

Rotavirus genotypes as etiological agents of diarrhoea in general populations of two geographic regions of Brazil

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ABSTRACT

Rotavirus is the main global cause of severe childhood diarrhoea among children. In 2006, Rotarix® (G1P[8]) was introduced into Brazil's National Immunization Program. The vaccine coverage rate was 84.4% in 2009. Evidences of increasing G2P[4] after 2006 opened up the discussion about the vaccine effectiveness to non-G1 strains. The aim of this study was to identify the circulating rotavirus genotypes in two Brazilian regions during 2009. A total of 223 positive samples by immunochromatography and latex agglutination assay from the Northeast (Bahia/Pernambuco States) and Southeast (São Paulo/Rio de Janeiro States) regions were included in the study. The samples were submitted to genotyping by nested-PCR according to VP7(G) and VP4(P) and 175 samples (78.5%) were able to be characterized. Considering the characterization of VP7, the G-types detected were G1, G2, and G4 in the Northeast, and G2, G3, G5, and G9 in the Southeast. Considering the characterization of VP4, the P-types detected were P[4], P[8], and P[6]/P[9] in the Northeast and the Southeast. The most frequent mixed types found were G2P[4]/G2P[NT](81.4%), G2P[6](5.2%), G1P[6](5.2%) in the Northeast, and G2P[4]/G2P[NT](78.8%), G2P[6](8.2%), G9P[8](4.7%) in the Southeast. Among immunized individuals whose age ranged from 0-4 years, the G2P[4]/G2P[NT] genotype was identified in 91.0% of cases, and among non-immunized individuals of the same age, the G2P[4]/G2P[NT] genotype was identified in 85.7% of the cases. In accordance with the high level of vaccine coverage, the data suggest that the circulation of G2P[4] in these regions had a considerable increase after the introduction of Rotarix®.

KEYWORDS: Rotavirus. Epidemiology. Genotype. Vaccine.

INTRODUCTION

Rotavirus infection is among the top six causes of severe diarrhoea in children, having already caused over two million deaths in children under five years of age¹. It is transmitted via the fecal-oral route or through the consumption of contaminated water or food, with a minimum infectious dose of 10 virions².

Rotavirus strains can be classified into 8 groups or species (A-H); of these groups only A, B, C, and H can infect humans. Recently, two newer groups of rotavirus have been proposed (I and J)^{1,3-5}. The molecular characterization of rotavirus is based on the gene that corresponds to VP4(P) and VP7(G) proteins. In Brazil, between the years of 2006 and 2009, the genotype G2 associated with P[4] or undefined VP4 was the most frequent strain. It was present in 49%, 66%, 85%, and 37.5% of the samples analyzed each year, respectively. In contrast, genotypes G1 and G3 associated with P[8] or undefined VP4 have declined in frequency since 2005⁶.

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Received: 27 October 2016

Accepted: 31 March 2017

Rotarix[®] vaccine, which was included in the Brazilian immunization schedule in March 2006, consists of a monovalent preparation of the G1P[8] strain. From 2006 to 2012, the vaccine coverage was 46.5%, 79.8%, 81.2%, 84.4%, 83.0%, 87.1%, and 86.4%, respectively⁷. Although Rotarix[®] has proven to be effective against infections by non-G1P[8] strains, recent studies have shown an increase in frequency and prevalence of the G2P[4] strain in Brazil and in Australia, and of the G9P[4] genotype in Mexico^{6,8-12}. In this context, the aim of the present study is to describe the circulating rotavirus genotypes in two Brazilian regions in 2009.

MATERIALS AND METHODS

The 223 samples used in this study were collected during the year of 2009 in the Southeast region [States of São Paulo (SP - latitude: -23° 32' 51", longitude: -46° 38' 10") and Rio de Janeiro (RJ - latitude: -22° 54' 10", longitude: -43° 12' 27")] and in the Northeast region [States of Bahia (BA - latitude: -12° 58' 16", longitude: -38° 30' 39") and Pernambuco (PE - latitude: -08° 03' 14", longitude: -34° 52' 52")] of Brazil. These samples come from patients of all ages with diarrhoea whose physicians requested rotavirus screening in feces. We do not have information about the hospitalization of these patients, because samples were collected in the outpatient clinic (approved by the Ethics Committee - CEP 0771/11).

Immunochromatographic analyses (Vikia[®] Rota-Adeno, bioMérieux SA, Marcy- l'Étoile, France) were conducted in 223 rotavirus positive stool specimens. Viral RNA (dsRNA) was extracted in the QIA Symphony (QIAGEN, Hilden, Germany) instrument with the QIA Symphony[®] Virus/Bacteria Mini kit (QIAGEN, Hilden, Germany) from the supernatant of a suspension containing 10% of feces in PBS (10 mmol/L of sodium phosphate and 0.15 mol/L of sodium chloride with a pH from 7.2 to 7.4). VP7 and VP4 genotyping were obtained with two PCR reactions for each one of the genes. In the first reaction, BEG/END (VP7) and CON3/CON2 (VP4) primer pools were used, whereas in the second one, pools 9CON1/9T1-1/9T1-2/9T-3P/9T-4/FT5/9T-9B (VP7) and CON3/2T-1/3T-1/1T-1/4T-1 (VP4) were employed. In the first PCR (RT-PCR), the mixture (5 µL dsRNA with 3 µL DMSO) was heated to 93 °C for 3 min, and cooled in ice bath for 5 min. Then, 42 µL of the following mixture were added [14.2 µL H₂O, 16 µL mix dNTP (1.25 mM of each dNTP), 5 µL 10 x reaction buffer (200 mM Tris-HCl/pH-8.4/500 mM KCl), 5 µL MgCl₂ (50 mM), 1 µL primer pool (20 µM of each primer), 0.4 µL Platinum Taq DNA polymerase (Invitrogen, California, USA) and 0.4 µL Reverse Transcriptase (Superscript II

– Invitrogen, California, USA)]. After that, the mixture was submitted to the following conditions: 42 °C/60 min, 35 cycles (94 °C/1 min, 42 °C/2 min, and 72 °C/1 min), and 72 °C/5 min. In the second PCR (semi-nested-PCR), 1 µL of the cDNA formed in the RT-PCR was added to 24 µL of the mixture [10.3 µL H₂O, 8 µL mix dNTP (1.25 mM of each dNTP), 2.5 µL 10 x reaction buffer (200 mM Tris-HCl/pH-8.4/500 mM KCl), 2.5 µL MgCl₂ (50 mM), 0.5 µL primer pool (20 µM of each primer), 0.2 µL Platinum Taq DNA polymerase (Invitrogen, California, USA)]. The mixture was then submitted to the following conditions: 25 cycles (94 °C/1 min, 50 °C/2 min, and 72 °C/1 min), and 72 °C/5 min. For all reactions, both negative (H₂O) and positive (genotyped sample) controls were used. PCR products were analyzed by electrophoresis in 1.2% GelRed[™]- Biotium-stained agarose gel (GelRed[™] - Biotium, California, USA) and genotype identification relied on the size of the DNA sequence obtained, using bands with previously defined molecular weight sizes as a reference [1 Kb plus and 50 pb DNA ladder (Invitrogen, California, USA)]¹³⁻¹⁵.

RESULTS AND DISCUSSION

Of the 223 samples, 48 were not PCR-positive for rotavirus, whereas 175 had the VP7 and/or VP4 genotypes identified. There was no significant difference of age, sex, place of origin and time of year of collection between the 48 samples that did not have the presence of viral RNA confirmed and the other 175 genotyped samples. The 223 stool specimens were collected from patients for rotavirus testing at the laboratory. The samples were kept refrigerated (4-8 °C) for 4-7 days until their inclusion in this study, when they were then frozen at -20 °C.

Rotavirus testing is performed through immunochromatography, which detects the presence of the VP6 protein. Therefore, sample stability is less critical than in genotype analysis, since RNA degrades in temperatures above -20 °C. In 2008, Téllez *et al.*¹⁶ observed that the specificity of the Vikia[®] Rota-Adeno kit was 24.2%, lower than the PCR's. Thus, the absence of RNA in those 48 samples may be related to sample stability differences between the two methods and to the lower specificity of immunochromatography. It is also important to note that 81.3% of the samples were from patients from 0-4 years of age and, in this age group, stool collection is more complicated and results are drawn from a reduced sample volume, making it harder to achieve PCR viral RNA stability. The absence of viral RNA may also be due to the presence of PCR inhibitors in the feces.

Of the 175 genotyped samples, 50.9% belonged to

women, 45% belonged to men, and 4% did not have the gender identified. Regarding the age group, 59.4% (-104/175) were children aged from 0-10 years, and of those 48 (27.4%; n=48/175) were children from 0-2 years (Table 1). The average age observed was of 15.6 years old, while the mean age was six years old. Concerning the Brazilian state of origin, 52.6% (-92/175) were from the Northeast (Pernambuco/Bahia States) and 47.4% (83/175) from the Southeast (São Paulo/Rio de Janeiro States). As for the time of collection, 58.9% (103/175) were collected in the winter, 14.9% (-26/175) in the summer, 13.1% (-23/175) in the spring, 10.9% (-19/175) in the autumn, and 2.3% (- 4/175) in an unidentified season (Figure 1). Rotavirus infection was observed in all age groups, but a higher incidence was found in children aged 0-2 years, confirming the suspicion that this infection is still more frequent in this age group of the population, and highlighting the need of effective vaccination. Infection in adults is mostly associated with the virus being transmitted

to them by infected children. Therefore, effective childhood vaccination may cause an indirect effect of reducing the number of infected adults^{10,12,17}. About the distribution throughout the year, 69.7% of the genotyped samples were concentrated in the colder and drier seasons. In this context, the distribution of cases throughout the year seems to be similar to that of temperate climate countries, where infections happen predominantly in the colder and drier months. The present study suggests, however, that rotavirus infection in these regions of Brazil is in-between the period observed in tropical climate countries where the cases are spread throughout the year, what is observed in temperate climate countries. Focusing on each one of the regions, we noticed that the Northeast region (Pernambuco/Bahia States) typically presenting tropical weather with higher average temperatures and milder temperatures in the winter has a higher concentration of cases in the winter, whereas the Southeast region (São Paulo/Rio de Janeiro States), characterized by lower temperatures in the winter had more evenly spread cases throughout the year. Therefore, one may state that curiously enough the Northeast region demonstrated a profile that is more similar to that of temperate climate countries. In agreement with this fact, Silva *et al.*¹⁸ observed that rotavirus infection in the State of Pernambuco in 2007 and 2008 also happened more frequently from May to September (autumn and winter). As a whole, it is expected that the general sanitary conditions are better in the Southeast region than in the Northeast region.

VP7 genotyping revealed the circulation of G1, G2, G3, G4, G5, and G9, though in 6 cases VP7 genotyping was not possible. The dominant VP7 genotype was G2 (88.0%; -154/175), followed by G1 (4.6%; -8/175) (Table 2). VP4 genotyping showed genotypes P[4], P[6], P[8], and P[9], though the genotyping of 26 samples

Table 1 - Classification of samples with VP7 and/or VP4 genotype identified by age group

Age group	Number of samples
0 - 2 years	48
3 - 4 years	25
5 - 10 years	31
11 - 20 years	12
21 - 30 years	19
31 - 40 years	8
41 - 50 years	7
> 50 years	16
No information	9
TOTAL	175

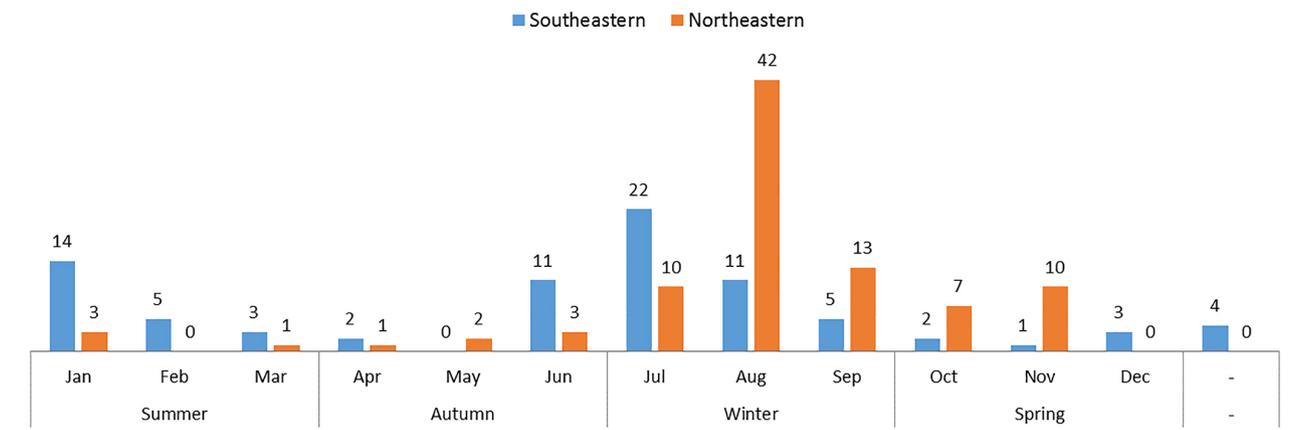


Figure 1 - Classification of samples from the Southeast and Northeast regions of Brazil that were PCR-positive for rotavirus by date of collection

Table 2 - Classification of samples confirmed as rotavirus-positive by VP7 genotype

Genotype	Number of samples
G2	154
G1	8
Unidentified	6
G9	5
G3	1
G4	1
G5	1

was not possible. The dominant VP4 genotype was P[4] (72.0%; n=126/175), followed by P[6] (10.3%; -18/175) (Table 3). Additional data are presented in Tables 2 and 3. Concerning the combinations of VP7 and VP4, 69.1% (-121/175) corresponded to G2P[4], 14.3% (-25/175) to G2 with unidentified VP4, 6.9% (-12/175) to G2P[6], and 13.7% (-24/175) to other strains (G1P[6], G9P[8], P[4] with unidentified VP7, G1P[8], P[8] with unidentified VP7, G1 with unidentified VP4, G1P[4], G3P[9], G4P[6], G5P[8], G9 with unidentified VP4, and P[9] with unidentified VP7) (Table 4). In the Northeast region, the most common rotavirus strains were G2P[4] (72.8%; -67/92), G2 with unidentified VP4 (13.0%; -12/92), and G2P[6] (5.4%; -5/92), whereas in the Southeast, the most frequently found strains were: G2P[4] (65.1%; n=54/83), G2 with unidentified VP4 (15.7%; n=13/83), and G2P[6] (8.4%; n=7/83) (Table 5). As for the presence of different genotypes in the same patient, there were four samples of G2P[4]P[6] (Pernambuco, São Paulo, and Bahia States), one of G1P[4]P[6] (Bahia State), one of G2/G9 with unidentified VP4 (SP), and one of P[8]/P[9] with unidentified VP7 (Bahia State).

Table 3 - Classification of samples confirmed as rotavirus-positive by VP4 genotype

Genotype	Number of samples
P[4]	126
Unidentified	26
P[6]	18
P[8]	9
P[9]	2

A pool of G1-, G2-, G3-, G4-, G5- and G9-specific primers was used for the VP7 genotyping. Of the 175 genotyped samples, six did not have the VP7 type identified. There was gene amplification in one of those samples in the first PCR, but not in the second one, which indicates that

Table 4 - Classification of samples confirmed as rotavirus-positive by VP7-VP4 combination. (* Unidentified P genotype/** Unidentified G genotype)

Genotype	Number of samples
G2P[4]	121
G2P*	25
G2P[6]	12
G1P[6]	5
G9P[8]	4
G**P[4]	4
G1P[8]	2
G**P[8]	2
G1P*	1
G1P[4]	1
G3P[9]	1
G4P[6]	1
G5P[8]	1
G9P*	1
G**P[9]	1

Table 5 - Classification of rotavirus strains by region of origin (Brazil) (* Unidentified P genotype/** Unidentified G genotype)

Genotype	Northeast	Southeast
	Number of samples	Number of samples
G2P[4]	67	54
G2P*	12	13
G2P[6]	5	7
G1P[6]	5	-
G9P[8]	-	4
G**P[4]	1	3
G1P[8]	2	-
G**P[8]	1	1
G1P*	1	-
G1P[4]	1	-
G4P[6]	1	-
G**P[9]	1	-
G3P[9]	-	1
G5P[8]	-	1
G9P*	-	1

the genotype is not among those chosen as the focus of this study, characterizing it as emerging or of low circulation, since this study focuses on the top circulating genotypes in the Brazilian population^{9,19}. The other five cases had no VP7 gene amplification in the first or second PCR, with the presence of the virus being confirmed by VP4 genotyping.

In the studied population, VP7 genotyping revealed the circulation of all investigated genotypes (G1, G2, G3, G4, G5 and G9), with G2 and G1 as the most frequent ones. On a global scale, Santos and Hoshino²⁰ noticed that from 1989 to 2004, the dominant VP7 genotype was G1. In Brazil, Gurgel *et al.*⁹ noticed that before the inclusion of Rotarix[®] in the immunization schedule, from 1986 to 2006, the most frequent VP7 genotype was G1, representing 44% of the total, followed by G9 with 19% and G2 with 17%. Still in Brazil, Leite *et al.*¹⁹ observed that from 1982 to 2005 G1 was the most common VP7 genotype, representing 43% of the cases and G2 representing 9%, whereas from 2006 to 2007 (approximately one year after the implementation of Rotarix[®]) G1 had its frequency reduced to only 3% of the cases and G2 became the most frequent genotype, representing 74% of the cases, followed by G9 with 11%. Focusing on the G2 frequency only, Leite *et al.*¹⁹ observed that from 1982 to 1995 this genotype was identified in 26% of the cases; from 1996 to 2005, this rate dropped to 2%, and from 2006 to 2007, it increased to 74%. One could assume that the VP7 profile observed in this study is similar to the one seen after the implementation of Rotarix[®].

The VP4 genotyping used the same methodology as the VP7 genotyping, with the primer pool of the second PCR corresponding to P[4], P[6], P[8], and P[9] genotypes. Of the 175 genotyped samples, 26 had unidentified VP4 and turned out negative in the first and second PCR rounds, being confirmed as rotavirus-positive by VP7 identification. The VP4 genotyping revealed the circulation of four of the studied genotypes (P[4], P[6], P[8], and P[9]), with P[4] as the most common one (70%). Santos and Hoshino²⁰ noted that from 1989 to 2004 the two most common VP4 genotypes in the world were P[8] and P[4]. Focusing on the period between 1986 and 2006 in Brazil, Gurgel *et al.*⁹ stated that before the introduction of Rotarix[®] in the official immunization schedule, P[8], P[4], and P[6] had frequency rates of 75%, 10% and 6%, respectively. In a different study, Leite *et al.*¹⁹ analyzed the Brazilian population from 1982 to 2007 and named the one-year period from 2006 to 2007 as the post-Rotarix[®] period. From 1982 to 2005, P[8] was present in approximately 70% of the cases and P[4] in 9%. From 2006 to 2007, in contrast, P[4] was identified in approximately 70% of the cases and P[8] in about 15%. Considering the descriptions in the literature, one could say that the prevalent profile observed in this study for the VP4 genotypes resembles that of the post-Rotarix[®] period.

Of the total genotyped samples, 3% and 15% were PCR negative for VP7 and VP4, respectively. This may be related to genetic drifts, genomic rearrangements (shifts), or genetic reassortments that take place in the primer binding site.²¹⁻²⁹

As for the historically usual VP7-VP4 combinations (G1P[8], G2P[4], G3P[8] and G4P[8]), there were no cases of G3P[8] and G4P[8], and only two of G1P[8]. G2P[4], on the other hand, it was observed in 69% of the samples. G9P[8], which was recently found with a higher frequency and classified as a usual strain, was reported in only 2.3% of the samples. Only one case of G5P[8] was found. Literature data indicate that G1P[8] was the most prevalent strain in the world from 1989 to 2004. In Brazil, G1P[8], G9P[8], G2P[4], G3P[8], G4P[8] and G5P[8] were, in ascending order, the most common strains from 1982 to 2005. After 2006, the year in which Rotarix[®] was included in the Brazilian immunization schedule, there was a change in the circulation of strains. G2P[4] took the lead as the most frequent type, while G1P[8] ranked third.^{9,11,19,20,30} Approximately 5% of rotavirus cases are caused by uncommon combinations of VP7 and VP4. In this study, we found the following uncommon combinations: G1P[4], G1P[6], G2P[6], G3P[9], and G4P[6], which altogether accounted for 10% of the cases (n=18).

Literature data suggest that the infection by two or more genotypes of rotavirus (mixed infection) has a frequency rate of 15% in South American countries. It is believed that those infections occur more often in regions where there is greater diversity and circulation of uncommon strains^{20,31}. In this study, seven cases of mixed infections (4%) were found. Of those, four samples had P[4]/P[6] associated with G2, one had P[4]/P[6] associated with G1, one had G2/G9 associated with unidentified VP4, and one sample had P[8]/P[9] associated with unidentified VP7.

Comparing the regions of study (Southeast and Northeast) by genotype, we see that the three most frequent ones (G2P[4], G2 with unidentified VP4, and G2P[6]) have similar rates in both regions, representing approximately 87% of the samples. Among the historically usual strains, G1P[8] was found only in two cases in the Northeast region, G2P[4] was the dominant type in both regions, and G9P[8] and G5P[8] were found only in the Southeast region in approximately 5% and 1% of the samples, respectively. As for the uncommon VP7-VP4 combinations, G1P[6], G1P[4], G4P[6], and G2P[6] were circulating in approximately 12% of the samples from the Northeast, whereas G3P[9] and G2P[6] were seen in about 9% of the samples from the Southeast. Regarding cases of mixed infection, there was a 5% rate in the Northeast samples against 2% in the Southeast ones. In the Northeast group, there were three samples with G2-associated P[4]/P[6], one with G1-associated P[4]/P[6], and one with P[8]/P[9] associated with an unidentified VP7. In the Southeast group, there was one sample with G2/G9 associated with unidentified VP4 and one with G2-associated P[4]/P[6].

Table 6 - Reports by selected authors on the circulation of rotavirus strains

Location	Period	Observed	Reference
Sergipe	Nov./06 - Feb./07	G2P[4] – observed in 100% of the samples	Gurgel <i>et al.</i> ⁸
Sergipe	Oct./06 - Apr./08	G2P[4] – observed in 95% of the samples	Gurgel <i>et al.</i> ³²
Recife	Mar./06 - May/07	G2 – observed in 100% of the samples	Nakagomi <i>et al.</i> ³³
Rio de Janeiro	2005 - 2007	G2P[4] – 1.4% (2005) / 44% (2006) / 96% (2007)	Carvalho-Costa <i>et al.</i> ³⁴
Parauapebas	2006 - 2008	G2P[4] – observed in 90% of the samples	Mascarenhas <i>et al.</i> ³⁵
São Paulo	2006 - 2009	G9P[8] – dominant type in 2006 G2P[4] – dominant type in 2007 / 2008 / 2009	Cilli <i>et al.</i> ¹¹
São Paulo	2006 - 2008	In hospitalized children: G2P[4] - 15% (2006) / 70% (2007) / 100% (2008)	Sáfadi <i>et al.</i> ³⁶
Belém	2008 - 2009	In hospitalized children: G2P[4] predominance	Justino <i>et al.</i> ³⁷
Minas Gerais	2009 - 2010	G2P[4] predominance	Dulgheroff <i>et al.</i> ³⁸
Curitiba	2001 - 2008	In hospitalized patients: G2P[4] predominance after 2006	Pereira <i>et al.</i> ³⁹
18 Brazilian states	2005 - 2009	G9 – dominant type in 2005 (52%) G2P[4] – 49% (2006) / 66% (2007) / 85% (2008) / 37%(2009)	Carvalho-Costa <i>et al.</i> ⁶
Australia	2007 - 2009	Increase in G2P[4] rates in most states where Rotarix® is in use	Kirkwood <i>et al.</i> ⁴⁰

Official data from the Brazilian Ministry of Health reveal that in 2009 the Rotarix® vaccine coverage in the states of Pernambuco, Bahia, Rio de Janeiro, and São Paulo was 88.6%, 78.5%, 80.5%, and 89.1%, respectively⁷. Corroborating literature findings (Table 6) for the post-Rotarix® period, this study found an approximate predominance of 83% of G2 associated with P4 or with an unidentified VP4, an absence of G3P[8] and G4P[8], and only two cases of G1P[8] (1% of the studied samples), which shows a significant increase in the G2P[4] predominance. Selective pressure exerted by the vaccination has eventually led the unvaccinated population to be affected by the strains that are predominant among vaccinated individuals.

CONCLUSION

Of the VP7 genotypes found, only G2 was circulating in both of the regions studied. G1 and G4 were circulating only in the Northeast region (Bahia and Pernambuco States) whereas G3, G5, and G9 were circulating only in the Southeast region (São Paulo and Rio de Janeiro States). The VP4 genotypes that were found (P[4], P[6], P[8] and P[9]), on the other hand, were all circulating in both regions. G2P[4] proved to be the most common combination in both regions (Bahia and Pernambuco – 72.8%; São Paulo and Rio de Janeiro – 65.1%). The G2P[4] predominance was seen in a population with a reasonable coverage of Rotarix®, but further studies are needed to understand the reasons that may have led to this increase of genotype two

and to define whether or not the vaccine formulation should be modified. According to our data, multivalent vaccines might have to be proposed in order to be useful to different regions of the country.

ACKNOWLEDGEMENTS

The authors thank the colleagues from the UNIFESP virology laboratory, from the Fleury Group's Institute for Research and Development, and from the Adolfo Lutz Institute, for their contribution and support in the development of this study.

We would also like to thank the Federal University of São Paulo and the Fleury Group for their technical and financial support.

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