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Norovirus in feces and nasopharyngeal swab of children with and without acute gastroenteritis symptoms: First report of GI.5 in Brazil and GI.3 in nasopharyngeal swab

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ABSTRACT

Background: Noroviruses (NoVs) are an important cause of acute gastroenteritis (AGE), worldwide.
Objectives: To evaluate the frequency, viral load and molecular profile of NoV in fecal and nasopharyngeal swab samples from hospitalized children, and to determine children's secretor status.
Study design: From May 2014 to May 2015, 219 children were included in the study, 96 with gastroenteric symptoms and 123 without gastroenteric symptoms. All fecal and nasopharyngeal swab samples were screened by TaqMan RT-qPCR duplex (GI/GII NoV) and quality samples were characterized by genomic sequencing.
Results: Norovirus positivity rate in feces was 15.4% in asymptomatic and 18.8% in the symptomatic group. The median viral loads in feces were 2.69×10^8 GC/g and 4.32×10^7 GC/g from children with or without AGE symptoms, respectively. In nasopharyngeal swab samples, the NoV positivity was 11.4% in symptomatic children, with a median viral load of 2.20×10^7 GC/mL and 6.5% in asymptomatic children, with an average viral load of 1.73×10^6 GC/mL. In only two cases NoV was detected in both samples. A considerable genomic variability was observed in feces, with six genotypes being detected, as follows: GII.4, GII.6, GI.3 and GII.3, GI.2 and GI.5. Two GI.3 was detected in nasopharyngeal swab.
Conclusions: Our data reveal considerable NoV frequencies in both nasopharyngeal and fecal samples from symptomatic and asymptomatic children. Higher viral loads were detected in samples from AGE symptomatic children, when compared to asymptomatic children. High genomic variability was observed, with this being the first report of GI.5 NoV in Brazil and of GI.3 in nasopharyngeal swab samples.

1. Background

Noroviruses (NoVs) are an important cause of acute gastroenteritis (AGE). In a systematic review it was estimated that the NoV account for 18% of all AGE cases in the world [1]. The *Norovirus* genus belongs to the *Caliciviridae* family, and is further subdivided into six genogroups and approximately 40 genotypes [2], based on the complete genomic sequence of the gene encoding the VP1 capsid protein [3]. Even though the viral receptor remains unknown, it is admitted that human histoblood group antigens (HBGAs) are putative receptors or co-receptors, at least for some NoV strains. Therefore, individuals who express such antigens in their mucosa (positive secretor status – Se +) would be more susceptible to NoV infection when compared to those that do not express those antigens in their mucosa (negative secretor status – Se –) [5].

NoVs are transmitted by the fecal-oral route, through direct person-to-person contact or contact with fomites, by ingestion of contaminated food or water, and ingestion of aerosolized particles from vomit of infected individuals [6–8]. One study reported NoV positivity in 0.5% (3/562) of nasopharyngeal swabs from children with respiratory symptoms, suggesting that NoV may be transmitted by the respiratory route [9].

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2. Objectives

The main objectives of this study were to evaluate the frequency, viral load and molecular profile of NoV in fecal and nasopharyngeal swab samples from children under six years of age, presenting or not AGE symptoms, hospitalized in a public child care referral hospital, and also to determine the secretor status of the children participating in the study.

3. Study design

3.1. Design and study population

This is an observational, cross-sectional study, conducted in samples obtained from children up to six years of age. Samples were collected from children hospitalized between May 2014 to May 2015 at the Materno Infantil Hospital, Goiânia, Goiás, Brazil. The study population was divided into two groups, the first group comprised children with AGE symptoms (diarrhea with or without vomiting and/or fever) at the time of sample collection and the second group included children who did not present any of those symptoms. The AGE asymptomatic population consisted of children that were attended for other reasons, such as surgery, congenital conditions, respiratory tract infections, and others.

The study population comprised 219 children between 0 and 70 months, with a mean of 15 months old. Considering the symptoms, 44% (96/219) of the children were included in the AGE symptomatic group. One fecal sample and one nasopharyngeal swab were obtained from each child participating in the study and all children's clinical data were obtained from medical records.

Samples were only collected after a consent form was signed by the parents or legal guardians of the child. The study was approved by the Ethics Committee on Research of the Clinical Hospital/Federal University of Goiás (protocol: 37305314.7.0000.5078).

3.2. Sample processing

All samples were stored at 4 °C until they were processed, stool samples and nasopharyngeal swabs were transported, processed, and tested separately. After collection, each nasopharyngeal swab was diluted in a sterile tube containing 2 mL of minimum essential media (MEM), resulting in an approximated dilution of 1:10, and transported immediately to the laboratory. The tubes were homogenized by vortexing, the swabs were removed from the tube that were centrifuged at 1300 × *g* at 10° C for 10 min. The supernatants were collected and used for viral RNA extraction. The cell pellets were stored for secretor status evaluation by genotyping and phenotyping. Stool suspensions were prepared (20% in PBS, pH 7.4), and all clinical samples were stored at −80 °C until further testing.

3.3. GI/GII NoV duplex RT-PCR TaqMan

Samples (20% fecal suspensions and swab supernatants) were extracted using a commercial kit (QIAamp Viral RNA Mini kit-Qiagen, Freigburg, Germany), following the manufacturer's instructions. Viral loads were determined by quantitative GI/GII NoV Duplex RT-PCR TaqMan (RT-qPCR) assay targeting the polymerase/capsid junction region (Table 1), using the AgPath-ID™ One Step RT-PCR kit (Life Technologies, Grand Island, NY), as it has done by Schultz et al. [13], adapted for a duplex reaction. For the standardization of the duplex RT-qPCR previously sequenced samples were used in previous studies conducted in our laboratory [14,15]. A negative control (MilliQ water) and a positive control were included in each run. To verify the presence of inhibitors TaqMan® Exogenous Internal Positive Control Kit (Life Technologies, Grand Island, NY) was used. Viral load was determined by a standard curve built with serial dilutions (10^7–10^1) of recombinant plasmids containing GI and GII NoV inserts (genotypes: GI.3 and GII.4). Curve validation was obtained by the correlation coefficient ($R > 0.99$) and efficiency of reaction. Samples with Cycle threshold (Ct) <40 cycles were considered positive.

3.4. Molecular characterization of NoV positive samples

To determine the NoV genotypes, samples with the highest loads were submitted to conventional RT-PCR assays using the same primer pair used in the RT-qPCR assay. Products that had sufficient DNA concentration were purified by using 65% isopropanol and 70% ethanol, and then submitted to sequencing reaction (Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA)), in duplicates, in an automatic sequencer (DNA ABI PRISM 3130, Applied Biosystems). Sequences quality analysis was determined by the interface phred/prhap [16]. Consensus genomic sequences and prototype sequences of each NoV GI and GII genotypes, obtained from GenBank, were subjected to alignment using Clustal X program [17]. Phylogenetic tree was constructed using the program MEGA version 7.0 [18]. Analysis were based on the neighbor-joining method, considering the nucleotide substitution model Kimura two parameters and 1000 replicates with boostraps values above 80%.

3.5. Determination of secretor status

Secretor status of the children were determined using nasopharyngeal swab cells by an enzyme immunoassay (EIE), using Lectin-UEA (Ulex europaeus agglutinin) specific for Fucα1-2Gal-R, which is secreted in saliva and mucous, only in secretors individuals, following the protocol described by Nordgren et al. [19], with modifications, instead of saliva epithelial cells from the respiratory tract were used as clinical material. Briefly, plates were sensitized with 100 μL of cells from nasopharyngeal swab pellet diluted 1:10 in carbonate-bicarbonate buffer (pH 9.6), and incubated at 37 °C for 2 h, followed by overnight incubation at 4 °C. Plates were washed with PBS (pH 7.4) and blocked at room temperature for one hour with PBS solution with 3% BSA. The conjugate (Lectin-UEA-HRP) diluted 1:1500 in PBS solution with 0.3% BSA was added to each well, and plate was incubated for 1.5 hs at 37 °C. The substrate was then added (H2O2 + TMB), followed by reading (absorbance determination at 450/620 nm). The cut-off value was determined by the mean of the absorbance of five negative controls plus the value of two standard deviations. An error margin of 10% above and below the cut-off value was considered. Two positive and negative controls were run on each plate. All samples were run in duplicate. Results from samples characterized as negative secretor phenotype were further confirmed by genotyping of the partial region of gene FUT2 as described by Lindesmith et al. [20], using cells from nasopharyngeal swab.

3.6. Data analysis

Data from the children's medical records were analyzed together with the obtained results. Statistical analysis were performed using IBM SPSS software, version 20, and the chi-square test ($\chi 2$) and Fisher's exact test were applied, when appropriate. Statistical significance was accessed, considering 95% confidence intervals, and p values < 0.05.

4. Results

The NoV positivity rate in feces was 15.4% (19/123) among children in the asymptomatic group and 18.8% (18/96) in the