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Original Article

Viral acute gastroenteritis: clinical and epidemiological features of co-infected patients

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ABSTRACT

Background: Acute gastroenteritis (AGE) is a common disorder that affects children worldwide. It is usually caused by viral agents, including rotavirus, enteric adenovirus, norovirus, and astrovirus groups. Currently, there are few reports about co-infection among these viruses, mainly in Brazil.

Methods: This is a retrospective study in which 84 rotavirus-positive samples from hospitalized patients at a teaching hospital in Southern Brazil, collected in the 2001-2010 period, were analyzed by polymerase chain reaction (PCR) and reverse transcription – polymerase chain reaction (RT-PCR), for the investigation of enteric adenovirus, astrovirus, and norovirus.

Results: In total, 12 of the 84 (14%) samples were positive to enteric adenovirus or norovirus. Clinical, laboratory, and demographic data showed statistically significant differences between mono and co-infected patients, including age and depletion rate.

Conclusions: These findings highlight the need for implementation of other enteric virus detection assays in clinical diagnosis for a complete laboratory investigation of hospitalized pediatric patients with AGE, in order to understand the impact of these pathogens on disease severity, spread within hospital, and consequently, prevent the dissemination of nosocomial infections.

Introduction

Acute gastroenteritis (AGE) is one of the most common diseases in children in developed and developing countries, and has been shown to be associated with significant morbidity and mortality rates. Almost two million children under 5 years of age die as a result of these infections across the world, which represents 19% of all deaths in this population.[1] In developing countries, the incidence rate of acute gastroenteritis is 2.1 to 3.8 diarrhea episodes per child between 11 and 48 months of age per year.[2] In Brazil, diarrhea presents higher morbidity rates and is the main cause of death in the first year of life.[3]

AGE can be caused by non-infectious and infectious agents, including bacteria, parasites, and viruses. More

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than 20 different viruses can cause AGE. Group A rotavirus (RV) has been reported as the leading cause of diarrhea in children under 5 years of age, and usually presents a seasonal pattern. Currently, an increase in the frequency of other enteric viruses, such as the norovirus, enteric adenovirus, and astrovirus has been reported and, more recently, bocavirus and human parechovirus were associated with gastrointestinal infections in pediatric and adult patients.[4-7]

Human adenoviruses are non-enveloped deoxyribonucleic acid (DNA) viruses and have been linked with a spectrum of clinical manifestations, including respiratory, gastrointestinal, ocular, neurologic, and urinary infections. So far, 51 different serotypes from six different subgenera (A-F)[5] were described. Enteric adenovirus (AdV40 and AdV41), subgenus F, was reportedly related to AGE in variable frequency (ranging from 1.4 to 10%), depending on the geographic settings.[5,8] It has been reported in many recreational water-associated outbreaks.[9] Norovirus (NoV) and sapovirus, of the Caliciviridae family, have been recognized as the main causes of nonbacterial acute gastroenteritis among all age groups in developed countries, being frequently associated with food- and water-borne outbreaks.[10,11] It has been reported that, as RV vaccine coverage becomes more widespread, NoV probably will be the most frequent pathogen associated with severe pediatric diarrhea.[12] It is classified into five genogroups (GI to GV); GI and GII are the most frequently associated with human infection, and these genogroups are subdivided into more than 25 genotypes. This genetic variability has an impact on outbreaks worldwide and on vaccine efficacy.[13,14] The Astrovirus (AstV), Astroviridae family, a small round virus, has been associated with AGE, with a frequency range between 2% to 26%.[15] The human astrovirus has been classified into eight serotypes (HAstV1 – HAstV8) based on antigenic variability of the capsid protein.[5] Outbreaks related to this virus have been reported in schools, neonatal intensive care units, and also in adult and elderly patients.[15-17] Serious complications, such as necrotizing enterocolitis, have been reported in children with astrovirus infections.[18]

Sporadic studies have shown the importance of these pathogens and their distribution in some regions of Brazil.[3,19-29] Although previous studies on the epidemiology of these viruses have detected co-infection between rotavirus, enteric adenovirus, astrovirus and norovirus, little is known about the frequency, distribution and severity of these viral co-infections associated to AGE in Southern Brazil.[30]

The diagnosis of these pathogens can be performed by antigen detection or by molecular methods, such as polymerase chain reaction (PCR), reverse transcription – polymerase chain reaction (RT-PCR), or multiplex RT-PCR. Various enzyme immunoassays to detect viral antigens of norovirus, astrovirus, and enteric adenovirus are available. This method is very sensitive and specific. However, its large-scale application is limited due to the high costs of the reagents and to lack of recognition of the importance of these agents as putative agents of AGE. Molecular studies using RT-PCR and sequencing techniques have significantly contributed to the improvement in the detection of these viruses and to the knowledge of their epidemiology and clinical importance.

This Teaching Hospital (Hospital de Clínicas/ Universidade Federal do Paraná, HC-UFPR) is a tertiary referral hospital for pediatric patients with severe infections, and AGE is one of the most frequent causes of pediatric hospital admissions. In such cases, RV antigens are routinely investigated and the detection rate of this virus has been approximately 28%.[31] This is a retrospective study aimed to identify the frequency of co-infections of RV and other viruses, like the enteric adenovirus, astrovirus, and norovirus groups, using PCR and RT-PCR. Furthermore, the clinical and epidemiological characteristics of these patients, and the impact of viral co-infections on their outcome were assessed.

Material and methods

Clinical samples

This study comprised 84 fecal samples previously found to be positive for rotavirus by enzyme immunoassay, latex agglutination, or PAGE. The samples were sent to the Virology Laboratory of HC-UFPR in the 2001-2010 period and stored at -80°C. The medical records of the patients were reviewed. The study had the approval of the Committee on the Ethics of Research on Human Beings of the HC-UFPR, under the registration number 0221.0.208.000-10.

Viral DNA/RNA extraction

DNA/RNA was extracted from 150 µL of 10% fecal suspension with Buffer Tris-HCl 0.01M – $CaCl_2$ 0.0015 M by using a commercial kit (iNtRON Biotechnology, Inc® – Jungang Induspia, South Korea) according to the manufacturer's instructions. In the lysis buffer, pseudorabies viruses (PRV) at a concentration of 1.74 e^{-8} ng/µL were added as extraction internal control.

Adenovirus detection

Generic primers and amplification tests were carried out as described by Avéllon et al.[32] with some modifications: 2.5 µL of extraction product were added to 22.5 µL of a PCR mix containing 2.5 mM dNTP's (Bioron® – Ludwigshafe, Germany), 1.25 U of Taq DNA Polymerase (Bioron® – Ludwigshafe, Germany), 160 mM $(NH_4)_2SO_4$ (Bioron® – Ludwigshafe,Germany), 670 mM Tris-HCl (pH 8.8) (Bioron® – Ludwigshafe, Germany), 25 mM $MgCl_2$ (Bioron® – Ludwigshafe, Germany), and primary amplification primer to adenovirus and PRV at a concentration of 10 pmol/µL. Amplification was carried out on a Mastercycler Personal (Eppendorf® – Germany) thermocycler with cycling conditions, as follows: 40 cycles of 94°C/60 s, 50°C/60 s, and 72°C/60 s, and one extension step of 72°C/6 min. An additional denaturation at 94°C for two minutes preceded the first cycle. For the second reaction, 1 µL of primary amplification product was added to 24 µL of a new PCR mix

similar to the primary amplification, though containing secondary amplification primers. The amplicons were subjected to electrophoresis in 1% agarose gels, stained with ethidium bromide, 0.5 g/mL.

Astrovirus and norovirus detection

The detection of astrovirus and norovirus was performed by RT-PCR. First-strand cDNA was synthesized using random primers and a reverse transcription system (SuperScript® III Reverse Transcriptase, Invitrogen® – Carlsbad, CA, USA). Generic primers and amplifications tests for the identification of astrovirus and norovirus were carried out as reported by Noel et al.[33] and Vinjé et al.[34], respectively 2.5 µL of extraction product were added to 22.5 µL of a PCR mix containing 2,5 mM dNTPs (Bioron® – Ludwigshafe, Germany), 1.25 U of Taq DNA Polymerase (Bioron® – Ludwigshafe, Germany), 160mM $(NH_4)_2SO_4$ (Bioron® – Ludwigshafe, Germany), 670 mM Tris-HCl (pH 8.8) (Bioron® – Ludwigshafe, Germany), 25 mM $MgCl_2$ (Bioron® – Ludwigshafe, Germany) and primary amplification primer to norovirus or astrovirus at a concentration of 10 pmol/µL. For norovirus, amplification was carried out on a Mastercycler Personal (Eppendorf® – Germany) thermocycler programmed with the cycling conditions: 94ºC/2 min followed by 30 cycles of 94°C/60 s, 37°C/30 s, 72° C/30 s, and an elongation step of 72ºC for six minutes. Astrovirus amplification was performed using the same cycling protocol described for the adenovirus. Both amplicons were subjected to electrophoresis in 1% agarose gels, stained with ethidium bromide, 0.5 g/mL.

The electrophoresis gels were photographed under UV light and the bands were analyzed on E-Capt Program.

Statistical analysis

Data were compiled using JMP Software Version 5.2.1 and analyzed by the software GraphPad Prism Version 5.03. Fisher's exact or chi-square tests were used to assess differences between groups, and the Mann-Whitney test was used for continuous variables, as appropriate. Results of continuous data are expressed as median ± interquartile range. All p-values are two-tailed, and a value of < 0.05 was considered significant.

Results

A total of 12 (14%) patients presented viral co-infection. Enteric adenovirus was found in 10% of these cases, and norovirus was observed in 4%. Although the patients were tested for the presence of astrovirus, no co-infection with this pathogen was found (Fig. 1).

Mixed-infections occurred during all the years of this study, with peaks in 2001 and 2010, with three and four cases, respectively, which also corresponds to the period when there was a greater number of samples analyzed (Fig. 2). Demographic, clinical, and laboratory data of mono- and co-infected patients are compared in Table 1. A significant difference ($p = 0.03$) was found in the median age of the children in the mono- and co-infected groups, indicating that viral co-infections tend to be more frequent in older children. However, concerning gender and length of hospital stay, no significant differences were found between the groups.

Low-grade fever and vomiting were the most common symptoms in both groups. Anorexia and depletion were less frequent, but depletion was more common in co-infected patients ($p = 0.048$). Considering the frequency of diarrheal episodes per day (episodes/d), although the co-infection group had less than five episodes/d and the mono-infection group had more than five episodes/d, the difference was not significant.

Analysis of laboratory data showed a significant difference in the number of lymphocytes between mono- and co-infection groups, while leukocytes and bands were similar in both groups. Similarly, there was a significant difference in the concentration of sodium and level of base excess (BE) between the groups, showing a more severe dehydration in the co-infected patients.

In the co-infected group, two (16%) cases of nosocomial infection were detected and two patients died, while in the other group there were 11 (15%) cases of nosocomial infection and three patients died ($p = 0.1469$).

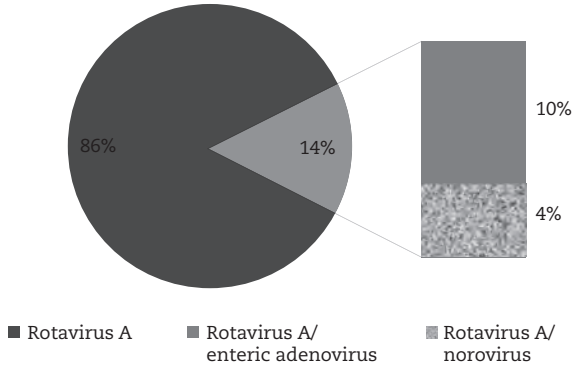

Fig. 1 - Total of samples analyzed and enteric viral co-infection.

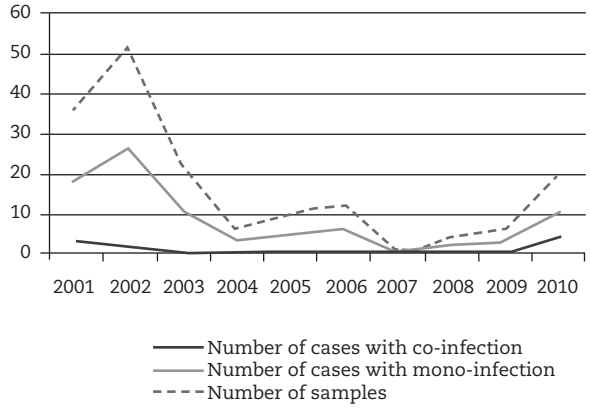

Fig. 2 - Distribution of mono-infection (rotavirus A) and co-infection (other enteric viruses) cases over the years.

Table 1 - Viral gastroenteritis: demographical, clinical and laboratorial characteristics of mono (rotavirus A) and co-infected (other enteric viruses) patients

Data	Co-infection n = 12	Mono-infection n = 72	p-value
Demographical data			
Age (months) Median (IQV)	18 Mo (12 - 46)	9 Mo (6 - 17)	**0.0365**
Gender (male/female)	8/4	47/25	0.925
Clinical data			
Fever (y/n)	8/2	39/21	0.3498
Vomiting (y/n)	5/2	54/5	0.1570
Anorexia (y/n)	1/3	10/43	1.000
Depletion (y/n)	4/5	46/13	**0.0483**
Episodes of diarrhea (episodes/day) (≥ 5 / < 5)	3/4	37/18	0.6800
Length of hospitalization (d) Median (IQV)	7 (3-21)	4 (3-7)	0.1143
Laboratorial data			
Leukocytes cells/mm^3 Median (IQV)	10,880 (5,160 - 15,120)	11,000 (8,680 - 14,200)	0.9098
Lymphocytes % Median (IQV)	22 (15 - 25)	31 (20 - 46)	**0.0391**
Bands % Median (IQV)	11 (4 - 14.5)	12 (6.5 - 20)	0.4241
BE mEq/L (-) Median (IQV)	4.8 (2.7 - 13.7)	15 (8.9 - 18.8)	**0.0284**
Sodium mEq/L Mean (± SD)	139.7 (± 7.36)	149.9 (± 12.94)	**0.0413**
Potassium mEq/L Mean (± SD)	3.8 (± 0.60)	3.81 (± 0.68)	0.7749

Mo, months; d, days; IQV, interquartile variation; y/n, yes/no; SD, standard deviation. Normal values: BE (base excess) = - 2 to +2 mEq/L. Sodium, serum = 136-145 mEq/L. Potassium, serum = 3.5-5.0 mEq/L. Bold, statistically significant.

Discussion

The prevalence of viral co-infection by enteric adenovirus, astrovirus, or norovirus was assessed in patients admitted to a tertiary hospital from 2001 to 2010, previously diagnosed with RV infection. Disease severity was evaluated comparing co-infected and mono-infected patients. Molecular methods were used to detect these enteric pathogens. Detection is crucial for the appropriate management of the pathogens, preventing the indiscriminate use of antibiotics, providing accurate information on the causal agent and on the role of pathogens in infectious diseases. Although RV is known to be the most frequent pathogen associated to AGE, the contribution of other enteric viruses remain relatively unclear.[5] To the authors' knowledge, this is the first report to study the impact of the association of enteric pathogens in patients with AGE in Southern Brazil.

Viral cross-infections have been reported worldwide with percentages ranging from 2% to 19%.[5,8,35-37] Despite the small number of samples analyzed, the incidence of cross-infections with rotavirus in the present samples was high (14%). Unlike the results of previous studies, rotavirus and enteric adenovirus appeared as the most frequent mixed infection, followed by norovirus.[8,30,37,38]

NoV has been reported as the second most frequent causative agent of AGE in young children after RV.[39] Moreover, severe clinical manifestations have been associated to certain specific genotypes, such as NoV GII.[4,39]

The adenovirus PCR diagnostic assay used in this study can detect all serotypes. Thus, the detection of a virus eliminated by this transmission route is possible. However, none of the patients presented clinical manifestations suggestive of other AdV infections, such as respiratory, neurologic, urinary, or ocular symptoms. The burden of the gastrointestinal disease associated with this virus on patients' health certainly has been lower than that of RV and NoV infection. It should be pointed out however that there are few studies on enteric AdV detection. Also, there is great variability among laboratory methods used. The regional importance of the pathogen should also be considered to stimulate studies in different settings. In a study conducted

in southern Vietnam,[5] HAstV was found to be the second most common agent detected in children with diarrhea and, similarly, Adv was the second most frequent pathogen detected in Ghana, in 19.9% of the cases.[5]

There are few reports on viral co-infection and gastroenteritis in Brazil. A previous study was conducted in the state of Espírito Santo, Southeastern Brazil, showed a frequency of 15% of NoV-RV mixed infections in symptomatic patients.[27] In the state of São Paulo, NoV co-infection was found with rotavirus, adenovirus, and astrovirus in a frequency of 1.8%, 2.1%, and 11.1%, respectively.[40] In the state of Rio de Janeiro, NoV-HAdV mixed infection was found in 3% of the samples during an outbreak investigation.[21] In Salvador, in the state of Bahia, Northeastern Brazil, the frequency of co-infections with NoV-RV and NoV-AdV in young adults were 7% and 2.3%, respectively.[41] Data regarding the incidence and prevalence of these viruses in other regions consists of isolated reports.[42]

Unlike the findings described by Román et al.[43] in Spain, in the present study viral co-infection was more prevalent in older children up to 18 months. It was observed that the symptoms, such as fever, vomiting, anorexia, as well as length of hospitalization and number of episodes of diarrhea were not significantly different in the comparison of mono- and co-infected groups. Co-infected patients had a lower level of base excess indicating metabolic acidosis, which resulted from increased dehydration and, consequently, severe depletion. Since 2004 there has been a significant decrease in the number of rotavirus-positive samples. There may be several explanations for this finding, including improvement of the sanitary conditions of the population, greater access to basic health care, change in the profile of the HC-UFPR (which has become a tertiary referral hospital only for pediatric patients with severe infections) and, also, the inclusion of rotavirus vaccination in the Brazilian National Immunization Program, in 2006. With the decrease in RT infection rates, other enteric viruses may become more common. Therefore, the development of more sophisticated diagnostic methods to investigate these pathogens is essential to prevent the spread of infection.

In conclusion, viral co-infection occurs at high levels and in some cases was associated to a severe disease, reinforcing the need to implement a diagnostic routine in the laboratory and epidemiological vigilance for these agents in a hospital setting. However, the molecular basis of such enhanced virulence remains speculative and warrants further study.

Conflict of interest

All authors declare to have no conflict of interest.

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