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Noroviruses associated with acute gastroenteritis in a children's day care facility in Rio de Janeiro, Brazil

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Abstract

Noroviruses (Norwalk-like viruses) are an important cause of gastroenteritis worldwide. They are the most common cause of outbreaks of gastroenteritis in the adult population and occur in nursing homes for the elderly, geriatric wards, medical wards, and in hotel and restaurant settings. Food-borne outbreaks have also occurred following consumption of contaminated oysters. This study describes the application of a reverse transcription-polymerase chain reaction (RT-PCR) assay using random primers (PdN6) and specific Ni and E3 primers, directed at a small region of the RNA-dependent RNA polymerase-coding region of the norovirus genome, and DNA sequencing for the detection and preliminary characterisation of noroviruses in outbreaks of gastroenteritis in children in Brazil. The outbreak samples were collected from children <5 years of age at the Bertha Lutz children's day care facility at Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, that occurred between 1996 and 1998, where no pathogen had been identified. At the Bertha Lutz day care center facility, only Fiocruz's employee children are provided for, and they come from different social, economic and cultural backgrounds. Three distinct genogroup II strains were detected in three outbreaks in 1997/98 and were most closely related to genotypes GII-3 (Mexico virus) and GII-4 (Grimsby virus), both of which have been detected in paediatric and adult outbreaks of gastroenteritis worldwide.

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- Day care center
- Gastroenteritis
- Genotypes

Introduction

The Norwalk-like viruses have recently been reclassified as noroviruses (1,2) by the International Committee on the Taxonomy of Viruses. They are also known as small round structured viruses (3), a name based on their morphological appearance as seen by electron microscopy. *Norovirus* (Norwalk-like virus) is a genus in the family *Caliciviridae* along with the other human calicivirus genus *Sapovirus* (Sapporo-like virus). The *Lagovirus* (rabbit haemorrhagic disease virus) and the *Vesivirus* (vesicular exanthema of swine virus) complete the *Caliciviridae*. The prototype strain of noroviruses is Norwalk virus, a genogroup I strain. The genomic diversity of noroviruses includes two genogroups (I and II) (4,5) and a number of genotypes, which have yet to be formally

agreed upon. The genogroup I strains include GI-1 (Norwalk virus), GI-2 (Southampton virus), GI-3 (Desert Shield virus), and GI-4 (Valetta virus) (6) and the genogroup II strains include GII-1 (Hawaii virus), GII-2 (Melksham virus), GII-3 (Mexico virus), and GII-4 (Grimsby virus) (6). However, genogroup II strains are the predominant strains responsible for outbreaks of gastroenteritis worldwide, including the United States of America (5,7), Germany (8), the Netherlands (9,10), United Kingdom (11-14), and South Africa (15,16).

Only a few studies have investigated the molecular and sero-epidemiology of noroviruses in South America. The genomic diversity of noroviruses in children in a Brazilian shantytown was investigated by Parks et al. (17). Sero-prevalence and sero-incidence studies for norovirus have shown a 71% rate in Brazilian children (18); 39 to 100% of Amazonian Indians were found to have antibodies to Norwalk virus (19) and 67 to 91% of Chilean adults and children had antibodies to Mexico and Norwalk virus (20,21). Norovirus studies of the paediatric population have been undertaken in various countries including Argentina (22), Japan (23-25), South Africa (26), Canada (27), Mexico (28,29), Spain (30), France (31), Germany (32), and Finland (33,34).

This study describes the application of a broadly reactive norovirus reverse transcription-polymerase chain reaction (RT-PCR) assay to investigate several large outbreaks of acute non-bacterial gastroenteritis in a children's day care facility in Rio de Janeiro, Brazil, from 1996 to 1998.

Material and Methods

Children's day care facility

Between April 1996 and March 1998, eight outbreaks of acute gastroenteritis occurred at the Bertha Lutz day care facility at the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. In four of eight outbreaks (April 1996, May and September 1997, and March 1998) no etiological agent had been identified. At the Bertha Lutz day care center facility, only Fiocruz's employee children are provided for, and they come from different social, economic and cultural backgrounds. A total of 132 fecal samples from children up to 5 years of age were obtained from these four outbreaks: 23 fecal samples in April 1996, 26 in May 1997, 71 in September 1997, and 12 from March 1998. All samples were stored as fecal suspensions at -20ºC and the study was undertaken between September and December 1999. Specific details on the outbreaks were not available.

Nucleic acid extraction and RT-PCR assay

Ten percent (weight/volume) fecal suspensions were prepared in 10 mM Tris-HCl Ca^{2+}, pH 7.2, and stool suspensions were concomitantly used for ssRNA extraction by the glass powder method as described by Boom et al. (35). Complementary DNA (cDNA) was prepared by adding 40 µl RNA (36) to 1 µl 50 µM random primers (PdN6, Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK), followed by incubation at 70ºC for 5 min and cooling on ice for 5 min. This was followed by the addition of 13.5 µl of a reverse transcription mix containing a final concentration of 10 mM Tris, pH 8.0, 50 mM HCl, 5 mM $MgCl_2$, 1 mM of each dNTP (Amersham Biosciences), and 150 units of MuMLV-reverse transcriptase (Invitrogen, Paisley, UK) in a final volume of 54.5 µl. Reverse transcription was performed at 37ºC for 1 h. The reaction was stopped by incubation at 95ºC for 5 min and then cooled on ice. cDNA (5 µl) was added to a 45 µl PCR mix containing a final concentration of 10 mM Tris, pH 8.0, 50 mM HCl, 1.5 mM $MgCl_2$, 1 mM of each dNTP (Invitrogen), 20 pmol of each primer Ni and E3 (36) located in the RNA-dependent RNA polymerase-coding region, and 1 unit of TAQ

DNA polymerase (Invitrogen). Products were amplified using the following conditions: 95ºC for 2 min and 31 cycles of 95ºC for 15 s, 40ºC for 45 s and 72ºC for 1 min, followed by 1 cycle of 72ºC for 5 min and holding at 15ºC (36). The amplicons were then electrophoresed on 2% agarose gel in Tris-borate-EDTA buffer in Wide Mini-Sub Cell GT (BioRad Laboratories, Hemel Hempstead, UK). The gel was stained with ethidium bromide (0.5 µg/ml) and PCR amplicons were visualized and photographed using a Gel Doc 1000 Polaroid system (BioRad). The Ni and E3 primers used in this study predominantly amplify GII strains, but will also amplify GI strains.

PCR amplicon cloning and sequencing

PCR amplicons for norovirus generated from fecal samples were cloned using a TA cloning system® (TOPO®; Invitrogen). Two microliters of unpurified PCR amplicons was ligated to a pCR®2.1-TOPO® vector, the ligation mix was incubated at room temperature for 5 min and a 2-µl amount was added to the 50-µl TOPO10' competent cells (Invitrogen) and incubated on ice for 30 min. The cells were then heat shocked at 42ºC for 30 s and immediately transferred to ice. Each transformation was incubated with 250 µl of SOC medium (Invitrogen) at 37ºC for 30 min in a shaking incubator. One hundred microliters of each transformation was spread on an L-agar plate containing ampicillin and X-gal/IPTG (Invitrogen) for blue/white colony selection and incubated at 37ºC overnight.

Three white colonies from each transformation were sub-cultured onto an L-agar plate and incubated at 37ºC overnight. The template for PCR was 1 µl of a 100-µl water/colony heat denaturation preparation. The colonies were screened with pCR®2.1-TOPO® vector primers (PTAG5' primer 5' GCT ATG ACC ATG ATT ACG CCA A 3', downstream of an M13 reverse primer and PTAG3' primer 5' TGT AAA ACG ACG GCC AGT GAA 3', which overlaps with the M13 (-20) forward primer) using the Expand High Fidelity PCR System™ (Roche Molecular Biochemicals, Lewes, UK). PCR amplicons were purified for sequencing PCR using a Geneclean® Spin kit (Anachem, Luton, UK). Purified DNA was sequenced in both directions using PTAG5' and PTAG3' primers, the Beckman Coulter™ CEQ2000 Dye Terminator Cycle Sequencing Quick Start kit (High Wycombe, UK), according to manufacturer instructions, and a Beckman Coulter™ CEQ2000 capillary sequencer. Generation of contiguous sequences and pairwise alignments of the 76-bp inter-primer region (Ni/E3) of the norovirus ORF1 sequences was performed using Genebuilder and Clustal in Bionumerics version 2.5 (Applied Maths, Kortrij, Belgium).

Results and Discussion

Noroviruses were detected in three of the four day care facility outbreaks examined for which no etiological agent had been identified. In outbreaks two (May 1997), three (September 1997), and four (March 1998), noroviruses were detected in 6/26 (23%), 27/71 (38%) and 8/12 (67%) samples, respectively. Noroviruses were not detected in samples collected from outbreak 1 (April 1996).

The analysis of PCR amplicons by partial DNA sequencing confirmed that all norovirus strains belonged to genogroup II and the nucleotide alignments of the strains designated Bertha Lutz (BL) day care facility strains (BL2, BL3, and BL4) were compared with other reference strains from GenBank and the Enteric Virus Unit, norovirus sequence database at SRMD, London, UK (Figure 1). The BL2 strains from outbreak 2 in May 1997 had 100% identity with Grimsby/1995/UK (Lordsdale/1995/UK-Genbank X86557), the BL3 strains from outbreak 3 in September 1997 had 90% identity with Mur1/

1997/JP (AB01929), and the BL4 strains from outbreak 4 in March 1998 had 95% identity with Mexico/1989/MX (Genbank U22498). The multiple strains from each outbreak demonstrated in Figure 1 were the only samples sequenced and all strains within an outbreak were identical.

This is the first report of noroviruses associated with outbreaks of gastroenteritis in day care facilities in Brazil. Further studies are needed to determine the extent of norovirus infection in outbreaks of food-related gastroenteritis and outbreaks in the paediatric and adult population in Brazil.

The low level of norovirus positivity in each outbreak was probably a result of the specimens being stored as fecal suspensions at -20ºC and examined 1-2 years after collection.

Small regions of the polymerase gene were used for analysis, as the primers used had to be broadly reactive in order to amplify the cDNA of a wide range of genogroup II norovirus strain types, and although sequence data generated from a small region of the RNA polymerase should be interpreted with caution, previous phylogenetic studies using related primers have revealed a robust relationship between this small region amplified and more extensive regions of the polymerase gene (11-13).

The norovirus strain in outbreak 2 (BL2/1997/BR) in May 1997 was identical to Grimsby/1995/UK (4), a genotype GII-4 (Bristol/1993/UK) which is the commonest circulating strain in the UK and Europe. GII-4 has been an endemic strain and the predominant strain in the UK since 1992, and has been responsible for 50 to 70% of norovirus outbreaks in each year except 1993/94 (14). The second outbreak of norovirus gastroenteritis which occurred in September 1997, was caused by a possible GII-4 (Bristol/1993/UK) variant strain (BL3/1997/BR) and is closely related to Mur1/1997/JP. The third norovirus outbreak was in March 1998 and

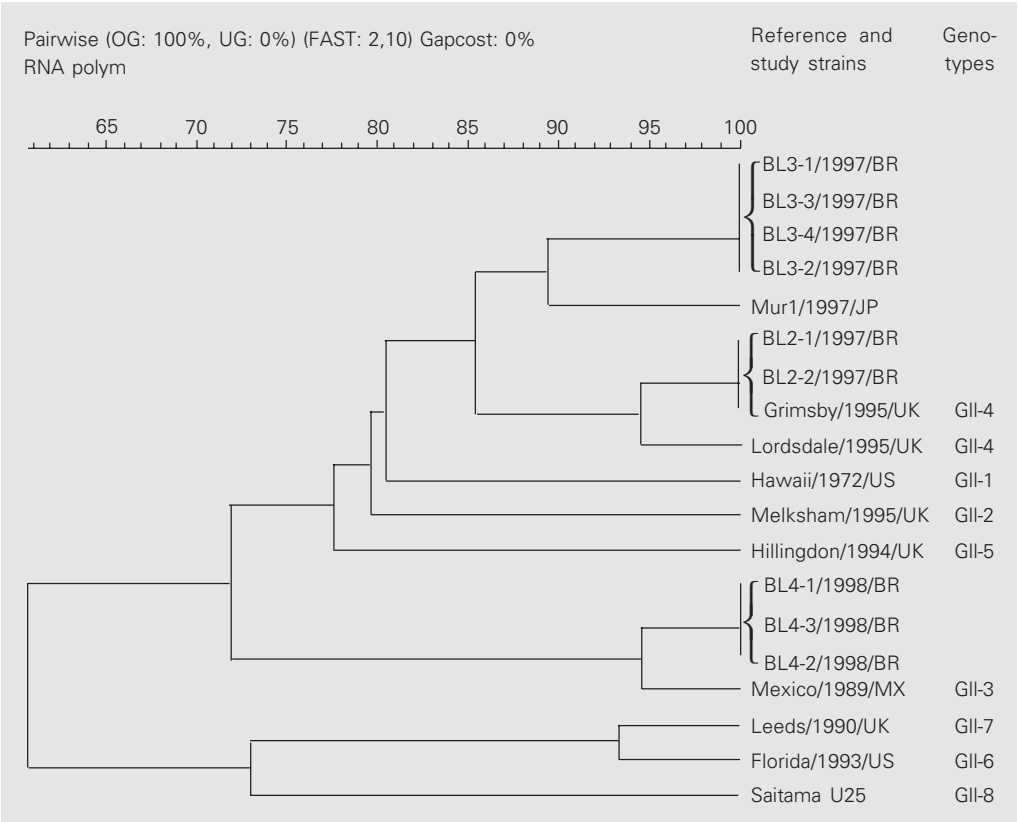

Figure 1. Comparison of norovirus strains detected in a children's day care facility in Rio de Janeiro, Brazil, with strains described in other parts of the world. BL2-1 and 2-2 are strains from patients in outbreak 2 in 1997, BL3-1 to 3-4 are strains from patients in outbreak 3 in 1997, and BL4-1 to 4-3 are strains from patients in outbreak 4 in 1998. Genbank strains are Hawaii/1972/US (U07611), Melksham/1995/UK (X81879), Mur1/1997/JP (AB01929) Lordsdale/1995/UK (X86557), Mexico/1989/MX (U22498), Florida/1993/US (AF414407), and Saitama U25 (AB039780). For Grimsby/1995/UK, Hillingdon/1993/UK and Leeds/1990/UK, see Vinje et al. (4). Sequences of strains from this study are available from the author of correspondence.

was caused by a GII-3-like (Mexico/1989/MX) strain (BL4/1998/BR). The Mexico/1989/MX strain was responsible for an epidemic of norovirus gastroenteritis in 1993/94 in the UK (14), but has not been seen recently in the UK. No capsid data were available for the confirmatory genotyping of these Brazilian strains.

The importance of noroviruses infection in children has been well demonstrated in developed countries; however, little is known about the role of those viruses in sporadic cases or outbreaks of paediatric gastroenteritis in developing countries in South and Central America, Africa and Asia (22,26,28). The strains mostly observed in Brazil and worldwide belong to genogroup II, previously in the North-East region of Brazil (17) and now in the South-East region in this study, as well as in other developing and developed countries (22-25,31-33). There is a need to examine the diversity of norovirus strains in populations in developing and developed countries for which data are not available, to better understand the virus epidemiology and its global spread.

The present study demonstrates that noroviruses are present in the paediatric population in Brazil. Further studies are needed to investigate the burden of gastroenteric illness due to noroviruses in the paediatric and adult population in Brazil.

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