Detection and Characterization of Rotavirus G and P Types from Children Participating in a Rotavirus Vaccine Trial in Belém, Brazil

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This study sought the characterization of rotaviruses in a trial with a tetravalent rhesus-human rotavirus vaccine in Belém, Brazil in children who received three doses of vaccine or placebo in the 1st, 3rd and 5th months of life. Rotavirus electropherotypes, subgroups, G serotypes, G, [P] and [P],G genotypes were determined in 93.3%, 95.9%, 93.3%, 73.3%, 95.5% and 92.2% of isolates, respectively. Serotypes G1, G2 and G4 were detected in 58.9%, 30% and 4.4% of the cases, respectively. Rotavirus genotype G5 was detected for the first time in Northern region in 4.4% of the infections. Rotavirus genotypes P[8], P[4], P[6] and P[8+6] were detected in 54.5%, 26.7%, 12.2%, and 2.2% of the cases, respectively. The predominant genotypes were P[8],G1 and P[4],G2 with 53% and 26.6% of the infections, respectively. Unusual strains accounted for 20.5% including P[4],G1, P[6],G1, P[6],G4, P[6],G5, P[8],G2, P[8],G5. Mixed infections involving P[8+6],G2 and P[8+6],G1 were also noted. The neonatal P[6] strains associated with diarrhea were detected among children aged 9-24 months. To our knowledge, this study represents the first in Brazil to analyse, on molecular basis, rotavirus genotypes from children participating in a rotavirus vaccine trial. These results are of potential importance regarding future rotavirus vaccination strategies in Brazil.

Key words: rotavirus - vaccine - genotype G - genotype P - Belém - Brazil

Rotaviruses A constitute the most important cause of severe gastroenteritis among infants and young children in developing and developed countries, accounting for about 680,000 deaths per year in developing countries (Kapikian & Chanock 1996, Miller & McCann 2000). Because of the high mortality-rate associated with rotavirus diarrhoea, particularly in the developing countries, the availability of an effective vaccine is a goal to be pursued.

The rotavirus belongs the *Reoviridae* family, genus *Rotavirus.* The complete viral particle is constituted by a triple-layered shell protein and the genome that consists of 11 segments of double-stranded RNA (dsRNA) each one coding a protein (Kapikian & Chanock 1996). The proteins VP4 and VP7 induce type-specific neutralising antibodies and are involved with the immunity protection. G serotype is associated with VP7 protein whereas P serotype refers to VP4 protein (Kapikian & Chanock 1996).

Fourteen G serotypes/genotypes, of which 10 (G1-G6, G8-G10 and G12) were described infecting humans (Parashar et al. 1998), and 20 P genotypes, as specified by the 4th dsRNA segment; 4 of these were described infecting humans, as follows: P[4], P[6], P[8], and P[9] (Estes 1996). The serotypes G1-G4, are the most common cause of disease worldwide (Kapikian & Chanock 1996, Gentsch et al. 1996). In developing countries other G serotypes/genotypes are important such as G5, G6, G8, G9, and G10 (Gentsch et al. 1996, Santos et al. 1998).

On the basis of the current binary proposed system for rotavirus characterization, the majority of isolates from diarrhoeic children fall into four groups: P[8],G1, P[4],G2, P[8],G3, and P[8],G4 (Bishop et al. 1991, Woods et al. 1992, Beards et al. 1995). Of these, P[8],G1 and P[4],G2 rotavirus strains are the most prevalent worldwide (Rasool et al. 1993, Das et al. 1994, Santos et al. 1994, Gentsch et al. 1996).

The present report documents the diversity of rotavirus G and P types from children participating in trial using the tetravalent rhesus-human reassortant rotavirus vaccine (RRV-TV) in Belém, Brazil.

MATERIALS AND METHODS

Patients and methods - This study was aproved by the Regional Council of Medicine of the State of Pará, the Secretary of Public Health of the State of Pará, and the Ministry of Health of Brazil, and by the Ethical Commitee of Instituto Evandro Chagas (Belém) and Ethical Review Commitee of the World Health Organization.

The study was a 2-year, prospective, double-blind, placebo-controlled, randomized trial in which infants received three doses of RRV-TV or placebo in the 1st, 3rd and 5th months of life (Linhares et al. 1996). In the present study we are considering 90 rotavirus positive cases diagnosed by enzyme immunoassay (DAKO[TM], Copenhagen, Denmark), involving 83 children with gastroenteritis wich received three doses of RRV-TV vaccine (270 children) or placebo (270 children).

G serotyping and subgrouping by enzyme immunoassay - Subgrouping and G-serotyping of rotavirus-positive samples were performed essentially as described by Taniguchi et al. (1987), by using monoclonal antibodies against each of the subgroups I and II and human G1, G2, G3, and G4 serotypes, which were kindly provided by Dr Shozo Urasawa, Department of Hygiene and Epidemiology, Sapporo Medical College, Sapporo, Japan.

Polyacrylamide gel electrophoresis - The double stranded RNA was extracted from 400 µl of positive suspensions follow the metodology described by Boom et al. (1990). The electrophoresis was performed using a 5% polyacrylamide slab gels (PAGE) and the gels were then stained with silver nitrate as previously described by Pereira et al. (1983).

Reverse transcription-polymerase chain reaction for G and P rotavirus genotyping - The reverse-transcription followed by polymerase chain reaction (RT-PCR) for G and P rotavirus genotyping was performed in two steps, as described by Gouvea et al. (1990, 1994) and Gentsch et al. (1992), respectively, with modifications introduced by Leite et al. (1996). In the first amplification, a mixture of consensual primers 9con1/9con2 or Beg/End9 (G genotype) and 4con2/4con3 (P genotype) was used. To assess both G and P broad reactivity the mixture was cycled in a termocycler (Perkin Elmer, GeneAmp PCR System 9600), being submitted to 30 cycles of amplification. The amplicons were then eletrophoresed on 1% agarose gel in Tris-Borate-EDTA buffer (TBE) in Wide Mini-Sub Cell GT (Bio-Rad Laboratories, Hercules, CA, USA). The gel was stained with ethidium bromide (0.5 µg/ml) and cDNAs visualized and photographed using a system Gel Doc 1000 (Bio-Rad, Laboratories, Hercules, CA, USA).

The nested-PCR was carried out using a mixture of G (G1-G5 and G9 and rotavirus animal primers Beg 9, d end 9, CRW8, end 9-6) (Gouvea et al. 1990, 1994), and P (P[4], P[6], P[8], and P[9]) (Gentsch et al. 1992) specific genotyping oligonucleotide primers by 30 cycles of amplification. All amplicons were subsequently subjected to electrophoresis on 1% agarose in TBE buffer containing ethidium bromide (0.5 µg/ml).

Southern hybridization and chemiluminescent detection - Southern hybridization with oligonucleotide probes was carried out to confirm the P (Ramachandran et al. 1996) and G (Leite et al. 1996) genotype-specificities according to the method described by Ando et al. (1995), with modifications introduced by Leite et al. (1996). Dig-probes were used to discriminate between porcine G5 OSU-like strains from G5 human strains isolated from Brazilian infants, as described by Alfieri et al. (1996).

RESULTS

The PAGE showed a clear profile in 84 (93.3%) out of 90 positive samples. The predominant electropherotype was long, accounting for 38 (70.4%) and 24 (66.7%) of tested samples in the placebo and vaccine groups (Table I).

The sub-group specificity was carried out in 70 out of 73 (95.9%) positive samples, of which, sub-group II was the most prevalent, followed by sub-group I in both groups (Table I).

Serotyping was carried out on 84 (93.3%) out of 90 rotavirus positive specimens. G1, G2 and G4 were detected and the most prevalent serotype was G1 in both placebo (58.2%) and vaccine (60%) groups (Table I). The usual correlation between subgroups and serotypes was noted in 80% of positive samples.

Nested-PCR was carried out in 90 samples to characterize the G rotavirus genotypes. Using 9con1/9con2 specific primers it was possible to characterize rotavirus G genotype in 66 (73.3%) of isolates. From 66 samples with defined G genotype, 43 (47.8%) were genotype G1, 18 (20%) G2, 1 (1.1%) G4 and 4 (4.4%) G5.

From the 24 remaining samples without G genotype, 19 with available specimens were tested using rotavirus animal primers Beg 9, d end 9, CRW8, end 9-6, obtained from rotavirus of animal origin, being all negatives.

From 86 (95.5%) samples with defined P genotypes, 24 (26.7%) were classified as genotype P[4], 11 (12.2%) P[6], and 49 (54.5%) P[8]. Two samples (2.2%) showed mixed

TABLE I
Characterization of electropherotypes, subgroups, G serotypes and P[],G genotypes of rotavirus A from a vaccine trial, Belém, PA, Brazil

	Children group				Total	
	Placebo	(%)	Vaccine	(%)	(%)	
Electropherotype						
Long	38	(70.4)	24	(66.7)	62	(68.9)
Short	14	(25.9)	8	(22.2)	22	(24.4)
Not determined	2	(3.7)	4	(11.1)	6	(6.7)
Total	54		36		90	
Sub-group						
I	10	(21.3)	6	(23.1)	16	(22)
II	33	(70.3)	16	(61.5)	49	(67.1)
I / II[a]	2	(4.2)	3	(11.5)	5	(6.8)
Not subgrouped	2	(4.2)	1	(3.9)	3	(4.1)
Total	47		26		73[b]	
G Serotype						
G1	32	(58.2)	21	(60)	53	(58.9)
G2	16	(29.1)	11	(31.4)	27	(30)
G4	3	(5.4)	1	(2.9)	4	(4.4)
Not serotyped	4	(7.3)	2	(5.7)	6	(6.7)
Total	55		35		90	
G Genotype						
G1	26	(47.3)	17	(48.6)	43	(47.8)
G2	11	(20)	7	(20.0)	18	(20)
4	1	(1.8)	-	-	1	(1.1)
G5	3	(5.4)	1	(2.8)	4	(4.4)
Not genotyped	14	(25.4)	10	(28.6)	24	(26.7)
Total	55		35		90	
P Genotype						
P[4]	16	(29.6)	8	(22.2)	24	(26.7)
P[6]	8	(14.8)	3	(8.3)	11	(12.2)
P[8]	28	(51.8)	21	(58.4)	49	(54.5)
P[8+6]	1	(1.9)	1	(2.8)	2	(2.2)
Not genotyped	1	(1.9)	3	(8.3)	4	(4.4)
Total	54		36		90	

a: reactivity with both subgroups (I and II); *b*: for sub-group were tested only 73 samples.

infections P[8+6] (Table I). The P[6] genotype was observed in both placebo and vaccine groups involving diarrhoeic children with ages between 9 and 24 months (data not shown).

The binary characterization [P],G was possible in 83 (92.2%) out of 90 positive samples. The predominant combination was the usual genotypes: P[8],G1 (53%) and P[4],G2 (26.6%). Unusual genotypes or mixed infections were observed in 20.5% of strains, such as: P[6],G1 (4.8%), P[6],G4 (4.8%), P[8],G5 (3.6%), P[4],G1 (2.4%), P[8],G2 (1.2%), P[6],G5 (1.2%), P[8+6],G1 (1.2%) and P[8+6],G2 (1.2%) (Table II).

Southern-hybridization using specific G1, G2, and G5 probes was carried out in 21 amplicons. Eight amplicons with expected size of genotype G1 and four with expected size of genotype G2 were confirmed as genotype G1 and G2 with the specific probes. However, only 4 out of 9 amplicons with expected size for G5 (742bp) were positive by Southern-hybridization (data not shown). Similarly, 11 samples were characterized by Southern-hybridization to

TABLE II

G and P genotyped rotavirus strains isolated from children who received a tetravalent rhesus-human rotavirus vaccine or placebo
in a clinical trial in Belém, PA, Brazil

Genotypes		Children group					
		Placebo	(%)	Vaccine	(%)	Total	(%)
Usual	P[8],G1	25	(49)	19	(59.5)	44	(53)
	P[4],G2	14	(27.4)	8	(25)	22	(26.6)
Unusual	P[6],G1	3	(5.9)	1	(3.1)	4	(4.8)
	P[6],G4	3	(5.9)	1	(3.1)	4	(4.8)
	P[8],G5	2	(3.9)	1	(3.1)	3	(3.6)
	P[4],G1	2	(3.9)	-	-	2	(2.4)
	P[8],G2	-	-	1	(3.1)	1	(1.2)
	P[6],G5	1	(2)	-	-	1	(1.2)
	P[8+6],G1[a]	1	(2)	-	-	1	(1.2)
	P[8+6],G2	-	-	1	(3.1)	1	(1.2)
Total		51		32		83	

a: P[8+6]: P mixed genotypes

P genotypes and, four reacted specifically with the probe P[6], three with probe P[8], two with probe P[4] and two with both probes P[8] and [6] (data not shown).

DISCUSSION

This is the first study in Brazil to determine the G and P rotavirus genotypes circulating among children participating in a vaccine trial carried out in Belém. To our knowledge, characterization of P genotypes has not been performed in previous vaccine trials worldwide (Clark et al. 1995, Lanata et al. 1996, Linhares et al. 1999).

In the present investigation it was observed an usual correlation of subgroup/serotype in 80% of positive cases. Similar rates were reported by Mohamed et al. (1994) in a study involving infants and young children with acute gastroenteritis in Saudi Arabia.

In this study, a high proportion (93.3%) of samples were G-serotyped using monoclonal antibodies. This rate is higher than those observed in previous studies carried out in Belém and Goiânia, Brazil, where only 50% and 61% of samples could be serotyped (Linhares et al. 1988, Cardoso et al. 2000). It is likely that examination shortly after collection has accounted for the higher rates of G-serotyping as compared to G- genotyping. This seems to be an unusual result since almost all published studies (including those from the Brazilian groups) found that RT-PCR typing greatly improves rotavirus G typing. Using primers Beg/End9 to amplify animal rotavirus strains in remaining 19 samples unsuccessfully amplified, we could postulated that those samples had mismatches in the consensual region for primer hybridization.

Among the four more epidemiologically important rotavirus G serotypes worldwide, three were identified in the present study: G1, G2 and G4. In addition, rotavirus genotype G5, not detected previously in the Northern Region of Brazil, occurred in 4.4% of isolates.

Santos et al. (1998), studying 49 rotavirus positive samples from diarrhoeic children from Rio de Janeiro, Brazil, found usual G1 and G3 genotypes in 27% and 12% of infection cases, followed by 61% of uncommon genotypes as G5 (25%), G10 (16%), G8 (5%), and mixed G types (16%). In the present investigation, the serotype G1 was detected in 58.9% of tested strains, whereas no G3 was recorded. In

contrast, rotavirus serotypes G2 and G4 accounted for 30% and 4.4% of isolates, respectively. The results obtained in the present study were similarly to those obtained in the United Kingdon by Beards and Graham (1995), who identified G1, G2, G3, and G4 in 93% of isolates.

Linhares et al. (1988), in a study carried out in Belém from December 1982 to March 1986, showed that serotype G1 was the most prevalent (50%), followed by G2 (30%), G4 (17%) and G3 (3%). In addition, Cardoso et al. (2000), during study conducted in Goiânia, from 1987 to 1994 recorded the following rotavirus serotypes: G1 (32%), G2 (46%), G3 (16%), G4 (2%), and G5 (4%).

This investigation showed relative frequencies of genotypes which differ from those found in another study carried out in Belém, from November 1992 to November 1994, when rotavirus serotype G2 was largely prevalent (80%) over the other serotypes in nosocomial infections (Gusmão et al. 1995).

The rotavirus genotype G5 was identified in 1991 (two isolates) and 1992 (two isolates), being detected at rates of 4.4%, three in the placebo and one in the vaccine group. Three samples were characterized as genotype P[8],G5 and one as genotype P[6],G5. The five remaining samples with expected size for G5, were negative by hybridization. Probably those samples had mismatches in the consensual region at probe hybridization since no amplification could be yielded.

To date, serotype G5 has largely been detected in pigs and, at lower rates, in equines, and also in humans. Gouvea et al. (1994) detected serotype G5 in 38 faeces of the 329 samples (12%) for Brazilian children with diarrhoea. Leite et al. (1996) recorded the genotype G5 circulating in several states of Brazil, suggesting a broad distribution of this unusual genotype in Brazil. Previous multicentric studies in Brazil have shown that rotavirus bearing P[8],G5 type-specificity may account for up to 9% of cases of acute gastroenteritis among infants and young children (Timenetsky et al. 1994, Leite et al. 1996, Cardoso et al. 2000).

Alfieri et al. (1996) by Southern-hybridization, sequencing, and RNA-RNA hybridization analysis, showed that rotavirus genotype G5 circulating in Brazil has homology with human rotavirus genotype P8 (Wa-like) and porcine

rotavirus genotype G5 (OSU-like). These results suggest that these strains have naturally reassorted, involving members of both P8 (Wa) and G5 (OSU) rotavirus genogroups.

With regards to the characterization of P genotypes in the present study, P[8], P[4] and P[6] accounted for 54.5%, 26.7% and 12.2% of isolates, respectively. These results were similar to those observed in South Africa, where genotypes P[8], P[4] and P[6] occurred in 64%, 22% and 8% of cases, respectively (Mphahlele & Steele 1995).

Working with hospitalized children in Belém, Mascarenhas et al. (1999) characterized 86% of isolates, in contrast with the present investigation when genotype P characterization was obtained in 95.5% of isolates.

Neonatal P[6] strains had been previously identified among asymptomatic neonates and has been regarded as avirulent (Bishop et al. 1983, Haffejee 1991). The results of the present study are, however, in contrast with those observations regarding two aspects: (i) P[6] was associated with diarrhoeic cases; and (ii) involved children older than 28 days.

In this study, genotypes P[8],G1 and P[4],G2 occurred in 53% and 26.6% of isolates, respectively. Leite et al. (1996), studying isolates from 9 states and the Federal District of Brazil, described the genotypes P[8],G1, P[4],G2, P[8],G3 and P[8],G4, in 43%, 12%, 6% and 6% of isolates, respectively. Ramachandran et al. (1998), in USA, detected P[8],G1, P[4],G2, and P[8],G3, at rates of 66.4%, 8.3%, and 6.9% of samples, respectively.

In the present investigation it was possible to characterize 79.5% and 20.5% of the samples as usual and unusual genotypes, respectively. Leite et al. (1996), in a countrywide study in Brazil, found genotypes P[8],G5, P[6],G2, P[9],G2 and P[9],G3 in 12% of the situations, and mixed infections in 21% of cases. Ramachandran et al. (1998), analyzing samples in a multicenter investigation in USA identified genotypes P[6],G9, P[8],G9, P[6],G1, P[8],G2 and P[4],G1 in 9.2% of the cases. In India Ramachandran et al. (1996) observed unusual strains in 43%, of tested strains with results similar to our study, if genotypes P[6],G1 and P[6],G4 are considered.

The rotavirus genotyping in Brazil demonstrated, in general, a high rate of mixed infection and samples frequently untypeable. These results shown a complexity of serotypes/genotypes G in children participating in a trial with a candidate rotavirus vaccine.

The rotavirus vaccine (RRV-TV Rotashield®) produced by Wyeth-Ayerst Research and licensed for use in the United States, has been suspended recently, following the occurrence of intussusception among vaccinated children (CDC 1999). A newly developed candidate rotavirus vaccine, P[8],G1, of human origin, has been recently administered to children, resulting in a rate of efficacy of about 90% against rotavirus gastroenteritis (Bernstein et al. 1999). Large-scale field trials are currently under way in developing countries.

These results described above have importance regarding a future rotavirus immunization strategies in Brazil, and underscore the need for a countrywide monitoring a rotavirus G- and P-types before the introduction of a rotavirus vaccine.

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