, Borges AMT, Da Costa PSS, Teixeira JMS, Giugliano LG, Leite JPG, Cardoso DDP 2007. Astrovirus infection in children living in the Central West region of Brazil. *Mem Inst Oswaldo Cruz 102*: 209-213.

Scaletsky ICA, Fabbricotti SH, Aranda KR, Morais MB, Fagundes-Neto U 2002. Comparison of DNA hybridization and PCR assays for detection of putative pathogenic enteroadherent *Escherichia coli*. *J Clin Microbiol 40*: 1254-1258.

Schnagl RD, Belfrage K, Farrington R, Hutchinson K, Lewis V, Erlich J, Morey F 2002. Incidence of human astrovirus in central Australia (1995 to 1998) and comparison of deduced serotypes detected from 1981 to 1998. *J Clin Microbiol 40*: 4114-4120.

Silva PA, Cardoso DD, Schreier E 2006. Molecular characterization of human astroviruses isolated in Brazil, including the complete sequences of astrovirus genotypes 4 and 5. *Arch Virol 151*: 1405-1417.

Traoré O, Belliot G, Mollat C, Piloquet H, Chamoux C, Laveran H, Monroe SS, Billaudel S 2000. RT-PCR identification and typing of astroviruses and Norwalk-like viruses in hospitalized patients with gastroenteritis: evidence of nosocomial infections. *J Clin Virol 17*: 151-158.

Victoria M, Carvalho-Costa FA, Heinemann MB, Leite JPG, Miagostovich MP 2007. Genotypes and molecular epidemiology of human astroviruses in hospitalized children with acute gastroenteritis in Rio de Janeiro, Brazil. *J Med Virol 79*: 939-944.

Walter JE, Mitchell DK, Guerrero ML, Berke T, Matson DO, Monroe SS, Pickering LK, Ruiz-Palacios G 2001. Molecular epidemiology of human astrovirus diarrhea among children from a periurban community of Mexico City. *J Infect Dis 183*: 681-686.