Quantitative and molecular analysis of noroviruses RNA in blood from children hospitalized for acute gastroenteritis in Belém, Brazil


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1. Background

Noroviruses (NoVs), members of the Caliciviridae family, are small, positive-polarity RNA viruses and were recently recognized as the second most common cause of severe childhood gastroenteritis [1–3]. Worldwide, NoVs are responsible for almost 50% of acute gastroenteritis (AGE) outbreaks and more than 90% of the non-bacterial AGE, being considered the major pathogens in outbreaks [1,4]. NoVs usually causes diarrhea and vomiting, and a number of other clinical manifestations are also observed: nausea, abdominal cramps, fever, headache, chills, and myalgias [5,6]. Although NoVs-infection usually causes a short-term and self-limiting disease, hospitalization is required when severe AGE affects the elderly [7] and immunocompromised patients [8]. Particularly in developing countries, where a high childhood mortality due to diarrhea is reported, NoVs have been estimated to cause up to 200,000 deaths each year in children less than 5 years old [9]. Despite a high genetic diversity, at least 25 human genotypes, the genogroup II, genotype 4 NoVs (GI.4) are responsible for the vast majority of AGE outbreaks caused by NoVs reported worldwide [1,5,6].

Particularly in the post-rotavirus vaccine era, NoVs have captured increasing attention as a major cause of AGE outbreaks and hospitalizations in children less than 5 years old [10]. In addition to the classic symptoms of AGE (watery diarrhea, vomiting, abdominal pain, and fever), NoVs infection has been associated, though to a lesser extent, with atypical clinical conditions such as seizures [11,12], encephalopathy [13], and necrotizing enterocolitis [14,15]. It is unknown whether a relationship exists between these atypical symptoms and NoVs dissemination to outside the digestive tract, and worldwide, few studies have detected NoVs in blood samples [12,13,16,17].
2. Objectives

In the present study, we aimed to investigate the presence of NoVs in stool and serum samples of children admitted to hospital with symptoms of AGE. We tested paired (stool/serum) samples to search for NoVs using an immune assay and molecular methods. Data from pediatric patients were recorded to evaluate a possible association between NoVs RNA in serum, stool viral load, and AGE clinical features.

3. Study design

3.1. Clinical samples collection

During seven months of a pediatric hospital-based surveillance study (March to September 2012), paired stool and serum samples were collected from children admitted in a pediatric clinic, located in Belém city, Pará state, northern Brazil. Children less than 6 years hospitalized for AGE (three or more liquid or semi-liquid evacuations in a 24 h period) were included in the study. Clinical samples collected after 48 h of admission and children presenting with persistent diarrhea (>14 days) were excluded. Serum and stool samples were stored at −20 °C until RNA extraction.

This study, designed with the primary objective of assessing rotavirus viremia, was performed in accordance to the ethical standards of the Declaration of Helsinki and ethical approvals were obtained from an independent Ethics Committee on Human Research of Evandro Chagas Institute, Brazilian Ministry of Health (IEC-CEPH, protocol No. 0039/2011). For all samples’ collection, a written informed consent was obtained from the patient’s parents or legal guardians.

3.2. Norovirus detection

Stool samples were initially tested for the presence of NoV antigen using the RIDASCREEN® Norovirus 3rd Generation (R-Biopharm AG, Germany) enzyme immunoassay (EIA) kit according to the manufacturer’s instructions. EIA positive stool and all serum samples were subjected to the reverse-transcriptase quantitative PCR (qRT-PCR) for NoV detection and quantification. Prior to reaction, RNA was purified from 140 µl of a 10% stool suspension or serum samples using the QIAamp Viral RNA™ extraction kit (QIAGEN, CA, USA), and immediately stored at −70 °C prior NoV detection/quantification.

Amplification, data acquisition, and analysis were performed on an ABI PRISM model 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using primers and probe designed by Kagayama et al. [18] and SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, CA, USA). A 10-fold serial dilution of a plasmid containing the ORF1/2 junction of NoVs GII was used to generate a standard curve for viruses’ quantification. Forty cycles were used in the reaction and the samples signals that crossed the threshold line, presenting a characteristic sigmoid curve, were regarded as positive.

For all molecular procedures, separate rooms were used among the different steps of viruses’ detection. To avoid cross contamination, RNA purification from stool and serum samples were performed separately. Samples were analyzed in duplicate; Positive clinical sample, DNase/RNase-free water and a no template control (NTC) were used as positive and negative controls in all procedures.

3.3. Molecular characterization and phylogenetic analysis

For NoVs GII genetic characterization and phylogenetic analysis, PCR (primers Mon431/432 [19] and G2SKR [20]) and semi-nested-PCR (primers COG2F [18] and G2SKR [20]) were performed targeting the 5’-end ORF2 region. Amplicons were purified and sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit® and the ABI Prism 3130xl DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Following the chromatograms analyses, consensual sequences were obtained using BioEdit [21]. A phylogenetic dendrogram was constructed by the neighbour-joining method using a matrix of genetic distances established under the Kimura-two parameter model using MEGA 5.05 [22]. NoV GI strains isolated from stool and serum samples were classified according to the genotyping tool [23] available on line and the sequences for 22 strains were deposited in the GenBank under accession numbers KC165031 to KC165052.

3.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism® software version 5. As appropriate, Mann–Whitney U test or Chi-square was used to assess significant difference between features recorded from children presenting AGE with and without NoVs RNA detection in serum. A p value inferior to 0.05 was considered to be statistically significant.

4. Results

4.1. Noroviruses detection

During this surveillance study, paired stool and serum samples were obtained from 85 children hospitalized for AGE. NoVs were detected in 34.1% (29/85) and 11.8% (10/85) of the stool and serum samples collected, respectively. Among the NoVs positive cases, NoVs RNA in serum was demonstrated in 34.5% (10/29) of the cases, suggesting viremia. Table 1 shows the clinical features of AGE and NoVs viral load in the stool samples from hospitalized children classified within two groups: NoVs AGE with and without RNA detection in blood. The duration of diarrhea was established from the onset of the clinical symptoms up to the interview with parents/legal guardians, in general at the first day of hospitalization. Co-infection involving NoVs and group A rotavirus (RVA) (data not shown) was observed in six AGE cases and were excluded from the analyses. All the six co-infection were detected within the group without NoVs RNA detection in serum.

The length of hospital stay (6.5 vs. 4.0 days; p = 0.006), and viral load quantified from the stool samples [3.9 × 10^11 vs. 1.1 × 10^11 genome copies per gram (GC/g); p = 0.0472] showed statistically significant difference when comparing patients with and without NoVs RNA in serum. We observed a lower level of NoVs GC among the group without viremia, represented by the Ct value (Fig. 1). For serum samples, NoVs was detected in a low level of GC per ml, ranging from 2.1 × 10^2 to 2 × 10^5 (mean of 4.9 × 10^4).

4.2. Norovirus characterization

NoVs was genotyped by sequencing the partial 5’-end of ORF-2 region [320 nucleotide (nt) in length]. A total of 69% (20/29) and 20% (2/10) of the stool and serum samples were sequenced, respectively. GII.4 was the most prevalent genotype detected (18/20–90%). Two samples were classified as genotype GII.7. Among the GII.4 strains detected, 100% were classified as 2010 variant (Fig. 2). The two NoVs strains sequenced from serum samples were classified as GII.4 and nt sequences matched 100%, comparing paired stool and serum sample each other.
Table 1
Clinical features and stool viral load in NoVs-infected children hospitalized with AGE with and without NoVs RNA in sera.

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>NoVs AGE with NoVs RNA in serum (n = 10)</th>
<th>NoVs AGE without NoVs RNA in serum (n = 13)</th>
<th>Statistical analysis (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of evacuations per day*</td>
<td>4 (3–8)</td>
<td>5 (3–8)</td>
<td>0.457</td>
</tr>
<tr>
<td>Duration of diarrhea, days**</td>
<td>3.5 (1–8)</td>
<td>4 (1–7)</td>
<td>0.535</td>
</tr>
<tr>
<td>Number of vomiting episode per day*</td>
<td>5.5 (3–8)</td>
<td>3.5 (0–8)</td>
<td>0.214</td>
</tr>
<tr>
<td>Duration of vomiting, days*</td>
<td>3 (1–10)</td>
<td>2.5 (0–6)</td>
<td>0.598</td>
</tr>
<tr>
<td>Fever (°C) (≥37.5)</td>
<td>4.5 (50%)</td>
<td>5 (50%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Length of hospital stay, days**</td>
<td>6.5 (4–11)</td>
<td>4 (1–6)</td>
<td>0.006</td>
</tr>
<tr>
<td>Leukocytosis (×10^3/mm^3)</td>
<td>3 (60%)</td>
<td>4 (33%)</td>
<td>0.861</td>
</tr>
<tr>
<td>Viral load** (GC/g)</td>
<td>3.9 × 10^11 (2.6 × 10^9–1.1 × 10^12)</td>
<td>1.1 × 10^11 (9.7 × 10^9–4.3 × 10^11)</td>
<td>0.047</td>
</tr>
</tbody>
</table>

NoVs, noroviruses; AGE, acute gastroenteritis; GC/g, genome copies per gram.

* Median value (range).
** No. (%) of patients.
† Mean value (range).
‡ The duration of diarrhea was established from the onset of clinical symptoms up to the interview with parents/legal guardians, in general during the first day of admission at the hospital.
§ Children with double infection of NoVs and group A rotavirus (n = 6) are not shown.

5. Discussion

The detection of RVA RNA and antigenemia among children with AGE is well established and documented [24–29]. However, few studies have investigated the presence of NoVs in serum samples of children with NoVs-positive AGE [12,16,17]. In the present study we found NoVs GII in 34.1% of the stool samples collected from children admitted to hospital with AGE. Among the positive samples, NoVs RNA was detected in the serum of a high proportion of the cases (43%), excluding six samples that were co-infected with RVA. Our results showed that AGE in the group that presented NoVs RNA detection in serum was not more severe in terms of frequency and length of diarrhea and vomiting, fever, and leukocytosis. However, we found a statistically significant difference in the duration of hospitalization (p = 0.006) and stool viral load (p = 0.047) when comparing the group of patients with and without NoVs RNA detection in serum.

We detected a high rate of NoVs viremia in patients with norovirus excretion, as compared to range of positivity rates (15% and 25%) found in previous studies [16,17]. There is a theoretical possibility that screening of the stool samples using an EIA selects for patients with a potentially high viral load, and therefore, the viremia rate (34.5%) might be overestimated. Takanashi et al. [16] also found a high NoVs' load in stool samples of serum-positive group, however with no statistically significant difference. In addition, Ramani et al. [29] showed a statistically significant correlation between the presence and levels of RVA antigen in serum samples and stool viral load.

Our results suggest that a high replication and resulting viral load could cause greater injury to the epithelium, leading NoVs to reach the intestinal lamina propria, and consequently blood vessels. A similar mechanism, concerning the RVA entry in blood stream, was also proposed by Ramani et al. [29].

Phylogenetic analysis of NoVs strains showed the high circulation of genotype GII.4 (90%) among the sequenced strains. This genotype was the most prevalent, as described in several studies in Brazil and elsewhere. Sub-typing of GII.4 demonstrated that all strains belonged to variant 2010. This GII.4 variant 2010 (also called New Orleans) was first detected in October 2009 in United States, and was responsible for 60% of the outbreaks during the 2009–2010 season [30]. This variant was also detected in several countries of Europe [31], confirming the global spread of this new variant; an evolution pattern previously demonstrated for others GII.4 variants. Two strains detected in stool samples without viremia were classified as GII.7. This genotype was previously detected in others Brazilian’s states in 2005 [32,33]. The two NoVs strains sequenced from sera matched 100%, at nt level, with their respective sequences obtained from faecal samples, corroborating results previously found [12,16]. The presence of the same strain in both samples reinforces the intestinal route as the entry site for NoVs to achieve the blood stream, as also suggested by Medici et al. [12].

An evaluation of a true viremia for NoVs (infectious viruses circulating in the blood stream) is hampered since no fully permissive cell system has been established for this agent to date. The present study describes preliminary results concerning NoVs from children hospitalized for AGE and is part of a major ongoing surveillance investigation with the primary objective of assessing RVA viremia. An additional limitation is that we did not search for enteropathogens other than rotavirus, for instance, bacteria, that might have a role in clinical severity.

Unlike RVA, few studies associate NoVs with extra-intestinal spread. We found here that NoV GII is not uncommonly disseminated to blood stream, since 34.5% (10/29) of children with NoV-positive AGE presented detectable viral level in serum samples. Our data suggest that the presence of RNA in serum correlates with a longer duration of hospital stay, as well as with a higher stool viral load. These results also suggest that NoVs GII could spread beyond the intestine and into the bloodstream. Our findings could help explain the relationship between severe AGE and viremia, and a possible link with systemic symptoms. This is the first study conducted in Brazil concerning the detection of NoV in
Fig. 2. Phylogenetic dendrogram based on partial capsid nucleotide sequences of noroviruses (NoVs) GI strains detected from stool (n = 14) and serum samples (n = 2) of hospitalized children with acute gastroenteritis. NoV prototypes were obtained from GenBank, and genotypes and their respective accession numbers are indicated at the right. Fecal (F) and serum (S) samples are marked with a filled and an unfilled diamond, respectively. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2000 replicates) are shown at the branch nodes and values lower than 60% are not shown.

Fig. 3. Bar graph showing the percentage of viremia detected in children under 14 years of age by the NoVs phylogenetic group. The number of viremia detected were: GI.1, 0%; GI.2, 5%; GI.3, 60%; GI.4, 90%; and GI.7, 100%.

serum samples. Future studies on children with NoV-positive AGE and viremia should be conducted for a clearer understanding of the NoVs pathogenesis.

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Competing interests
No conflicts of interest have been identified.

Ethical approval
The research was performed in accordance to the ethical standards of the Declaration of Helsinki and ethical approvals were obtained from an independent Ethics Committee on Human Research of Evandro Chagas Institute, Brazilian Ministry of Health (IEC-CEP, protocol No. 0039/2011).

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References


