

Molecular Characterization of Group A Rotavirus Isolates Obtained from Hospitalized Children in Salvador, Bahia, Brazil

Karina Serravalle¹, Norma Santos², Silvia Inês Sardi¹, Sarah Peregrino Santos Silva¹, Hugo da Costa Ribeiro Junior³, Ângela Peixoto de Mattos³ and Gúbio Soares Campos¹

¹Laboratory of Virology, Institute of Science Health, Federal University of Bahia, Salvador, BA; ²Laboratory of Virology, Federal University of Rio de Janeiro, RJ; ³Metabolic Department Fima Lifshitz, Hosannah de Oliveira Pediatric Hospital; Salvador, BA, Brazil

Rotavirus is a major cause of infectious diarrhea in infants and young children. The objective of this study was to characterize the genotypes of Human Rotavirus found in children hospitalized with acute diarrhea in the Pediatric Hospital Prof. Hosannah de Oliveira of the UFBA in Salvador, Bahia, Brazil, during the years of 1999, 2000 and 2002. Fecal samples were analyzed (n=358) by methods EIARA and SDS-PAGE for detection of Rotavirus. Positive samples of one or two of these methods (n=168) were submitted to RT-PCR and Multiplex-Nested PCR to determine genotypes G and P. A hundred sixty-eight (46.9%) samples were positive and 190 (53.1%) negative. Only 17 (4.7%) samples had divergent results. The distribution of genotypes G during the first year, showed that the genotype G9 was present in 96,8% of the analyzed samples, in the second year, it was responsible for 96% and in the third year, 88,1%. The characterization of genotypes P demonstrated that the genotype P1A[8] was the most outstanding in all years. In this study we discuss the benefit to control the genotypes of Rotavirus through the molecular characterization for the development of potential vaccines.

Key-Words: Rotavirus, PCR, genotype, children.

Rotavirus is the most common cause of gastroenteritis in children under five years of age. Between 400,000 and 600,000 children are estimated to die annually worldwide as a result of this infection [1-3].

In Brazil, the first registered case of rotavirus occurred in 1976, in the city of Belém, state of Pará [4], when the virus was detected by electronic microscopy in the feces of children with diarrhea. Since that time, it has been diagnosed in various Brazilian states, confirming the importance of this virus in the etiology of childhood diarrhea [5-7].

Rotavirus is an icosahedral virus measuring 70 nm in diameter with a double capsid and a viral genome composed of 11 segments of double-stranded RNA. Rotaviruses are classified in seven groups (A-G) based on the antigenic characteristics of the capsid proteins. Group A has a wide antigenic and genomic diversity, with various serotypes and genotypes, mainly due to variations in the VP4 and VP7 proteins of the external capsid. The types specified by the VP7 protein are denominated G serotypes/genotypes (glycoprotein) and those specified by the VP4 protein are denominated P serotypes/genotypes (sensitive to proteolysis) [8].

To this date, 14 G serotypes/genotypes (G1-G14), of which G1, G6, G8, G10 and G12 infect humans, and 21 P genotypes (P1-P21), of which P1A[8], P1B[4], P2A[6], P3[9], P3B[13], P4[10], P5[3] and P8[11] infect humans [8,3,9], have been described.

Based on a binary classification system of the combination of P and G, the majority of genotypes isolated in children with diarrhea caused by rotavirus infection are P1A[8]G1, P1B[4]G2, P1A[8]G3 and P1A[8]G4 [10,11,7]. Recent studies show that the incidence of P1A[8]G9 and P2A[6]G9 genotypes as a cause of severe diarrhea in children all over the world, including Brazil, has been increasing. For this reason, studies on the molecular epidemiology of this virus are important in order to determine the genotypes circulating within a community and to enable the possible implementation of vaccines [12-24].

The objective of this study was, therefore, to characterize the genotypes of Human Rotavirus detected over a period of three years in children admitted to a pediatric hospital with acute diarrhea caused by this virus. Data resulting from this study would contribute towards epidemiological investigation by characterizing the rotavirus genotypes detected in hospitalized children, reflecting the presence of the virus in the community.

Materials and Methods

Samples for Rotavirus Testing

During the years of 1999, 2000 and 2002, fecal samples were collected from children under five years old (n=358) with acute diarrhea, in the *Fima Lifshitz* Metabolic Unit of the *Hosannah Oliveira* Pediatric Hospital in Salvador, Bahia, Brazil. The feces were collected in sterile containers and kept frozen at -20°C until analysis.

Rotavirus Detection

Two different detection methods were used to verify the presence of the virus in the fecal samples (n=358).

Enzyme Immunoassay (EIARA – Combined Enzyme Immunoassay for Rotavirus and Adenovirus). The presence of the virus in the fecal samples was detected using a double-

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Address for correspondence: Dr. Gúbio Soares Campos. Laboratório de Virologia. Instituto de Ciências da Saúde, Universidade Federal da Bahia Av. Reitor Miguel Calmon s/n, Vale do Canela. Zip code: 40110-100. Salvador, Bahia, Brazil. E-mail: gubio@ufba.br. Telephone/Fax: (55-71) 3235-0937.

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sandwich, enzyme-linked immunosorbent assay (ELISA), (Fundação Oswaldo Cruz, Ministry of Health, Brazil), according to the instructions provided by the manufacturer [25].

Polyacrylamide Gel Electrophoresis (SDS-PAGE): Electropherotypes. The fecal samples were submitted to RNA extraction using Trizol LS reagent (*Life Technologies Inc., Grand Island, NY*) followed by precipitation in ethanol. The precipitated viral RNA was re-suspended in denaturing Laemmli buffer [26] and submitted to vertical electrophoresis in SDS-PAGE 7.5%. The presence of viral RNA in the fecal sample was confirmed following staining with a 7 mM silver nitrate solution [27].

Cell Culture: Viral Isolation

MA-104 cell culture maintained in Eagle's minimum essential medium (GIBCO-BRL), supplemented with 10% fetal bovine serum (GIBCO-BRL) and antibiotic-antimycotic solution (GIBCO-BRL) was used in this study. When direct genotyping was unsuccessful, positive fecal samples (n=60) were centrifuged at 10,000 g and the supernatant used to inoculate the MA-104 cell monolayers, carrying out up to three consecutive passages following 72 hours of culture. The infected cell cultures were then used for the extraction of RNA as described later.

Multiplex RT-PCR Assays: G and P Genotyping

The samples that were classified as positive by ELISA or SDS-PAGE (n=168) were used to determine G and P genotypes. These samples were submitted to the Polymerase Chain Reaction (PCR) technique with reverse transcriptase (RT) for the genes that codify VP4 and VP7, and later to a Multiplex-Nested PCR combining specific primers to amplify the G1-G10 or the P1-P4 genotypes [28].

In summary, a 10 µL aliquot of the viral RNA extracted using the Trizol method (*Life Technologies Inc., Grand Island, NY*) from the fecal samples (n=108) or from the infected cell culture (n=60), was denatured at 97°C for 5 minutes and then added to a tube containing the Beg9 and End9 primers (1µM) for VP7 or Con3 and Con2 (1µM) for VP4, with a Master Mix solution, containing Taq DNA polymerase (0.5 U), dATP, dGTP, dCTP, dTTP (200 µM) and MgCl₂ (1.5mM) (*Promega Corporation, Madison, USA*) and 5 units of the reverse transcriptase enzyme (*Promega Corporation, Madison, USA*), with a final reaction volume of 50 µL. This final mixture was submitted to 30 cycles at 94°C for 2 minutes, 55°C for 1 minute and 72°C for 5 minutes, followed by a final extension cycle of 72°C for 5 minutes (*GeneAmp PCR System 2400, Perkin Elmer*). This first RT-PCR was the basis of the subsequent genotyping (Multiplex-nested PCR) and determination of the G genotype. The product of RT-PCR (5 µL) was added to a solution containing each one of the primer sets, H2, A, C [29] (1µM), together with the Master Mix solution (composed of Taq DNA Polymerase (5U), dATP, dGTP, dCTP, dTTP (200 µM) and MgCl₂

(1.5mM), (*Promega Corporation, Madison, USA*) to a final volume of 50 µL. The reaction was submitted to 15 cycles at 94°C for one minute, 55°C for 2 minutes, 72°C for 1 minute, followed by 72°C for 7 minutes (extension), (*GeneAmp PCR System 2400, Perkin Elmer*). To determine the P genotype, the procedure was the same, using the P primer set [28,29] (1 µM) submitted to 25 cycles at 94°C for one minute, 55°C for 2 minutes, 72°C for 3 minutes, followed by 72°C for 7 minutes.

The PCR products were submitted to an agarose gel electrophoresis, placed in an ethidium bromide solution (1µg/mL) (*Sigma Chemical Company*) for 10 minutes and photographed using a Polaroid film in an ultraviolet light transilluminator.

Statistical Analysis

Analysis of the results was carried out using the statistical software programs SPSS version 9.0, and Epi-Info, version 6.04.

Results

Detection of Rotavirus by ELISA/SDS-PAGE (Electropherotypes)

Table 1 compares the results of the ELISA and SDS-PAGE techniques. During the years of 1999, 2000 and 2002, the analysis of the 358 samples using the ELISA (EIARA) and SDS-PAGE techniques resulted in 168 positive samples (46.9%) and 190 negative samples (53.1%). Agreement between the results obtained by these two methods was 95.3%, only 17 samples having discordant results: 13 were positive according to EIARA but negative according to the SDS-PAGE technique, while 4 were positive according to the SDS-PAGE technique but negative according to EIARA.

Figure 1 shows the electrophoretic profile of the rotavirus-positive samples, showing that the migration of the 11 segments of RNA followed a 4:2:3:2 pattern that is characteristic of the group A rotavirus. Interestingly, due to duplication of one viral segment, one sample had 12 RNA segments suggesting a possible genetic recombination.

Genetic Analysis of the VP4 and VP7 Genes: RT-PCR Genotyping

The results obtained by RT-PCR are shown in Figure 2. The presence of the 1062-bp consensus fragment corresponds to the amplification of the gene that codifies for VP7 and the 876-bp consensus fragment corresponds to the gene that codifies for the VP4 protein. Of the samples that were positive according to at least one of the methods used (ELISA or SDS-PAGE), (n=168), it was possible to amplify VP7 and VP4 using the RT-PCR technique in 163 samples (97.0%).

The result of Multiplex-nested PCR genotyping for the G (VP7) and P (VP4) genotypes is shown in Tables 2 and 3. Table 2 shows the distribution of G genotypes. In a total of 157 samples, 96.8% (30/31) belonged to the G9 genotype group during the first year of the study, while the G1 genotype was present in only one sample (3.2%). In the second year, G9 was

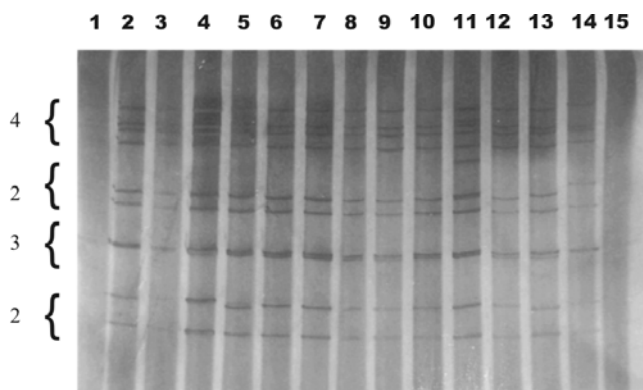
Table 1. Detection of Rotavirus by ELISA and SDS-PAGE in the feces of children hospitalized in the city of Salvador, Bahia, Brazil, 1999, 2000 and 2002

ELISA•	SDS – PAGE*		Total
	Negative	Positive	
Negative	190 (53.1%)	4 (1.1%)	194 (54.2%)
Positive	13 (3.6%)	151 (42.2%)	164 (45.8%)
Total	203 (56.7%)	155 (43.3%)	358 (100.0%)

•Enzyme immunoassay (EIARA – Combined Enzyme Immunoassay for Rotavirus and Adenovirus).

*Polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 1. 7.5% Polyacrylamide gel electrophoresis, (SDS-PAGE), of RNA from Human Rotavirus, extracted from fecal specimens from children during the years of 1999, 2000 and 2002 in Salvador, Bahia Brazil. Column 1: SA-11; 2-13 virus, fecal samples positive for Rotavirus; 14, RNA of Bovine Rotavirus; 15, negative fecal sample. Column 11: Fecal sample with 12 segments of RNA. All samples presented an electrophoretic profile of 4:2:3:2, characteristic of viral RNA of the group A Rotavirus.



present in 96.0% (24/25) of the samples analyzed and genotype G4 in 4.0% (1/25). In the third year, G9 was found in 88.1% (89/101) of the samples, genotype G1 in 10.9% (11 samples), and genotype G4 in 1% (1 sample). Although not statistically significant ($p=0.097$), the incidence of the G9 genotype would appear to be declining over the years.

Table 3 shows that the P genotypes were found in 144 (85.7%) samples. Analysis of the distribution frequency of the genotypes in the two first years shows that the P1A[8] genotype was present in 100% of samples. In the studies carried out in the third year, this same genotype was present in 98.9% (89/90) of samples, while genotype P1B[4] was present in only 1.1% (1/90) of samples.

Figure 3 shows the amplified products of the P1A[8], G1 and G9 genotypes.

Discussion

Studies carried out in Brazil in different geographical regions of the country indicate that the frequency of diarrhea associated with rotavirus in children under the age of 5 admitted to hospital or receiving care in clinics ranges from 12%-42% [29,30], similar to those found in our study in which the presence of rotavirus was confirmed in 46.9% of the samples analyzed. The genetic characteristics of the virus provide characterization of the molecular aspects of the infection, thereby providing new data on the evaluation of the predominant genotypes of the isolated virus. In this study, rotavirus genotyping, using RT-PCR and Multiplex-nested PCR revealed the presence of genotypes G and P of the virus present in the feces of infected children. Of the G genotypes in this study, genotype G9 was identified as being predominant (85.2%), followed by G1 and G4. Other recent studies in Brazil have also shown emergence of genotype G9 [31,32]. The G9 genotype was considered an uncommon genotype, reported for the first time in 1983/1984 in a pediatric hospital in Philadelphia, USA, with a frequency of only 9.2%, whereas later studies (1995-1996) showed a much higher incidence (56%) [33]. Following this finding, several countries were alerted to the presence of G9 and current percentages range

Table 2. Distribution of the G genotypes of Human Rotavirus detected in the fecal samples of children hospitalized in the city of Salvador, Bahia, Brazil, 1999, 2000 and 2002

Year	Viral detection	G1	Genotypes G4	G9	Samples that did not undergo genotyping	Total
1999	31	1 (3.2)*	-	30** (96.8)	2 (6.1)	33
2000	25	-	1 (4.0)	24 (96.0)	1 (3.8)	26
2002	101	11 (10.9)	1 (1.0)	89 (88.1)	8 (7.3)	109
Total	157 (93.5)	12 (7.1)	2 (1.2)	143 (85.2)	11 (6.5)	168

*Numbers in parentheses, percent.

**The incidence pattern of G1 and G9 was similar during 1999, 2000 and 2002 ($p=0.29$).

Figure 2. Products of amplification of the VP7 and VP4 Rotavirus genes by RT-PCR. Line 1: 876-bp consensus fragment for VP4; 2 – 1062-bp consensus fragment for VP7; 3- Negative control; M – Marker of molecular weight of 50 bp (Promega). DNA molecular marker 50 bp.

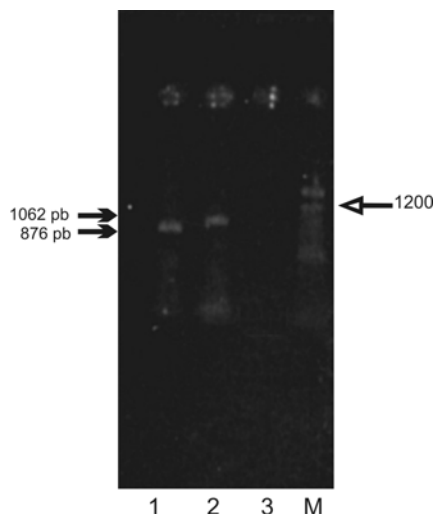


Figure 3. Electrophoretic profile of the amplification products of the genes (VP7/VP4) of Rotavirus by Multiplex-Nested RT-PCR in fecal samples from children hospitalized during the years of 1999, 2000 and 2002 in Salvador, Bahia, Brazil. Lines 1-2: Genotype G3 (582 bp); 3: Genotype G1 (749 bp); 4: Genotype P1A[8] (345 bp); 5: Genotype G9 (306 bp); 6: Negative control; M: Marker of molecular weight of 50 bp. DNA molecular marker 50 bp.

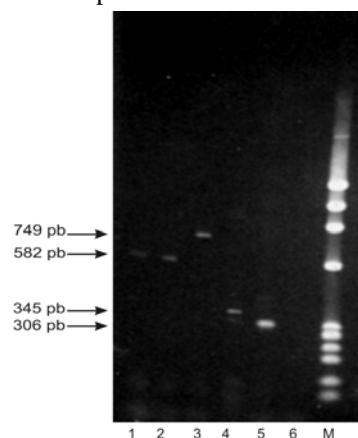


Table 3. Distribution of the frequency of the P genotypes of Human Rotavirus in fecal samples of children hospitalized in the city of Salvador, Bahia, Brazil, 1999, 2000 and 2002

Year	Viral detection	Genotypes		Samples that did not undergo genotyping	Total
		P1A[8]	P1B[4]		
1999	30	30 (100.0)*	-	3 (9.1)	33
2000	24	24 (100.0)	-	2 (7.7)	26
2002	90	89 (98.9)	1 (1.1)	19 (17.4)	109
Total	144 (85.7)	143 (85.1)	1 (0.6)	24 (14.3)	168

*Numbers in parentheses, percent.

from 0.4% to 78.4% [14,34,35,23,15]. The existence of these uncommon genotypes raises the question of whether they really represent emerging infections or if they were simply not diagnosed appropriately until now [34,36,37,16,38,18,39,23,24].

With respect to the characterization of P genotypes, the P1A[8] and P1B[4] genotypes were the most relevant, and these data are in agreement with results from other studies in which the predominant genotypes found in humans were P1A[8] and P1B[4] followed by P2A[6] [40,32].

In our study, it was not possible to characterize the genotypes in some samples. The presence of enzyme inhibitors or variations in the succession of nucleotides in the hybridization area of the primers may be responsible for inadequate amplification. Today, the majority of studies on the molecular characterization of rotavirus use, as in our case, more than one group of primers able to identify genotypes of human or animal origin, such as G9 of bovine origin [31]. Nevertheless, the lack of characterization of some samples may suggest the presence of new genotypes.

The results of this study show the molecular characterization of the rotavirus over a period of three years

in hospitalized children with acute diarrhea, emphasizing the presence of the P1A[8] and G9 genotypes as being predominant in this area. The occurrence of uncommon P and/or G genotypes, reinforces the need for constant surveillance of the molecular epidemiology of rotavirus with a view to developing a possible vaccine, and to initiate new discussions with respect to whether the clinical manifestations may be associated with certain genotypes.

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