Journal of Clinical Virology 58 (2013) 378–384

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Molecular epidemiology of norovirus strains in Paraguayan children during 2004–2005: Description of a possible new GII.4 cluster

Maria Eugenia Galeano [a,b], Magaly Martinez [b], Alberto A. Amarilla [b,1], Graciela Russomando [b], Marize Pereira Miagostovich [a], Gabriel I. Parra [b,*], José Paulo Leite [a]

[a] Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Ministry of Health, Rio de Janeiro, RJ, Brazil
[b] Molecular Biology and Genetics Department, Health Sciences Research Institute, National University of Asunción, Asunción, Paraguay

ARTICLE INFO

Article history:
Received 20 March 2013
Received in revised form 28 June 2013
Accepted 12 July 2013

Keywords:
Norovirus
Acute gastroenteritis
Paraguayan children
GII.4 norovirus

ABSTRACT

Background: Noroviruses (NoV) have been shown to be an important cause of morbidity and mortality in children worldwide, only second after Group A rotaviruses (RVA). In Paraguay, acute gastroenteritis (AGE) is the third cause of mortality in children ≤5 years old.
Objectives: To analyze the presence and diversity of NoV in Paraguayan children ≤5 years old presenting AGE.
Study design: Three hundred seventy eight fecal samples, negative for pathogenic bacteria and RVA, were collected from children admitted as ambulatory and hospitalized patients in a large private hospital from Asuncion, Paraguay from 2004 to 2005. The presence and diversity of NoV was determined by two different RT-PCR strategies and nucleotide sequencing.
Results: One hundred and sixty one samples were positive for NoV by partial amplification of the viral polymerase gene (RdRp). No seasonality or differences in the viral prevalence for the different age-groups were detected. GII and GI NoVs were associated to 58% and 42% of the infections, respectively. The genotype was determined in 18% (29/161) NoV-positive samples. The genotypes detected were: GII.4 (18%), GII.17 (18%), GII.6 (14%), GII.7 (14%), GII.3 (10%), GII.5 (3%), GII.8 (3%), GII.16 (3%), GI.3 (14%) and GI.8 (3%). Amplification of the ORF2 from the GII.4 strains showed the presence of a new GII.4 variant.
Conclusions: The results showed a continuous circulation of NoV in children throughout the two years of study and an extensive diversity of genotypes co-circulating, highlighting the need for better surveillance of NoV in Paraguayan children.

1. Background

Acute gastroenteritis (AGE), both epidemic and sporadic, are common causes of morbidity and mortality in people of all ages [1]. According to Black et al., 1.336 million of deaths occur annually in children ≤5 years old due to diarrheic infections worldwide [2]. Noroviruses (NoV) are considered the most common cause of AGE outbreaks in adults worldwide and the second most frequent viral agent causing AGE in young children after group A rotavirus (RVA) [3,4]. In developing countries, NoV have been estimated to cause around 1.1 million hospitalizations and up to 200,000 children deaths annually [5].

NoV are small (27–38 nm in diameter), non-enveloped viruses that belong to the *Caliciviridae* family. The single-stranded, positive-sense genome is organized in three open reading frames (ORFs): ORF1 encodes for 6 nonstructural (NS) proteins, including the RNA-dependent RNA polymerase (RdRp); ORF2 encodes the major capsid protein (VP1); and ORF3 encodes the minor capsid protein (VP2) [6]. The marked genetic diversity presented by NoV prompted the development of multiple protocols for detection and molecular characterization, which target different regions of the ORF1 and ORF2 [7–11].

Based on sequence differences on the VP1, NoV have been classified into six genogroups (GI–GVI), and multiple genotypes [11,12]. NoV from GI, GII, and GIV have been shown to infect humans, being the GII.4 strains the most prevalent. Although GII.4 strains have been shown to be the main cause of AGE outbreaks worldwide,

* Corresponding author. Present address: Caliciviruses Section, Laboratory of Infectious Diseases, NIAID, National Institute of Health, Bethesda, MD, USA. Tel.: +1 301 402 1258; fax: +1 301 480 5031.
E-mail addresses: gabriel_parra@hotmail.com, parrag@niaid.nih.gov, parrag@niaid.nih (G.I. Parra).
[1] Present address: Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.

1386-6532/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jcv.2013.07.008

other NoV strains (e.g. GII.3) have been shown at high incidence in sporadic cases of diarrhea in children ≤5 years old [13].

In Paraguay, AGE is considered the third cause of mortality in children ≤5 years old [14]. Since 1998, epidemiological studies have been carried out to determine the role of RVA in the etiology of non-bacterial AGE infections in Paraguayan children, indicating that is responsible for approximately 25% of all AGE infections in that population [15,16].

2. Objectives

To analyze the presence and diversity of NoV in children ≤5 years old with AGE, previously determined negative for RVA and enteric pathogenic bacteria.

3. Study design

3.1. Sample collection

A total of 927 fecal samples were collected from children ≤5 years old with non-bacterial AGE who were admitted as ambulatory and hospitalized patients in a large private hospital from Asuncion, Paraguay between January 2004 and December 2005. All samples were screened for RVA [17]. Approximately 50% of the RVA-negative samples from each month were randomly selected for NoV screening (378 out of 701 RVA-negative samples; 161 collected during 2004 and 217 during 2005). In months were a low number of samples was originally collected, at least 10 samples were selected if enough material was available. In only two months (March and May, 2004) less than 10 samples were selected (6 and 8, respectively) for NoV screening. No data on whether the patients were linked to outbreaks or serious cases of gastroenteritis was collected.

3.2. RNA extraction and PCR amplification

Viral RNA was extracted from 10% fecal suspension in Tris–HCl–Ca^{++} [0.01 M] (pH: 7.2) buffer using guanidine isothiocyanate/silica method, as described by Boom et al. [18]. Reverse transcription was carried out using random primers (Invitrogen®, Carlsbad, CA, USA). For NoV detection, generic primers (Mon431/Mon432 and Mon433/Mon434) that target the region B of the ORF1 (RdRp gene) [7], were used according to the conditions described by Beuret et al. [19]. The molecular characterization was performed by using degenerated primers CapA/CapB1/CapB2 and CapC/CapD1/CapD3 that target the VP1 gene (region D) as described by Vinjé et al. [9]; and the primers JV12Y, JV12i, Ni-R and G1 that target the RdRp gene as described previously by Boxman et al. [10]. PCR products were resolved on 2% agarose gel electrophoresis, followed by ethidium bromide staining, and the images were obtained using the Kodak Gel Logic 212 image capture system (Carestream Health Inc., Upland, CA, USA).

3.3. Nucleotide sequencing and bioinformatic analyzes

PCR products from the region D were purified directly from the agarose gels using the QIAquick® Gel extraction kit (QIAGEN®, Valencia, CA, USA) in accordance with manufacturer's instructions. Nucleotide sequencing was carried out using both primers and Big Dye Terminators v3.0 and an automated sequencer ABI PRISM 3730 Genetic Analyzer (Applied Biosystems®, Foster City, CA, USA). The GII.4 strain characterization was performed by sequencing the PCR product of the entire ORF2 with GII.4-specific primers that target the 5′ and 3′ end of the ORF2. Internal primers were design to cover nearly the full ORF2 (primers available upon request). All sequencing reactions were repeated at least twice to verify the accuracy of the differences. The sequences obtained were analyzed with BioEdit v.7.0.9.0 software, using Clustal W for sequence alignment [20]. The phylogenetic trees were constructed using the Neighbor-Joining method and Kimura 2-parameter as a model of nucleotide substitution using the MEGA v4.0 software package [21]. The statistical significance was estimated by bootstrap method (1000 pseudo-replicates). The nucleotide sequences (≥200 pb) reported in this study are available at GenBank database under the accession numbers: KC736563–KC736586. The X-ray solved structure of the VP1 from TCH0_5 (Protein Data Bank [PDB] accession number: 3SKB) was used to identify the antigenic sites and differences among GII.4 strains. Figure was rendered using MacPyMol (DeLano Scientific LLC).

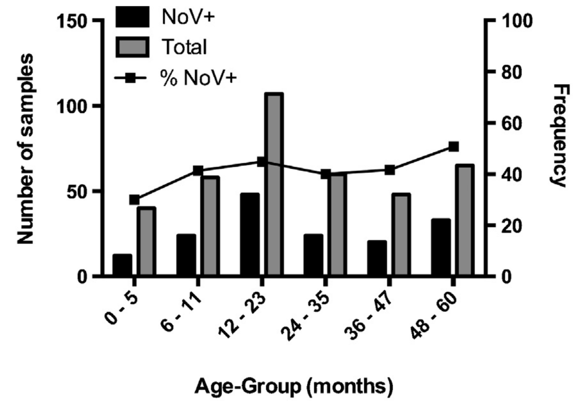

Fig. 1. Frequency of norovirus infections in children with acute gastroenteritis in Asuncion, Paraguay by age-group.

4. Results

4.1. Viral detection

NoV genome was detected in 161 (43%) of 378 analyzed samples. All age groups presented similar frequencies of NoV infection (Fig. 1). NoV infection was detected throughout the year, with peaks distributed across different months (Fig. 2). Climatic data like mean temperature, relative humidity and rainfall was obtained from the Climate Department of the Meteorology and Hydrology Service from the Ministry of Defense (Paraguay); and no relationship between NoV infections and those factors was observed in this study (data not shown).

4.2. Noroviruses characterization

NoV genogroup was determined in 84 out of 161 positive samples using region B and/or region D amplification. GII strains were detected in 54.7% (46/84) of the samples, GI in 40.5% (34/84), and GI/GII mixed infections were observed in 4 samples (4.7%). Partial sequence of the ORF2 was obtained from 29 samples (Table 1). Paraguayan GI strains were classified into two genotypes: GI.3 and GI.8 (Fig. 3). Norovirus GI.3 strains were grouped in two phylogenetic clusters: GI.3a and GI.3b [22]. Three out of the four GI.3 Paraguayan strains grouped with the prototype strain Hu/Osaka/010314/2001/JP (EF547393) within the cluster GI.3b [23]; while the strain Py830SR05 was related to Brazilian GI.3a samples detected during 2006 and 2007 [24,25] and the prototype strain Hu/DesertShield395/1993/US (U04469), described in USA soldiers in Saudi Arabia in the 1990s [26]. The strain Py698SR04, was related to the Boxer strain responsible for viral AGE outbreaks on USA Navy Ships in 2001, and was, therefore, classified as genotype GI.8.