Identification of Two Sublineages of Genotype G2 Rotavirus Among Diarrheic Children in Parauapebas, Southern Pará State, Brazil

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On a world scale, group A human rotaviruses are the most common cause of severe acute gastroenteritis during infancy and childhood, including five (G1, G2, G3, G4, and G9) epidemiologically important genotypes. Among these, G2 denotes a different genogroup which appears to have a cyclic pattern of occurrence and yet little information is available about its genetic variability. The aim of this report was to characterize the emergence of G2 genotype in Parauapebas, Southern Pará State, Brazil, some of which detected after introduction of rotavirus vaccine. A total of 241 fecal specimens from young children with acute gastroenteritis were collected from the “Yutaka Takeda Hospital,” a Municipal Hospital, and at the Parauapebas’ Health Unit, Pará, from January to September 2006 and during March to November 2008. All samples were tested for rotavirus using immunochromatography, polyacrylamide gel electrophoresis (PAGE), and RT-PCR, yielding an overall positivity of 12.45% (30/241). Rotavirus G2P[4] was identified in 27 of 30 samples (90%), followed by G1P[8] (2/30, 6.67%) and G9P[8] (1/30, 3.33%). Phylogenetic analysis was performed in 15 of the G2 strains, all of which grouped into lineage II. Four of these strains clustered into sublineage II-a (year 2006) and 11 into one possible new sublineage named II-c (year 2008, except SAL-1920-C). The recent re-emergence of G2 genotype associated with lineage II in Brazil warrants the continuous monitoring of circulating rotavirus strains following the nationwide universal use of rotavirus vaccine. J. Med. Virol. 82:712–719, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: rotavirus; G2 genotype; diarrheic children

INTRODUCTION

Rotaviruses are the major cause of severe gastroenteritis in infants and young children worldwide, being associated with 611,000 deaths annually, 80% of which occurring in the low-income countries [Parashar et al., 2006]. Human rotaviruses (HRV) are members of the Reoviridae family, genus Rotavirus with a genome that consists of 11 segments of double-stranded RNA (dsRNA), encoding six structural (VP1–VP4, VP6, and VP7) and six non-structural (NSP1–NSP6) proteins. Its classification is essentially made on the basis of a binary system represented by VP4 (gene 4) and VP7 (gene 9) proteins [Estes and Kapikian, 2007], even though a more complete classification system is currently being proposed by Matthijnssens et al. [2008], using all 11 genome RNA segments. The four globally more common rotavirus combinations, P[8]G1, P[4]G2, P[8]G3, and P[8]G4, are likely to be responsible for 88.5% of the rotavirus diarrheal episodes among children worldwide. The P[8]G9 genotype is an important new type causing acute gastroenteritis in children worldwide [Kirkwood et al., 2004]. Of interest is the fact that P[4]G2 genotype strains show a continent/subcontinent wide variation when analyzed from 1973 to 2003, as follows: South America (23%), Africa (2%), Asia (13%), North America (11%), Europe (9%), and Australia/Oceania (14%) [Santos and Hoshino, 2005].

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Epidemiological studies carried out in Belém, Northern Brazil, including 1,240 rotavirus strains identified in 12 studies have shown that G2 displays a cyclic pattern of occurrence over time [Oliveira et al., 2008]. Also, other epidemiological studies carried out in Belém, Northern Brazil demonstrated the variability of antigenic and genetic diversity of rotavirus strains showing a higher frequency of mixed infections and unusual genotypes [Gusmão et al., 1999; Linhares and Bresee, 2000; Mascarenhas et al., 2002, 2006, 2007].

In this study we report the emergence of G2 genotype in Paraúapebas, Southern Pará State, Brazil, some of which detected after introduction of rotavirus vaccine.

MATERIALS AND METHODS

Patients and Specimens

The present study was conducted in areas under direct influence of the Salobo Project, where copper mining area is located in the National Forest of Tapirapé-Aquiri, of Marabá city and the Park Zoobotânico of Carajás, in Paraúapebas, Southern Pará State (Fig. 1). The city of Paraúapebas belongs to the southeastern Mesoregion Paraense and Micreoregion of Paraúapebas and is about 700 km from Belém with 91,000 inhabitants approximately. It has farming and lumber activities as its economic base and is subject to migratory flows from all parts of Brazil and shelters world’s biggest mineral province, Carajás, with 411,948 ha. The rainfall season denote a 2,000–2,400 mm annual precipitation, and the annual average temperature is around 23–25°C. The driest period includes July to September. The relative humidity of average air is of 80% with the predominant vegetation of the Savannah type.

The surveillance was made in a hospital based for gastroenteritis conducted from January to September 2006 and March to November 2008 in both the Yutaka Takeda Hospital, a Municipal Hospital, and the Paraúapebas official Health Unit, located in Serra dos Carajás, Southern Pará State, Brazil.

For children up to 5 years old acute diarrhea was defined as the passage of three or more liquid or semi-liquid stools in a 24-hr period. The study was conducted following signature of informed consents by parents or legal guardians, as well as approval from ethical review committees.

Antigenic Test

Immunochromatography. All fecal samples were tested promptly in the field for group A HRV using the commercial kit Rota-Strip (CORIS, BioConcept, Gembloux, Belgium), following the manufacturer’s recommendations.

Genome Amplification and Nucleotide Sequencing

RNA extraction. The viral dsRNA was extracted from 10% fecal suspensions by using guanidinium isothiocyanate–silica nucleic acid extraction, as described previously [Boom et al., 1990], including modifications proposed by Araújo et al. [2001]. Polyacrylamide gel electrophoresis (PAGE) was carried out in Tris–glycine buffer and rotavirus genome profile was defined following electrophoresis of extracted dsRNA through vertical 5% acrylamide bisacrylamide gels [Pereira et al., 1983].

Reverse transcription-polymerase chain reaction. All HRV-positive samples by immunochromatography and PAGE were subjected to RT-PCR using SuperScript™ (Invitrogen, Carlsbad, CA) and the resulting cDNA was amplified to generate fragments of 904 and 876 bp corresponding to partial region of genes that encode VP7 and VP8* proteins, respectively. The primers used in the first amplification were 9con1/9con2 and 4con3/4con2 for G and P types, respectively, followed by a second round using specific primers 9T-1, 9T-2, 9T-3P, 9T-4, and 9T-9B for G types 1, 2, 3, 4, and 9, respectively [Das et al., 1994]. Characterization of P genotypes was performed following a strategy similar to that used for G typing, using type-specific primers 1T-1 (P[8]), 2T-1 (P[4]), 3T-1 (P[6]), and 4T-1 (P[9]), as described by Gentsch et al. [1992].

DNA Sequencing of VP8* and VP7 Genes

The nucleotide sequencing was carried out as reported by Mascarenhas et al. [2006], using the primers 9con1/9con2 and 4con3/4con2 and the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The products were analyzed using an automatic ABI Prism 3100 DNA sequencer (Applied Biosystems).

Multiple Sequence Alignment and Phylogeny

Sequence data from both strands were aligned and edited using the BioEdit Sequence Alignment Editor (version 7.0.5.2) program and compared with G2 and P4

prototype sequences as follows: G2: DS-1 (AB118023), TA3 (AF106280), Australia/5/77 (X00572), 410GR (AY261335), TF85 (AF106299), D00107 (FJ436812), RUS-Nov06-885 (FJ447565), Matlab6-04 (EF690797), MMC84 (EU839924), Dhaka26-06 (EF690795), KY3303 (AY2-61350), TA26 (AF106284), TA65 (AF106288), Bangla81 (EF690808), TB-Chen (AY787646); G4: DS-1 (AB118025), KO-2 (AF401755), 1-2003 (DQ172841), MMC6 (EU839950), RUS-Nov06-885 (FJ447575), 6199 (FJ410001), IS-2-1992 (X82323), H41-1993 (DQ172839), I200-1997 (DQ172840), rj5619 (DQ857927), and Wa (L34161).

Phylogenetic tree was constructed using the MEGA software version 3.1 [Kumar et al., 2004] by neighbor-joining (NJ) method. For NJ, a distance matrix calculated from the aligned sequences by Kimura two-parameter formula [Kimura, 1980] was used. For determining the reliability of tree topology, bootstrap analysis [Felsenstein, 1995] was carried out on 2,000 replicates.

Nucleotide Sequence Accession Number
The nucleotide sequences determined in this study have been deposited in the GenBank (http://www.ncbi.nlm.nih.gov) and assigned the accession numbers: FJ492764–FJ492780.

RESULTS
Characterization of Rotavirus Strains by Immunochromatography, PAGE, and PCR Analysis
From January to September 2006 and March to November 2008, 241 stool specimens were screened for rotavirus by immunochromatography and subsequently by PAGE, yielding 12.45% (30/241) and 12.03% (29/241) positivity rates, respectively. Of the 30 positive samples, 27 (90%) displayed a clear short electrophoretic RNA profile. Two additional samples showed a long electrophoretic pattern and bands could not be identified in another sample tested by PAGE (data not shown). All 30 rotavirus-positive samples were genotyped by RT-PCR, with G2P[4] genotype being identified in 27 of 30 samples (90%) of strains followed by G1P[8] (2/30, 6.67%) and G9P[8] in one case (1/30, 3.33%). Of a total of 30 rotavirus-positive samples, 16 strains were collected from children after introduction of Rotarix™ in Brazil (GlaxoSmithKline Biologicals, Rixensart, Belgium) and 14 from 2008. Of the 14 positive strains from 2008, 4 (28.6%) children received rotavirus vaccine, 2 (14.3%) did not receive rotavirus vaccine, and for 8 (57.1%) information was not available with respect to vaccination against rotavirus. There was no significant difference (P = 0.6624) when rates of G2 strains were compared between vaccinated and non-vaccinated infants.

No other enteropathogen such as astrovirus, calicivirus, adenovirus, bacteria, and parasites were detected in positive rotavirus strains from Parauapebas, suggesting that rotavirus was the sole etiological agent of diarrhea.

Age Distribution of Rotavirus Infection
Rotaviruses were detected throughout the study period from March to September 2006 and March to November 2008 in children aged less than 5 years in 30/241 (12.45%) [<3 years (13/144, 9.9%) and 4–5 years (3/24, 14.3%) in 2006 and <3 years (13/47, 27.6%) and 4–5 years (1/9, 11.1%) in 2008] as shown in Figure 2.

Comparison of Amino Acid Sequence of VP7 Gene From Rotavirus G2, G1, and G9 Genotypes
The nucleotide sequencing included 18 selected rotavirus strains, 15 G2 (5 from 2006 and 10 from 2008), 2 G1 (1 from 2006 and other from 2008), and 1 G9 (2006). The analysis of the VP7 gene, genotype G2 allowed classification of these strains into lineage II using the Page and Steele [2004] classification (Fig. 3). Other G2 genotypes previously reported and recovered from diarrheic and non-diarrheic neonates and children from Belem were also included in this analysis for comparing the data [Mascarenhas et al., 2007].

Four strains from Parauapebas clustered into sublineage II-a, and 11 grouped into a possible new sublineage II-c. The two new sublineages (II-c and II-d) are being proposed based on a sequence variation of ≥3.5%, as analyzed by Page and Steele [2004] and supported by bootstrap values of 77% and 75%, respectively.

In relation to the G1 genotype the analysis was made based on Jin et al. [1996]. Two samples were detected in this study and assigned to lineage I with bootstrap value

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Fig. 2. Positivity observed to rotavirus strains in accordance to age in years.
of 82% (data not shown). The samples from the G9 genotype were compared with worldwide samples including strains circulating in Brazil and Belém as defined by Santos et al. [2005] and they clustered into lineage III with a 99% bootstrap value (data not shown).

Alignment of VP7 amino acid sequences from G2 strains showed some significant amino acid changes in antigenic regions (Fig. 4). The rotavirus-specific glycosylation site at aa positions 69–71 (N–S–T), was mostly conserved in all G2 samples analyzed. The glycosylation site specific to serotype G2 at aa 146–148 (N-T-S) was conserved in all serotype G2 isolated in Parauapebas. Similarly, the second rotavirus-specific glycosylation site at aa 238–240 (N–I–S) was also conserved in all G2 strains from Parauapebas.

Analysis of the aa sequences of the antigenic regions A, B, C, and F revealed different amino acid substitution patterns among G2 strains isolated in Parauapebas to each lineage or sublineage, as described by Page and Steele [2004]. As shown in Figure 4 this pattern is evidenced on positions 87, 96, 213, and 242.

This analysis was expanded to other regions of aa sequence of VP7 protein, which is been observed as other aa residues characteristics of each lineage: (a) to lineage I was observed the residues in the positions 75-P, 113-I, 125-N, and 129-V; (b) the lineage II, 75-S and 178-N were typical for these strains; (c) the lineage III presented specific aa residues on the positions 35-Y, 40-V/I, 42-V, 55-I, and 75-Y/N/I.

Serotype G2 strains isolated in Parauapebas during 2006 that were classified as sublineage II-a displayed an
Amino acid change at residue 242 that not was observed in the strains from sublineage II-c, recovered in 2008. Interestingly, we observed leucine, at residue 37, only in samples of Paraupebas classified as II-a. In addition, lysine at position 49 alteration was observed in two another human analyzed sequences (Matlab6-04 and PAK468) and in porcine strains.

Two aa differences were observed for Paraupebas strains if compared to prototype DS-1 with lineages II and III, as shown in aa 96 (D→N), aa 213 (N→D) as shown in Figure 5.


Twelve P[4] strains were analyzed to VP8* and were classified in accordance with Arista et al. [2005]. All strains fell into lineage III with bootstrap value of 99% (Fig. 5). The samples of this lineage seem to be divided into two subgroups, named III-a (nine samples; six from 2008 and three from 2006) and III-b (three samples; 2008). In general, these samples were well conserved showing medium values of similarity of 99.6% and 98.9%, respectively. These strains show divergence of...
2.7%. With regard to the lineage I and II prototypes, these diverge in more than 5%. With regard to the amino acid sequences, the samples of Parauapebas of the subgroup III-a differ to that from the subgroup III-b, as they present an 130-I. Furthermore, samples from Parauapebas collected during 2008 and clustered into subgroup III-a (except SAL-2084-D) have shown a 147-G. Unlike these latter samples from year 2008 in the subgroup III-b did not display change in the 147-G. Interestingly, the samples SAL-3273-F and SAL-3282-E share three amino acids with the prototype Wa G1P[8] (114-P, 160-D, and 189-S) which are not observed in any other P[4] sample analyzed (data not shown). With regard to the P[8] genotype the Parauapebas strains clustered into lineage VI, with a bootstrap value of 100% (data not shown).

**DISCUSSION**

In the present study, the rotavirus positivity in children was of 12.45% observing a good correlation between immunochromatography and PAGE (both techniques agreed in 93.7%) showing the importance of using the former procedure in studies carried out in the field and at hospitals.

Some reports have shown the predominance of specific G-type in hospitalized children. Santos et al. [2005] reported gastroenteritis by rotavirus in 208 of 648 (32%) from hospitalized children under 5 years of age during a 3-year period (1999, 2000, and 2002) in the city of Salvador, Brazil. The serotype G9 was associated with 164 of 208 (78.8%) rotavirus-positive samples followed by G1 (12.0%) and G4 (1.4%). In Belém, Gusmão et al. [1999] determined G serotypes in 55 (91.7%) of samples. Of these, the G2 type 2 was present in 83.6% (46/55) of samples and serotypes G1 and (mixed) G1 and G4 in 14.5% and 1.8%, respectively, recorded in cases of nosocomial diarrhea, community-acquired diarrhea, and controls. In the present report it was evident that G2P[4] rotaviruses were largely predominant and noticeably displayed identical electrophoretic patterns. Furthermore, VP7 genes of G2 rotaviruses detected from children denoted extremely high sequence identities, therefore clustering into a single branch into the dendrogram.

G2 genotype has been recorded recently in a study conducted by Rahman et al. [2007] in Bangladesh involving hospitalized children aged less than 5 years. This serotype accounted for 43.2% of gastroenteritis cases from 2005 to 2006 and 15.4% of cases from 2001 to 2005. In Brazil, in a recent cross-sectional survey of children with diarrhea attending emergency services in Aracaju, Brazil, between October 2006 and April 2008, of the rotaviruses detected, 95% (56/59) were P[4]G2 genotype [Gurgel et al., 2009]. In the present study, P[4]G2 genotype was detected in 87.5% (14/16) in 2006

In Belém, a recent review of 12 studies conducted over 26 years and gathering 1,240 rotavirus strains showed that 958 (77.3%) were successfully typed. Among these, 308 (32.1%) were G2. G2 type rates varied from 14.8% to 26.3% during 1981–1992. G2 became predominant (76.7%) in the 1992–1994 period and accounted for a high proportion (60.7%) of infections among hospitalized neonates during 1996–1998. There was an abrupt decrease in prevalence rates (2.3%) of G2 during the following 5 years and it re-emerged (91–95.9%) in early 2006, prior to the introduction of rotavirus vaccine. G2 serotype displayed a cyclic pattern of occurrence over time and recently re-emerged as the most prevalent genotype. This suggests that current predominance of G2 in parts of Northern Brazil and elsewhere in the country likely reflects a fluctuation due to natural variation over time [Oliveira et al., 2008].

Although of a different genogroup of the vaccine Rotarix™, currently used throughout Brazil and other countries, several studies have clearly shown this vaccine to confer heterotypic protection against this and other non-G1 serotypes [Vesikari et al., 2004; Salinas et al., 2005; Ruiz-Palacios et al., 2006]. It is worth, continuing, however, monitoring of circulating rotavirus strains following introduction of rotavirus vaccine, as stressed by WHO [2007].

During surveillance for rotavirus infection [Mascarenhas et al., 2006], conducted from May 1996 to May 1998 in neonatal care unit wards at a public hospital in Belém, Brazil, three neonatal strains (NB-301, NB-162, and NB-308) clustered into other new sublineage, named II-d. In addition, the sample HST-369, collected in 1999 from diarrheic children with diarrhea during a 2-year surveillance study in Belém, Brazil [Mascarenhas et al., 2007], clustered into lineage III. In contrast, the samples analyzed in the present study were classified into sublineages II-a (year 2006) and II-c (year 2008, except SAL-1920-C).

Of note, children from 2006 did not receive rotavirus vaccine because they were not age-eligible for vaccination (6 months to 4 years), when Rotarix™ was introduced in the Brazilian National Immunisation Program. The differences observed between samples from 2006 and 2008 would in principle raise a hypothesis of a possible vaccine-induced mutation. Nevertheless, we did not observe significant differences in amino acid composition between vaccinated and non-vaccinated children (SAL-3222-D, SAL-3273-F, and SAL-3284-F). The existence of different lineages and the re-emergence of G2 genotype made the surveillance for this genotype needed to detect possible changes with regard to circulating or regional differences in strains as well as differentiation in amino acid pattern observed among strains collected in different periods and localities. The current predominance of G2P[4] detected in the present study is likely to reflect the cyclic pattern of occurrence of this serotype in our region and elsewhere in the Latin America.

It is worth mentioning that Iturriza-Gómez et al. [2001] showed that residue 96-N at antigenic region A is associated with mutants escape strains. In our study, we noted that samples from 2006 (non-vaccinated infants) and 2008 (vaccinated and non-vaccinated children) had 96-N residue, whereas samples recovered from 1997 to 2000 (NB-162, NB-301, NB-308, and HST369) did not present such a substitution (Fig. 4). The potential importance of this substitution warrants further studies.

This statement reinforces the need for further investigations to assess the cyclic circulation of G2 genotype considering the recent introduction of rotavirus vaccine in Brazil, which is P[8]G1 genotype.

In summary, we detected and characterized rotavirus isolates in Parauapebas, Brazil, and then identified possible two distinct new sublineages for G2 genotype and reinforced the need of surveillance of rotavirus in our region aiming to detect the possible appearance of a mutant escape that eventually may pose a challenge to vaccine strategies, probably reflecting a selective pressure induced by the vaccine.

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