

Hospitalization due to norovirus and genotypes of rotavirus in pediatric patients, state of Espírito Santo

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Viruses are the leading cause for hospitalization due to gastroenteritis worldwide. Group A rotaviruses (RV) are the most prevalent and are assorted in glycoproteins (G) and protease sensitive (P) dual genotypes based on polymorphic genes that encode the external VP7 and VP4 capsid proteins, respectively. Noroviruses (NoV) have increasingly answered by sporadic gastroenteritis. This study aimed to determine the prevalence of NoV and RV in 68 hospitalized children, between July 2004 and November 2006, at a pediatric hospital in Vitória city, state of Espírito Santo, Southeastern Brazil. Nucleic acid was extracted from fecal suspension following the guanidine-silica procedure. Reverse transcriptase-polymerase chain reaction (RT-PCR) and polyacrylamide gel electrophoresis were employed for NoV and RV detection, respectively. RV genotyping was accomplished using RT-PCR followed by heminested multiplex PCR with specific primers for the most prevalent types of G and P. Fecal samples were positive for NoV and RV in 39.7% (27/68) and 20.5% (14/68), respectively and together were responsible for 60% (41/68) of the cases. RV genotypes were: 50% G9P[8], 28.7% G2P[4], 7.1% G1P[8], G2P[8] and G?P[8]. Vomit was a prominent manifestation observed in 92% and 85% of the NoV and RV cases, respectively. The median hospitalization was 5 and 5.5 days for the patients infected with NoV and RV, respectively. The data showed that NoV prevailed over RV and it also corroborated the emergence of RV G9 genotype followed by G2P[4], reinforcing the need for RV genotype surveillance.

Key words: noroviruses - group A rotaviruses - sporadic gastroenteritis - hospitalizations

Acute gastroenteritis remains a leading cause of morbidity and mortality worldwide, especially among those up to five years old. The global mortality is estimated at two million per year, mainly in developing countries (Okitsu-Negishi et al. 2004). Many infectious agents (bacteria, parasites and viruses) can be associated, but viruses are mainly responsible for endemic and epidemic gastroenteritis, mostly represented by group A rotaviruses (RV) and noroviruses (NoV). Although with low mortality in developed countries, gastroenteritis is one of the most common illnesses requiring hospitalization in an estimated one out of five cases, making this a serious public-health matter (Glass et al. 2006).

RV, a member of Reoviridae family, is recognized as the most significant cause of diarrheic illness attacking children up to the age of three, worldwide (Glass et al. 2006). RVs are classified into seven groups (A-G) based on the VP6 intermediary capsid protein, of which three (A-C) are human pathogens; group A is responsible for 95% of infections (Kapikian et al. 2001). The genome

consists of 11 segments of dsRNA enclosed by three concentric capsids. The migration pattern in acrylamide gel electrophoresis serves to diagnose and permits inference of the RV group. The proteins VP4 and VP7 form external capsids and their respective genes classify group A RV in protease sensitive (P) and glycoprotein (G) genotypes, respectively. Until now, 27 P genotypes (Martella et al. 2006) and 15 G genotypes have been described, 11 and ten of P and G, respectively, are associated with human infection. The mostly commonly reported combinations are: G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (Santos & Hoshino 2005). However, uncommon genotypes or combinations are described throughout the world as well as in Brazil (Leite et al. 1996, Santos et al. 1998, Pietruchinski et al. 2006). Monovalent live oral RV vaccine (G1P[8], Rotarix™) recently introduced into the routine program of childhood immunization in Brazil, will potentially reduce the burden of severe diarrheic illness and hospital admission. However, continuous investigation of the group A RV genotypes is of extreme importance for surveillance of vaccine efficacy.

Norovirus, one of the two genera belonging to *Caliciviridae* family which causes human infection, is a small non-enveloped single strand RNA virus that requires special cell systems for in vitro replication (Straub et al. 2007). Human NoV strains belong to GI, GII or GIV genogroups from the five existing ones. NoVs are the major cause of epidemic gastroenteritis worldwide and are related to the consumption of food and water or aquatic

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recreational activities attacking persons of all ages (de Wit et al. 2003), as well as outbreaks in nurseries, hospitals, daycare centers and hotels (Gallimore et al. 2004, de Wit et al. 2007). However, since the 1990s, molecular procedures have recognized this virus as a common agent of sporadic self-limiting gastroenteritis (de Wit et al. 2001, Marshall et al. 2003, Oh et al. 2003).

The aim of this study was to determine the frequency of sporadic NoV and RV infection in children admitted to a hospital in Vitória, Espírito Santo. RV genotype was also accessed in the period before vaccine introduction.

PATIENTS, MATERIALS AND METHODS

From July 2004 to November 2006, 68 diarrheic stool samples were obtained (6 in 2004, 33 in 2005 and 29 in 2006) from 68 infants (63 under 4 and 5 between 4 and 12 years old), 64 of whom had been hospitalized with gastroenteritis. The patients were accessed at a pediatric hospital belonging to the UNIMED network, Centro Integrado de Assistência à Saúde (CIAS), in Vitória city, Southeastern Brazil. The samples, with free consent from the parents, were obtained after an average of 4.6 days of patient hospitalization. A questionnaire was applied for clinical and socio-demographic information and patient's records were accessed for complementary information (days of hospitalization, bacterial enteric pathogens). This study was approved by Ethical Research Committee from CIAS-UNIMED.

RNA was extracted using the guanidine isothiocyanate-silica method (Boom et al. 1990) from 10% fecal suspension in pH 7.2 Tris-calcium buffer (0.01M/0.0015M). Biphasic polyacrylamide gel electrophoresis (PAGE) was performed to search for the 11 segments of RV dsRNA, silver stained, and the electropherotype was determined (Herring et al. 1982, Pereira et al. 1983).

Complementary DNA (cDNA) was obtained in a reverse transcription reaction using 20 mU pd(N)₆TM (Amersham Bioscience, UK) hexanucleotide random primer (Iturriza-Gomara et al. 1999), after denaturation with dimethylsulfoxide for 7 min at 97°C. The cDNA was then submitted to nucleic acid amplification for NoV detection in all samples and for RV genotypes of those that were positive on PAGE. To investigate NoV infection, polymerase chain reaction (PCR) was carried out with two primer pairs (MON 431/432 and MON 433-434) for the NoV polymerase gene region (Beuret et al. 2002),

that amplify more than 90% of the strains from genogroups I and II (Blanton et al. 2006). Amplification protocol was according to Victoria et al. (2007). Consensus gene sequence related to external group A RV proteins was detected with a pair of consensus primers for VP7 (9con1-9con2) or VP4 (4con2-4con3) genes (Gentsch et al. 1992, Das et al. 1994). Reaction products of the first RV PCR were genotyped by heminested multiplex-PCR with two sets of specific primers for the G1-G5, G9 and P[4], P[6], P[8] and P[9] types described by Gentsch et al. (1992) and Das et al. (1994), with modifications by Leite et al. (1996). All the amplicons were carried out on 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide and visualized with an Eagle-Eye-IITM imager using an UV transilluminator.

RESULTS

NoV and RV infection were detected in 39.7% (27/68) and 20.5% (14/68) of the cases, respectively, showing that NoV and RV together affected 60% (41/68) of the hospitalized children. NoV was positive in 50% of the cases negative for RV. Viral gastroenteritis was responsible for hospital admission in 85% (23/27) of the NoV cases, the remaining 15% (4/27) were acquired in hospital. All cases positive for RV were the cause for hospitalization. No mixed infection was observed between the viruses or with enteropathogenic bacteria.

RV was detected only between June and August in all three years. NoV was detected between July and September 2005 and between May and July 2006, a period that corresponds to 89% of the strains obtained in this latter year. The majority (66.6%) of NoV cases occurred in 2006.

Eighty five percent and 93% of the NoV and RV infections, respectively, occurred in children up to two years old (Table). One RV case occurred in a child between three and four years old. NoV infection occurred in two of the 5 children who were more than four years old.

Of the positive cases, diarrhea and vomit were observed in 96% and 100% of NoV and in 92.6% and 86% of RV infections, respectively. The median hospitalization was 5 and 5.5 days for NoV and RV, respectively.

All RV belonged to group A, based on the migration pattern on PAGE, ten of these showed a long and four a short profile. RV genotypes were distributed as shown: 50% G9P[8] (7/14), 28.7% G2P[4] (5/14), 7% G1P[8], 7%

TABLE

Distribution of Noroviruses (NoV) and group A Rotaviruses (RV) frequency according to age group, at a pediatric hospital in Vitória, Espírito Santo, July 2004 to November 2006

| Age group | NoV (n = 27) | | | RV (n = 14) | | |
|-----------|--------------|------|--------------|-------------|------|--------------|
| | Frequency | % | Cumulative % | Frequency | % | Cumulative % |
| 0-1 y | 14 | 51.9 | 51.9 | 5 | 35.7 | 35.7 |
| > 1-2 y | 8 | 29.6 | 81.5 | 7 | 50.0 | 85.7 |
| > 2-3 y | 2 | 7.4 | 88.9 | 1 | 7.1 | 92.9 |
| > 3 y | 3 | 11.1 | 100 | 1 | 7.1 | 100 |

G2P[8] and 7% G9P[8] (1/14 each). All short profiles were G2P[4] genotype. G9P[8] genotype was observed during 2004 and 2005, while G2P[4] appeared only in 2006.

DISCUSSION

Acute gastroenteritis is one of the most common illnesses and can be caused by several infectious agents (bacteria, parasites and virus), whose etiology and prevalence change among developed and developing countries. However, RV, NoV, astroviruses and enteric adenoviruses are pointed out as important morbidity agents, independent of improvements in basic sanitation and hygiene (Clark & McKendrick 2004).

Besides RV, NoV that before was recognized as the major cause of epidemic nonbacterial gastroenteritis (Blanton et al. 2006), has now been shown to be an important agent of sporadic gastroenteritis worldwide (Oh et al. 2003, Kirkwood et al. 2005). However, little information is available concerning sporadic NoV infection in clinical or in hospitalized patients in developing countries (Gallimore et al. 2004, Borges et al. 2006, Soares et al. 2007, Victoria et al. 2007).

This work is the first report of NoV and RV in hospitalized children with gastroenteritis in Vitória, state of Espírito Santo and a high rate of NoV and RV infections were observed highlighting their importance in childhood hospitalization. NoV was found to be more important than RV in hospitalization-requiring admission due to gastroenteritis, at least in the period of study, on contrary to other reports that usually show NoV as the second cause after RV (Atmar & Estes 2006).

The high prevalence of NoV reported here, contrasts with other reports elsewhere dealing with sporadic cases in hospitalized children, which vary from 5.4% to 30% in pediatric patients (Subekti et al. 2002, Oh et al. 2003, Hansman et al. 2004). Nevertheless, prevalence as high as 48.4% and 53% have also been related (Kirkwood & Bishop 2001, Colomba et al. 2007). In Brazil, two studies conducted in hospitalized children revealed 8.6% of NoV infection in two cities in the West Central region (Borges et al. 2006); and a prevalence of 20% in Rio de Janeiro (Victoria et al. 2007). Considering gastroenteritis in cases negative for RV, we observed NoV in 50%, which contrasts with the 15.4% observed by Soares et al. (2007) in Rio de Janeiro.

On the contrary to NoV studies in Brazil, RV has been researched for some time. The prevalence of RV in hospitalized Brazilian children varies according to similar studies from 14% to 48% (Cardoso et al. 2003, Carvalho-Costa et al. 2006) and points out the importance of RV as hospital admission due to gastroenteritis, although, in the population and in the period studied, NoV was more prevalent. Although the period of samples obtained was partially coincident with the beginning of RV vaccination in Brazil, we do not believe that it is responsible for the lower prevalence in relation to NoV, partially because the age of the immunized children at the end of the study in the hospital would have to be about five months old. No case of RV infection occurred in infants less than six months old and only one case appeared at six months, of a child that had not been vaccinated.

Few reports concerning NoV and RV co-infection are available and they refer to rates from 2.1% to 19% (Oh et al. 2003, Medici et al. 2004). Although the present study was conducted in a developing region, co-infection was expected but was not observed, probably due to the few cases obtained; however, 4% of dual infection was observed by this research group in children who attended the emergency sector of a pediatric reference hospital in our region (Spano and colleagues, unpublished observations), the same rate as recently reported in hospitalized children in Rio de Janeiro, Brazil (Victoria et al. 2007).

Most of the children in this study were up to four years old and in the others over four RV was not observed as gastroenteritis cause. As has been well established, 90% of group A RV infection occurs during the first three years of life, whereas NoV attacks individuals of all ages (Kapikian et al. 2001, Atmar & Estes 2006). However, half of the NoV cases occurred up to one year old and most of them (81.5%) up to two years old, similar to the previous report from Rio de Janeiro (Victoria et al. 2007).

The average number of hospitalization days for NoV and RV infections was similar for both in this study, close to five days, as also reported by Rockx et al. (2002) in a prospective study about the natural history of calicivirus infection. The duration of NoV infection was previously determined to be short (1-3 days); however, reports of sporadic cases have shown longer periods (Atmar & Estes 2006). Thus, NoV-associated diseases can last longer than they were previously recognized. Some reports give further evidence that the duration of symptoms reduces with increasing age (Rockx et al. 2002). On the other hand, Lee et al. (2007) provides information on higher viral concentration with prolonged diarrhea (≥ 4 days).

Vomit was a prominent clinical signal observed for both virus infections in 90% of the all cases. Clinical reports describe vomit as an important manifestation for both RV and NoV infection, but less than we observed (Estes et al. 2001, Kapikian et al. 2001, Rockx et al. 2002). While Rockx et al. (2002) described that vomit was less common in NoV-infected children aged < 1 year (59%), 85.7% of the patients in the present study, also infected with NoV at this same age group, presented vomit. Our results are also in contrast with those reported in children infected with NoV at a hospital in Rio de Janeiro that reported vomit in 33.3% of the cases (Victoria et al. 2007). According to Moreno-Espinosa et al. (2004) vomit occurs more frequently in children than diarrhea does once more in disagreement with our results. At present, there is no explanation for these data.

Although NoV is primarily transmitted by the fecal-oral route, vomit carries the additional risk for nosocomial transmission through generation of infectious aerosol, an important airborne source of transmission (Marks et al. 2003). Four cases positive for NoV occurred in children admitted to the hospital for causes other than gastroenteritis, suggesting nosocomial transmission.

Most of the studies on seasonality describe winter

peaks in NoV-associated outbreaks or sporadic cases (Fankhauser et al. 1998, Fretz 2005) and some reports, in spring and summer, varying according to the NoV genogroups (Marshall et al. 2003). Prevalence of NoV at the beginning of the dry season was previously observed in Vietnam (Hansman et al. 2004). In contrast to countries with a temperate climate, the region of the present study does not have a clear variation in temperature. According to meteorological data, NoV infections occur in a period with a lower rainfall, which is in agreement with the recently published data concerning the dry periods of the year in Rio de Janeiro, the same Southeastern region as ours (Victoria et al. 2007), while Soares et al. (2007) did not find any seasonal pattern in the same region in an eight year study. On the other hand, Borges et al. (2006) related seasonality in the period of higher humidity in the West Central region, sub-tropical zone Brazil. Therefore, these data denote that more studies are needed to better elucidate this subject.

RV cases coincided with dryer months and are similar to other studies in Southeastern Brazil (Araujo et al. 2002, Rosa e Silva et al. 2002, Carvalho-Costa et al. 2006). The seasonality related to group A RV infection is in winter and dry months, although this pattern does not happen uniformly in tropical countries (Kapikian et al. 2001).

Taking the group A RV genotypes into account, the epidemiologic importance of G9P[8] found in 43% of the cases must be emphasized, overcoming the classic G1-G4 genotypes. This data reinforces the emergence of G9 on the Brazilian scenario, described for the first time in Rio de Janeiro by two research groups (Araujo et al. 2001, Santos et al. 2001) and at present, considered one of the most frequent genotypes in Goiás, Salvador and Rio de Janeiro (Costa et al. 2004, Santos et al. 2005, Carvalho-Costa et al. 2006, Volofão et al. 2006).

Among RV strains, a short profile in PAGE was observed in 28% of the cases, all of them corresponding to the G2P[4] genotype, which was observed among the strains obtained during 2006 but not during 2004 and 2005. Furthermore, this genotype was not detected in a previous study realized by this same research group in children attended to in an emergency room between 2003-2004 (Spano and colleagues, unpublished observations). This fact points out the temporal fluctuation of RV circulation strains in consecutive years and in a same geographic area, as related in a review (Santos & Hoshino 2005).

An extensive clinical assay conducted in Latin America, gave evidence of the protection conferred against the genotypes of group A RV observed here, with only a minor response to G2 (Linhares & Villa 2006). Recently published results show the detection of the single G2P[4] genotype in vaccinated patients in Northern Brazil (Gurgel et al. 2007). It thus becomes clear that RV genotype surveillance is beyond doubt a somewhat pressing question in order to observe it as a gastroenteritis agent and its clinical severity as well as to evaluate the vaccine immunization efficacy.

In conclusion, the present results revealed that, during the period of study, NoV was more frequent than RV

as the hospitalization cause due to gastroenteritis; a long period of hospitalization was observed not only for RV, but also for NoV infection; the occurrence of NoV and RV infection was mainly in the first two years of life, albeit NoV has been detected up to 12 years; prominent diarrhea and vomit was observed for both infections; RV genotype was represented mainly by G9 and followed by G2. It should be pointed out that there is a need to accomplish further studies on NoV and on genogroup determination, due to the scarce data on this subject in Brazil and to better determine the real impacts of this infection.

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