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BRIEF REPORT

Identification of lineage III of G12 rotavirus strains in diarrheic children in the Northern Region of Brazil between 2008 and 2010

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Abstract This study reports on the surveillance for rotavirus genotypes and the identification of G12 human rotavirus in the Northern Region of Brazil. Rotavirus-positive samples were collected from children <5 years of age with acute diarrhea from January 2008 to October 2010. G2P[4] was the most prevalent genotype, accounting for 45.6% (126/303) of cases. Five rotavirus strains bearing G12P[6] genotype specificity were detected. Phylogenetic analysis of the VP7 gene showed that G12 strains clustered into lineage III. This is the first detection of G12 strains from lineage III in Latin America, broadening the current evidence for the worldwide emergence of this genotype.

Keywords Acute gastroenteritis · G12 rotavirus · Genotypes

Worldwide, rotaviruses are recognised as a major cause of acute gastroenteritis in infants and young children, and in the young of many mammalian and avian species. It is estimated that rotavirus disease is responsible for approximately 36% of hospitalizations for diarrhea among children less than five years of age, resulting in 527,000 deaths annually, mostly (85%) in developing countries [1–3]. Efforts toward the development of effective rotavirus vaccines have been made during the past two decades to reduce the global burden of rotavirus diarrhea. In 2009, the World Health Organization (WHO) recommended inclusion of rotavirus vaccination in all national immunization programs [4]. Currently two live, attenuated, oral-administrable rotavirus vaccines, Rotarix^{TM} (Glaxo SmithKline) and RotaTeq^{TM} (Merck & Co., Inc.), are licensed in >100 countries and have been incorporated into the childhood immunization programs of several countries [5–8].

Rotavirus is a member of the family *Reoviridae*, and its genome consists of 11 double-stranded (ds) RNA segments, which encode 12 proteins: 6 structural and 6 non-structural proteins. Rotavirus particles are composed of three concentric protein layers: outer capsid, inner capsid and core [9]. Two viral surface proteins, VP7 and VP4, which make up the outer capsid shell, allow classification of rotavirus in G and P genotypes, respectively. To date, 27 G and 35 P genotypes have been reported by sequence analysis. Recently, a novel classification system has been proposed based on the nucleotide sequences of all rotavirus genes to provide a complete characterization of strains and possibly identify reassortment events [10, 11].

The most common G/P combinations of human rotaviruses are G1P[8], G3P[8], G4P[8], G9P[8] and G2P[4]. Despite the high prevalence of such strains, several studies have described the circulation of unusual and/or novel G and P genotypes, such us G5, G8 and P[9], mainly in African, Asian and American countries [12–17]. The occurrence of mixed infections involving G and P genotypes appears to be more common in developing countries where, in general, a broad genotype diversity can be seen.

G12 is currently recognised as a globally emerging rotavirus genotype that appears to be spreading more rapidly in recent years. G12 rotavirus was first identified in the Philippines in 1987, causing diarrhea in children [18, 19]. Subsequently, G12 rotavirus was detected in the United

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States, in several Asian countries, in Europe and in South America [20–23]. Four lineages of the VP7 gene of G12 strains have been described. Lineage I comprises the prototype L26 with P[4] specificity detected in the Philippines; lineage II clusters G12P[9] strains isolated in Asia and South America; lineage IV comprises prototype RU172 with P[7] genotype-specificity, the only porcine G12 strain; and lineage III consists of G12P[8] and G12P[6] human rotaviruses that are currently circulating and have been able to spread across the world [21, 24–27].

Current rotavirus vaccination programs require a continuous monitoring of circulating rotavirus strains in order to detect the emergence of possible uncommon and novel types, as well as to assess their potential impact on the effectiveness of vaccines. It has been argued by some authors that the increasing use of rotavirus vaccines might have an impact on viral ecology through a potential replacement of circulating genotypes [28]. The oral, live attenuated, monovalent (G1P[8]) rotavirus vaccine Rotarix™ was adopted by the public-health sector in Brazil in March 2006 to target an annual 3,000,000-birth cohort. Since then the Brazilian Ministry of Health (MoH) has implemented a nationwide surveillance network to assess the burden of rotavirus disease, as well as to monitor circulating strains. Taking advantage of this country's surveillance system, we were able to gather data on the occurrence of rotavirus genotypes among diarrhoeic children and, in particular, could characterize the emergence of G12 human rotavirus isolates in the Northern Region of Brazil.

Between January 2008 and October 2010, a total of 787 stool samples were collected from children with acute gastroenteritis through a surveillance program carried out by Instituto Evandro Chagas, a Brazilian MoH's Rotavirus National Reference Center located in Belém, Pará state. These specimens were obtained from five states in the Northern Region of Brazil (Acre [110 samples], Amazonas [325], Amapá [33], Pará [86] and Roraima [233]) and screened routinely for the presence of rotavirus using a commercial ELISA kit (Premier Rotaclone, Meridian Bioscience, Inc). Rotavirus-positive samples were further subjected to dsRNA extraction using guanidinium isothiocyanate-silica, as described previously [29]. Polyacrylamide gel electrophoresis (PAGE) was carried out in Tris-glycine buffer, and the rotavirus genome profile was determined following electrophoresis of extracted dsRNA through vertical 5% acrylamide bisacrylamide gels [30].

In order to determine the rotavirus genotype specificity, stool samples that were positive by both ELISA and PAGE were subjected to RT-PCR using Super-Script TM (Invitrogen, Carlsbad, CA), and the resulting cDNAs were amplified to generate fragments of 1062 and 876 bp, corresponding to portions of the genes encoding the VP7 and VP4 protein, respectively. The primers used in the first amplification were Beg9/End9 and 4con3/4con2 for the G and P type, respectively [31, 32]. G-typing was done using primer RVG9 in combination with primers BT1 (G1), CT2 (G2), ET3 (G3), DT4 (G4) and FT9 (G9) [31]. Characterization of P genotypes was performed using primer 4con3 in combination with type-specific primers 1T-1 (P[8]), 2T-1 (P[4]), 3T-1 (P[6]), and 4T-1 (P[9]) as described elsewhere [32].

The VP7 and VP4 (VP8* portion) genes of G12 strains were partially sequenced using the primers Beg9/End9 and 4con3/4con2 and a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The products were analyzed using an automatic ABI Prism 3130*xl* DNA sequencer (Applied Biosystems). The nucleotide sequences of these genes were aligned and edited using the BioEdit Sequence Alignment Editor (version 7.0.5.2) program and compared with the corresponding gene fragments of rotavirus strains available in GenBank. Phylogenetic analysis was performed with MEGA software version 4.0.1 by the neighbour-joining (NJ) method. For NJ, a distance matrix calculated from the aligned sequences using the Kimura two-parameter formula was used [33]. To determine the reliability of the tree topology, a bootstrap test of 2,000 replicates was performed [34]. The partial nucleotide sequences of the VP7 gene determined in this study have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) and assigned the accession numbers GU250828 (RV98670), GU250829 (RV98660), JF980340 (RV102320), JF980341 (RV105659), and JF980342 (RV108214).

Of the 787 stool specimens screened for rotavirus antigen by ELISA, 318 (40.4%) were positive for rotavirus. PAGE was performed with 687 samples, of which 214 (31.1%) displayed a typical RNA electrophoretic migration pattern. Of these, 119 (55.6%) specimens showed a short profile and 95 (44.4%) showed a long profile. All G2 rotavirus strains exhibited identical short electropherotypes, and G12 strains yielded a long pattern. Among the rotavirus-positive children, 93 (29.3%) received rotavirus vaccine (Rotarix®), 92 (28.9%) were not vaccinated, and for 133 (41.8%), information on rotavirus vaccination was not available. There was no significant difference ($P = 0.1280$) when rates of occurrence of genotypes were compared between vaccinated and unvaccinated infants.

The G genotype could be determined for 303 (95.3%) samples. G2 was the most prevalent type (47.5%, n = 144), followed by G1 (29.4%, n = 89) and G9 (12.5%, n = 38). With regard to the VP4 gene, 290 strains were P-typed (91.2%); the most frequent genotypes were P[4] (47.2%, n = 137), P[8] (38.2%, n = 111) and P[6] (6.2%, n = 18). Using the binomial (G and P genotype) nomenclature system, it could be seen that the G2P[4] was the most

prevalent genotype, responsible for 45.6% (n = 126) of cases, followed by G1P[8] (22.1%, n = 61) and G9P[8] (12.7%, n = 35). Mixed infections, as defined by the identification of more than one G- or P-type specificity in the same sample, were detected in 11.6% (n = 32) of the strains (Fig. 1).

Of a total of fifteen G non-typeable samples, seven showed the full-length VP7 gene amplified in the first round of PCR. They were selected for VP7 gene sequencing, and all of them were recovered from unvaccinated children. Two samples not could be characterized due to their low cDNA concentration, five G12 rotaviruses displayed long RNA patterns, and the VP7 gene partial sequences (924 bp) demonstrated levels of nucleotide and amino acid homologies of 99–100% and 98.3–100%, respectively. G12 isolates grouped into VP7 lineage III, showing a high degree of nucleotide homology (>87.9%) and amino acid homology (>91.4%) when compared with other human G12 rotavirus strains reported worldwide (Fig. 2). Alignment of VP7 amino acid sequences from G12 strains did not show any major amino acid changes in protein coding (data not shown). The nucleotide sequences of the VP4 genes of G12 strains were also determined. The VP4 gene of the five G12 strains belonged to lineage P[6]-Ia, as proposed by Martella et al. [35] (data not shown).

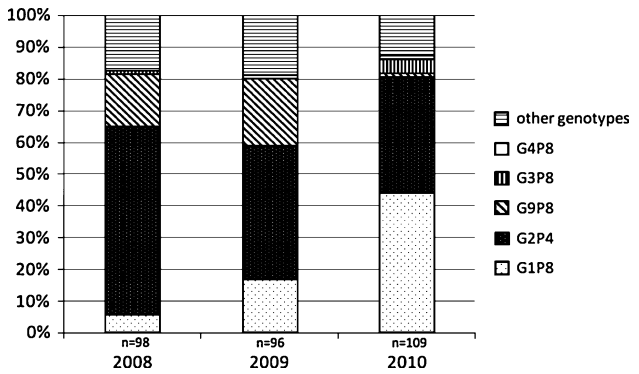

Fig. 1 Distribution of rotavirus G and P genotypes in Northern Region, Brazil, during 2008-2010

In the present study, G2P[4] was found to be the predominant genotype, accounting for 45.6% of isolates, mostly in 2008, when 59% of strains belonged to this genotype. Recently, a systematic review and meta-analysis study reported that the G2P[4] genotype accounted for 85% of circulating serotypes in the post-rotavirus-vaccine era in Latin America [36]. Although some studies in Latin America have suggested that the predominance of G2P[4] genotype in recent years may be associated with vaccine (Rotarix®)-induced selective pressure, such a continental phenomenon could merely reflect a natural fluctuation in co-circulating serotypes over time. The latter hypothesis is supported by the fact that G2 has also been a dominant strain in some Latin American countries where universal rotavirus vaccination has not yet been introduced into the public sector [15, 36–40].

Our study shows a significant increase in the prevalence of G1P[8] strains in 2010 compared to 2009 and 2008. Several studies have demonstrated that continuous circulation of G1 rotavirus strains may be due to their broad genetic diversity, as demonstrated by the occurrence of

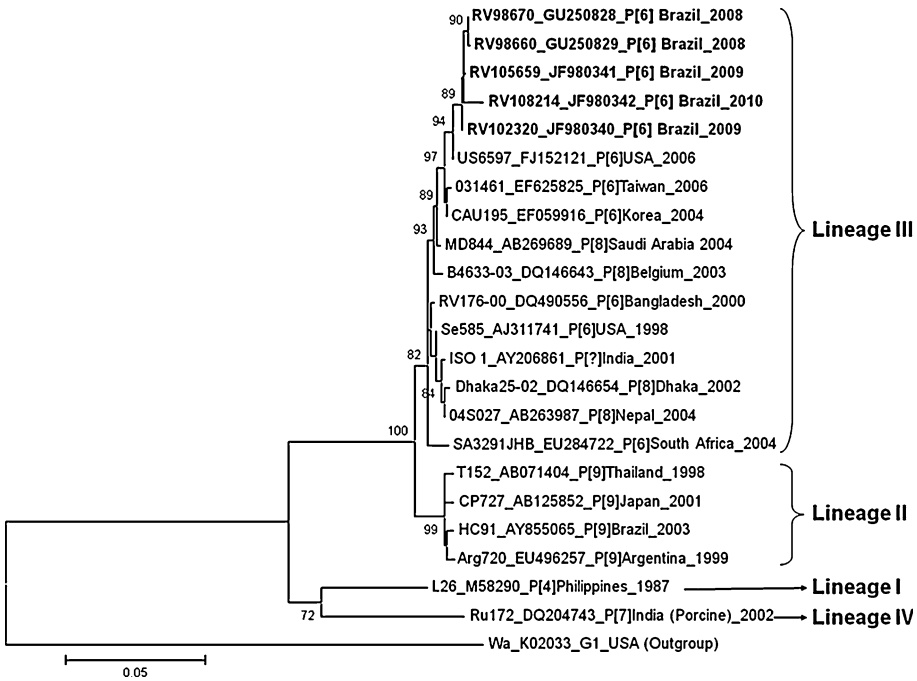

Fig. 2 Dendrogram based on the partial nucleotide sequences of the VP7 gene (924 bp; nt 1-924; aa 1-308). The numbers adjacent to the nodes represent the percent bootstrap support for that cluster. Bootstrap values lower than 70% are not shown. The calibration bar indicates substitutions per nucleotide. G12 strains analyzed in this study are in bold

strains with VP7 genes of different lineages and sublineages in the population [41, 42].

Five strains were typed as G12P[6] by sequence analysis. These samples were recovered from diarrhoeic children aged 16 months to 3 years and with no history of rotavirus vaccination. These G12-positive specimens were collected in Amazonas (3 samples) and Pará (2 samples) states between 2008 and 2010. Initially these samples were found to be G-untypeable by conventional RT-PCR methods, and they were genotyped by nucleotide sequencing of the VP7 fragment. Nucleotide homology and phylogenetic analysis of these samples showed 98.8% homology with US6597 (FJ152121), a strain from the USA that was recently isolated from a child with diarrhea [43].

Here, we describe the spread of G12P[6] rotavirus strains (clustering in lineage III of the VP7 gene) into Latin America. G12 rotavirus with P[4] specificity was first isolated in the Philippines in 1987 from children < 2 years, [18, 19]. In Brazil, the first G12 strain, bearing the P[9] genotype and displaying a long electropherotype, was reported in 2003 as having been recovered from an 11-month-old boy with diarrhea in Paraná State, Southern Brazil [23]. Unlike these findings, the recent isolates were combined with the P[6] genotype and demonstrated a high degree of homology with G12 strains currently circulating elsewhere. These findings are suggestive of a recent introduction of G12P[6] rotaviruses in the Northern Region of Brazil. In addition, the combination of the G12 and P[6] genotypes in the new isolates strongly suggests the possibility of a zoonotic transmission, as described elsewhere [44]. Some studies suggest that G12 strains are emergent worldwide [12, 27, 45]. In a pattern of occurrence similar to that noted for G9, the G12 genotype was found rarely for several years after its first detection, with subsequent increasing prevalence rates during the past few years. It should be considered whether G12 rotaviruses potentially challenge current rotavirus vaccination strategies, since the G12P[6] genotype rotavirus is not included in the composition of the two currently licensed vaccines [7].

The fact that G12 has been found to have been circulating in most parts of the world during the past decade and that it will probably soon become the sixth major human rotavirus genotype warrant the inclusion of G12 primers in routine genotyping procedures. Furthermore, complete genome characterization of G12 rotavirus strains should be encouraged in order to broaden our understanding on their evolution and possible impact on vaccine development. Surveillance studies to monitor prevalent genotypes of rotavirus after vaccine introduction are crucial, mainly to detect the spread of unusual genotypes, such as G9 and G12, as well as to detect the emergence of strains of possible novel genotype composition.

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