Prevalence and genomic characterization of G2P[4] group A rotavirus strains during monovalent vaccine introduction in Brazil

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ABSTRACT

This study aims to: estimate the prevalence of G2P[4] rotaviruses in Brazil between 2001–2011 from patients with acute gastroenteritis; perform phylogenetic analyses of G2P[4] Brazilian strains (from vaccinated and non-vaccinated children) based on VP7 and VP8* encoding genes and analyze the antigenic regions of these proteins comparing with RV1; and assess the full genetic background of eleven selected Brazilian strains. The G2P[4] detection rate among RVA positive samples was 0/157 in 2001, 3/226 (1.3%) in 2002, 0/514 in 2003, 0/651 in 2004, 31/344 (9%)/2005, 112/227 (49%)/2006, 139/211 (66%)/2007, 240/284 (85%)/2008, 66/176 (37.5%)/2009, 367/422 (87%)/2010 and 75/149 (50%)/2011. For the VP7 and VP8* encoding genes, 52 sequences were analyzed and shared up to 99% nucleotide identity with other contemporary G2P[4] strains detected worldwide, grouping into different clusters. Most differences inside antigenic epitopes of VP7 and VP8* have been maintained in the G2P[4] Brazilian strains along the years, and all were present before RV1 introduction. Eleven G2P[4] strains (4-vaccinated/7-non-vaccinated) were completely characterized and possessed the typical DS-1-like genotype constellation (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2) sharing up to 99% of nucleotide identity with contemporary worldwide strains. Reassortments between Brazilian G2P[4] human strains were observed. In conclusion, the data obtained in the current study suggests that implementation of RV1 vaccination might not influence the genetic diversity observed in G2P[4] analyzed strains. Several factors might have contributed to the increased prevalence of this genotype in Brazil since 2005: the introduction of RV1 into the Brazilian National Immunization Program has resulted in a decrease in the relative prevalence of predominant Wa-like RVA strains facilitating the increase of the heterotypic (DS-1-like) RVA strain G2P[4] in the Brazilian population; the genetic diversity found in different geographical regions throughout the years before, and after the introduction of RV1; the long period of low or no circulation of this genotype in Brazil previous to RV1 introduction could have created favorable conditions for the accumulation of immunological susceptible individuals.

1. Introduction

Group A rotaviruses (RVAs) were responsible for approximately 453,000 deaths worldwide among children ⩽5 years old in 2008, mainly (>80%) in countries in Asia and Sub-Saharan Africa (Tate et al., 2012). RVA belongs to the *Reoviridae* family and possesses a segmented dsRNA genome (11 gene segments) encoding six structural proteins (VP1-4, VP6-7) and six non-structural proteins (NSP1-6). Based on their two outer capsid proteins, VP7 and VP4, RVAs have been classified into G (Glycoprotein) and P (Protease-sensitive) genotypes, respectively. Up-to-date there are 27 G types and 37 P types reported (Matthijnssens et al., 2011; Trojnar et al., 2013). However, the (intra-)genotype diversity can change from one RVA season to the other and between different geographical locations. In addition, sporadic emergence of novel strains and/or

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uncommon genotype combinations have also been reported (Santos and Hoshino, 2005; Leite et al., 2008; Matthijnssens and Van Ranst, 2012).

The binary genotype classification system has been extended to the entire genome to better characterize RVA strains specifying the genotype of all 11 genome segments (Matthijnssens et al., 2011). Based on this classification, most of the human RVA detected worldwide possess either the Wa-like genotype constellation (I1-R1-C1-M1-A1-N1-T1-E1-H1) or the DS-1-like genotype constellation (I2-R2-C2-M2-A2-N2-T2-E2-H2) also called as genotype 1 and 2, respectively (Heiman et al., 2008; Matthijnssens et al., 2008; McDonald et al., 2009; Matthijnssens and Van Ranst, 2012).

Universal vaccination against RVA has been considered strategic in order to reduce both mortality and hospitalization due to diarrheal diseases, along with other measures such as oral rehydration, breastfeeding, zinc administration and improvement of sanitation in developing countries (WHO, 2013). In Brazil, RV1, an attenuated human monovalent (G1P[8]) vaccine has been included in the National Immunization Program (NIP) since March 2006. Official data from the NIP indicated that vaccine coverage in Brazil ranged between 46.5% (2006) and 87% (2011) (http://pni.datasus.gov.br/). An increase in the relative frequency of G2P[4] RVAs occurred in Brazil in the immediate post-vaccine years (Leite et al., 2008; Carvalho-Costa et al., 2005; Correia et al., 2010; Dulgheroff et al., 2012). As Brazil, Belgium and several Australian states implemented the use of RV1 in the NIPs since 2006 and 2007, respectively (Matthijnssens et al., 2012). After introduction of RV1, the three countries shown a relative increase in strains with the G2P[4] genotype compared with previous RVA seasons, and in the case of Australia also compared with states using RV5 (Leite et al., 2008; Correia et al., 2010; Carvalho-Costa et al., 2005; Kirkwood et al., 2011; Dulgheroff et al., 2012; Matthijnssens et al., 2012).

Some studies argued that both events, vaccine introduction and increase prevalence of G2P[4], were associated suggesting that the selective pressure generated by RV1 would select heterotypic G2P[4] strain over (partially) homotypic G1P[8], G3P[8], G4P[8], G9P[8] and G12P[8] RVA strains (Nakagomi et al., 2008; Gurgel et al., 2009; Matthijnssens et al., 2012). However, other Latin American countries that had not yet introduced universal vaccination also reported increased detection of G2P[4] at that time (Amarilla et al., 2004; Ferrera et al., 2007; Patel et al., 2008). Consequently, it has been suggested that this could be due to natural genotype fluctuation (Leite et al., 2008; Carvalho-Costa et al., 2009, 2005; Matthijnssens et al., 2009). As described by Matthijnssens et al. (2012), several factors might influence in RVA genotype distribution, including natural factors (acquired homotypic and heterotypic immunity, migration of people, emergence of new RVA strain variants, genetic diversity present in a particular genotype, among others) and the introduction of a RVA vaccine into NIPs.

The evolving prevalence rates of different RVA genotypes during and after vaccine introduction, as well as their epidemiological characteristics are important issues and represent a challenge to RVA vaccination programs. In this context, monitoring the emergence of genotypes and escaping strains associated to breakthrough infections is considered important in order to assess the impact of universal vaccination in RVA circulating strains. In the current study the prevalence of RVA genotype G2P[4] in Brazil between 2005 and 2011 was assessed. Furthermore, G2P[4] strains detected in the five regions of Brazil from 2005 to 2011 have been analyzed for the VP7 and VP8* encoding genes, and compared with RVA strains available in the Genbank database, including the RV1 vaccine. The complete genome characterization of eleven G2P[4] Brazilian selected strains was also performed.

2. Material and methods

2.1. Laboratory based RVA surveillance and specimen collection

Laboratory based RVA surveillance was performed with fecal deidentified samples obtained from patients with acute gastroenteritis. Samples were sent to the Regional Rotavirus Reference Laboratory – Laboratory of Comparative and Environmental Virology (RRRL-LVCA) by a network of state public health laboratories. These labs, in turn, receive samples of health centers and hospitals in the National Health System. We studied samples from the following Brazilian states: Acre ($n = 372$), Alagoas ($n = 151$), Amazonas ($n = 6$), Bahia ($n = 3495$), Ceará ($n = 667$), Distrito Federal ($n = 84$), Espírito Santo ($n = 483$), Goiás ($n = 44$), Maranhão ($n = 557$), Minas Gerais ($n = 373$), Mato Grosso do Sul ($n = 47$), Mato Grosso ($n = 3$), Paraíba ($n = 25$), Pernambuco ($n = 195$), Piauí ($n = 1$), Rio de Janeiro ($n = 4401$), Rio Grande do Norte ($n = 50$), Rondônia ($n = 51$), Rio Grande do Sul ($n = 3197$), Santa Catarina ($n = 281$), Sergipe ($n = 631$).

We studied 15,114 fecal samples, from which 3361 (22.2%) were RVA-positive. Among RVA-positive patients, 361 were fully (two doses of RV1) vaccinated children. Table 1 presents the number of samples per year, the RVA detection rates and prevalence of G2P[4] among RVA-positive patients.

2.2. Group A rotavirus detection and G- and P-genotyping

In order to detect RVA in stool samples, polyacrylamide gel electrophoresis (PAGE) (Pereira et al., 1983) and enzyme immunoassay (EIA, Premier Rotaclone®, Meridian Bioscience, Inc.; Ridascreen®, R-Biopharm), according to the manufacture's protocols. Nucleic acid was extracted from 10% fecal suspensions by the glass powder method described by Boom et al. (1990), including modifications as described by Gómez et al. (2013) and the QIAamp Viral RNA mini kit (Qiagen®/Westburg, The Netherlands) according to the manufacturer's instructions. In RVA-positive samples, the extracted RNA was reverse transcribed and G- and P-genotyping was performed using semi-nested multiplex PCRs as previously described (WHO/IVB/ 08.17, 2008). G and P RVA genotypes were confirmed by sequencing for 52 samples. Criteria used to select these samples were: number of positive samples per year (vaccinated and non-vaccinated), amount of fecal sample, and quality of extracted RNA. In addition, eleven selected strains representative from the surveillance period (2005–2011), based on clusters observed in VP8* and VP7 phylogenetic analysis, were investigated by whole genome analysis.

2.3. Genome segments amplification and sequencing

The amplification of the eleven genome segments from selected strains were performed using a OneStep RT-PCR Kit (QIAGEN®)

Table 1
Detection rate of group A rotavirus (RVA) and prevalence of G2P[4] genotype per year in Brazil.

Year	Studied samples, n	RVA-positive samples, n (%)	G2P[4] detection rate among RVA-positive samples, n (%)
2001	801	157 (19.6)	0 (0)
2002	840	226 (26.9)	3 (1.3)
2003	1856	514 (27.6)	0 (0)
2004	2544	651 (25.6)	0 (0)
2005	1292	344 (26.6)	31 (9)
2006	1576	227 (14.4)	112 (49.3)
2007	1232	211 (17.1)	139 (65.8)
2008	1013	284 (28)	240 (84.5)
2009	980	176 (17.9)	66 (37.5)
2010	1863	422 (22.6)	367 (86.9)
2011	1117	149 (13.3)	75 (50.3)

following manufacturer's instructions and the following amplification conditions: (i) for VP1-2: 50 °C/30 min (min) – 95 °C/15 min – 35 cycles of 94 °C/30 s (sec)/45 °C/30 s/72 °C/6 min, 72 °C/10 min; (ii) VP3-4: annealing temperature changed to 47 °C; (iii) for VP6, VP7, NSP1-4: annealing temperature changed to 50 °C (except for NSP5, 45 °C), and extension time to 3 min. Primers used to amplify the 11 gene segments are listed in Supplementary Material 1. The cDNA products were resolved on agarose gels electrophoresis and purified using the ExoSAP-IT PCR Product Cleanup Kit (Affymetrix, Miles Rd., Cleveland, OH, USA). Sequencing was performed with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit™ on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Instituto de Tecnologia em Imunobiológicos (Bio-Manguinhos/FIOCRUZ), and an ABI Prism 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA) at the Rega Institute of Medical Research (University of Leuven, Belgium).

2.4. Sequence and phylogenetic analysis

Sequences obtained in the current study were deposited in the GenBank database under the following accession numbers: KJ721696–KJ721783; KJ940055–KJ940118. Nucleotide BLAST searches were performed and multiple sequence alignments were carried out using the ClustalW program (Thompson et al., 1994). Phylogenetic trees were constructed using the Neighbor-Joining method with the Kimura-two parameter model in MEGA5.0 (Tamura et al., 2011). The statistical significance of the branch was assessed by bootstrap resampling analysis (2000 replicates).

Deduced amino acid sequences of the VP8* and VP7 proteins of the Brazilian G2P[4] RVA strains were compared with the RV1 strain, and RV5 strain (just VP7) using the Bioedit v.7.2.3 softwear (Hall, 1999).

3. Results

3.1. RVA Surveillance and G2P[4] prevalence

The frequency of G2P[4] RVA detection between 2001 and 2003 was very low, more specifically only three of the analyzed samples were positive for this genotype among 801 samples tested in 2001, 840 in 2002 and 1856 in 2003. During 2004, none of the 651 RVA positive samples analyzed among the 2.554 patients with gastroenteritis belonged to this genotype. After 2004, the first G2P[4]

RVA samples detected in the RRRL-LVCA were in August, 2005 in Western Brazil (Mato Grosso do Sul State), bordering Paraguay. Between August and September 2005, 22 additional G2P[4] samples were detected in the same Brazilian region. In September 2005, seven G2P[4] samples were detected in the State of Acre (Northern Brazil) bordering Bolivia. In December, 2005, one RVA positive sample was characterized in Rio de Janeiro, the second largest Brazilian city, which attracts people from Brazil and foreign countries (Fig. 1). Therefore, the frequency of detection of G2P[4] in 2005 was 31 out of 344 RVA positive samples (9%) (Table 1).

In the post vaccination era, detection rates of G2P[4] among RVA positive children with acute diarrheal disease were as follows: 112/227 (49%) in 2006, 139/211 (66%) in 2007, 240/284 (85%) in 2008, 66/176 (37.5%) in 2009, 367/422 (87%) in 2010 and 75/149 (50%) in 2011 (Table 1). Although the lower part of the 5570 Brazilian municipalities have been studied, and the cities surveyed varied from year to year according to the demands of local health authorities, it is observed that the proportion of municipalities with detection of G2 increased between 2005 and 2008, decreasing in 2009 and increasing again from 2010. As presented in Fig. 1, G2P[4] has spread from Western cities to the majority of surveyed municipalities from 2006 on. The proportion of surveyed municipalities with G2P[4] detection were as follows: 8 municipalities with G2P[4] detection/98 surveyed municipalities (8%) in 2005, 24/164 (15%) in 2006, 35/137 (26%) in 2007, 70/173 (40%) in 2008, 22/146 (15%) in 2009, 86/222 (39%) in 2010 and 23/168 (14%) in 2011.

3.2. VP7 and VP8* sequence and phylogenetic analysis

Fifty-two VP7 and VP8*/VP4 sequences (21 and 22 derived from vaccinated children for VP7 and VP8*, respectively) were obtained from children with acute gastroenteritis in 13 Brazilian states (four Brazilian regions) between 2005 and 2011 (Supplementary Material 2). Nucleotide and amino acid sequence identity among Brazilian analyzed strains are shown in Table 2. Brazilian G2P[4] obtained sequences shared a high level of nucleotide identity (98–99%) with other G2P[4] strains detected worldwide in the last decade, revealing a close phylogenetic relationship with such strains, including strains previously reported in Brazil (Gómez et al., 2005).

VP7 phylogenetic analysis revealed that all G2P[4] Brazilian strains analyzed belonged to lineage II, and grouped in several clusters: (a) strains detected between 2005 and 2007 in the
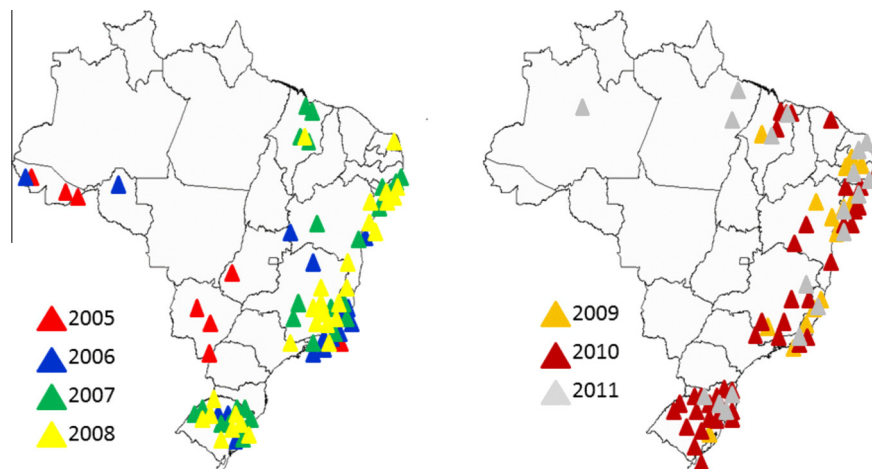

Fig. 1. Spatial spread of G2P[4] group A rotaviruses (RVA) in Brazil. The triangles indicate the cities where G2P[4] strains were detected. The first G2P[4] strains were detected in August/September 2005 (6 months before the implementation of universal vaccination) in western states and in December 2005 in Rio de Janeiro city (red triangles). In the following years, G2P[4] spread in vast areas in many surveyed states. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Nucleotide (nt) and amino acid (aa) identity values among Brazilian G2P[4] group A rotavirus (RVA) strains analyzed in the current study. Values for VP7 and VP8* are based on all the RVA Brazilian strains analyzed in the current study, while values for the rest of the genes/proteins (NSP1-5, VP1-4, and VP6) are based on the RVA Brazilian strains from which all 11 genes were obtained.

Encoding gene	% nt identity	% aa identity
VP7	95.9–100	97.2–100
VP8*	96.7–100	96.8–100
NSP1	96.9–100	96.7–100
NSP2	97.1–99.8	97.1–100
NSP3	97.4–100	97.7–100
NSP4	88.2–99.7	96.5–99.4
NSP5	97.2–100	99–100
VP1	94.8–99.7	98.6–99.7
VP2	97.6–99.8	99.1–100
VP3	87.3–99.9	93–100
VP4	97.5–99.7	98.7–99.7
VP6	96.7–99.9	98.9–100

Southern, Central Western and Northeastern regions (colored in cyan); (b) strains detected in 2007 in Rio Grande do Sul state (Southern Brazil) (colored in violet); (c) strains detected between 2009 and 2011 in the Southeastern, Northeastern and Southern regions (colored in blue); (d) strains detected in 2010 in the Northeastern and Southeastern regions (colored in orange); (e) strains detected between 2006 and 2009, and in 2011 in the Southeastern, Northeastern and Southern regions (colored in red) (Fig. 2).

VP8* phylogenetic analysis revealed that all studied strains belonged to lineage V (Fig. 2). Furthermore, Brazilian G2P[4] strains analyzed in the current study grouped into several clusters formed by: (a) strains detected throughout the entire study period in Southeastern, Northeastern, Central Western and Southern regions; (b) strains detected in 2007 in Rio Grande do Sul state (Southern); (c) strains detected between 2009 and 2011 in the Southeastern, Northeastern and Southern regions; (d) strains
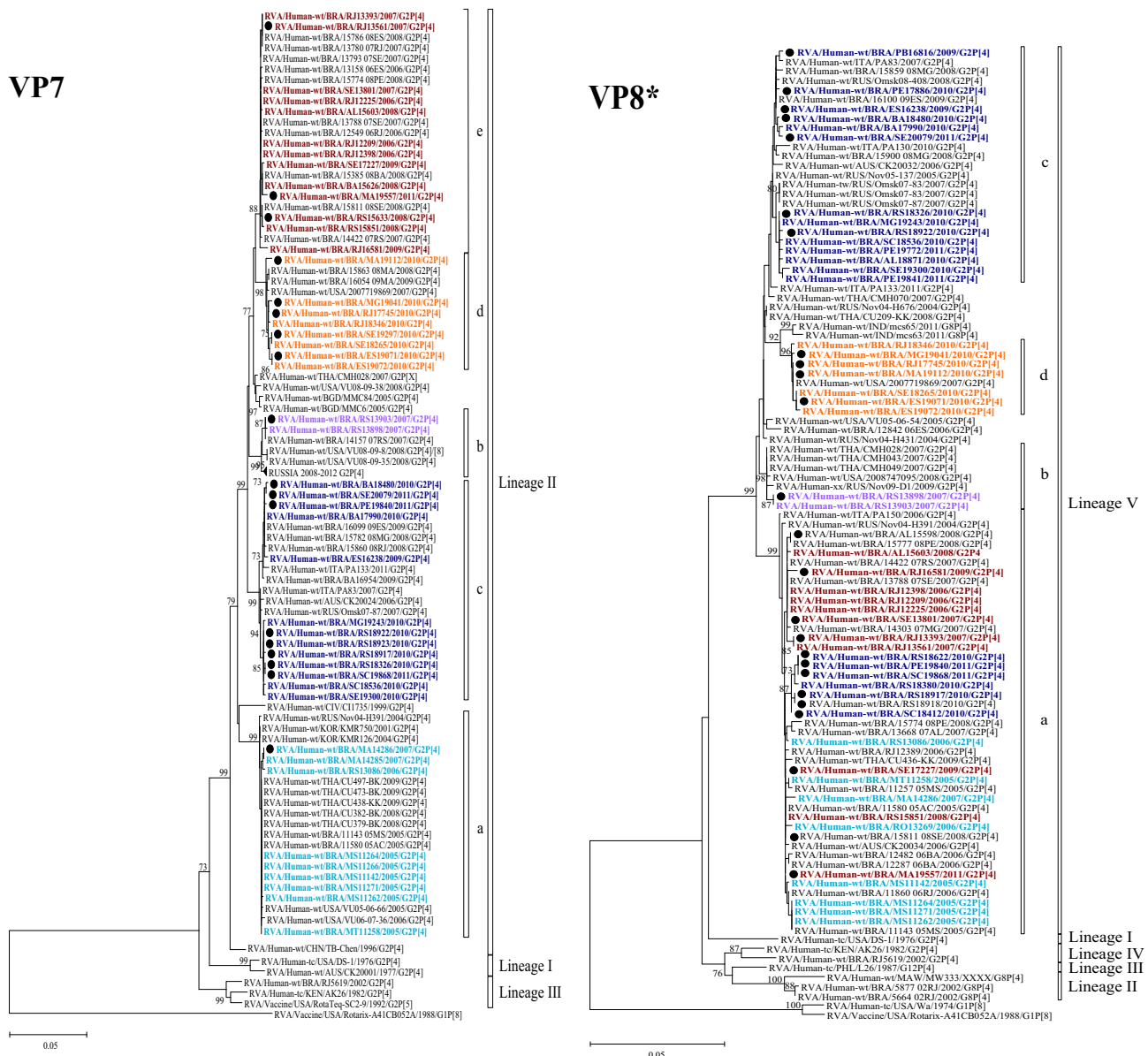

Fig. 2. Phylogenetic analyses based on VP7 and VP8* nucleotide sequences from Brazilian G2P[4] strains detected between 2005 and 2011, and group A rotavirus (RVA) strains available from GenBank. Brazilian G2P[4] strains analyzed in the current study are colored, and strains from vaccinated children are marked with a filled circle. Bootstrap values under 70% are not shown. G2 and P[4] lineages are shown at the right-hand side of each phylogenetic tree. Scale bars represent 0.05 nucleotide substitution per site.

detected in 2010 in the Northeastern and Southeastern regions (Fig. 2).

Phylogenetic analysis based on VP7 and VP8* encoding genes suggested the occurrence of reassortment among G2P[4] Brazilian analyzed strains colored in red, blue and cyan (Fig. 2).

Comparison of the deduced amino acid sequences of VP7 Brazilian G2P[4] strains and the RV1 strain revealed that Brazilian G2P[4] strains showed amino acid substitutions inside antigenic epitopes at positions, as well as when comparing with RV5 G2 strain, showing 38% and 86–93% of identity, respectively (Fig. 3).

As observed for VP7, Brazilian G2P[4] strains showed the amino acid substitutions inside antigenic epitopes of VP8 protein when comparing with RV1 strain revealing 42–50% (Brazilian G2P[4] strains that grouped inside cluster "a", "b", and "c") and 54% (Brazilian G2P[4] strains that grouped inside cluster "d") of amino acid identity with RV1 (Fig. 3).

RVA detected from vaccinated and non-vaccinated children shared the same amino acid differences when compared with RV1 strain for both proteins analyzed.

3.3. Whole-genome characterization of G2P[4] Brazilian strains

Eleven G2P[4] Brazilian strains, representative of the different clusters observed when analyzing the VP7 and VP8* encoding genes, and with sufficient stool material, were selected to perform complete genome analysis. Four of these strains were detected in vaccinated children, and seven in unvaccinated children and all were hospitalized with acute gastroenteritis between 2005 and 2011.

All analyzed strains showed a I2-R2-C2-M2-A2-N2-T2-E2-H2 genomic background (Table 3). Although all strains displayed the same genotype constellation, different genetic variants of circulating G2P[4] strains during the study period in Brazil were observed (Figs. 4 and 5).

The majority of the G2P[4] Brazilian strains analyzed in the current study (colored in red) showed close phylogenetic relationship with G2P[4] human strains detected mainly in Australia (1999, 2000, 2007), USA (2005, 2007), Italy (2006), and Thailand (2009) (Figs. 4 and 5). In addition, strains RVA/Human-wt/BRA/ES16238/ 2009/G2P[4], RVA/Human-wt/BRA/SE19300/2010/G2P[4] (colored

	Antigenic residues in VP7 protein																													
	7-1a															**7-1b**						**7-2**								
	87	91	94	96	97	98	99	100	104	123	125	129	130	291		201	211	212	213	238	242		143	145	146	147	148	190	217	221	264
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]	T	T	N	G	E	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T		K	D	Q	N	L	S	M	N	G
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P[5]	A	N	S	D	.	.	E	N	.	D	T	M	N	.		.	D	.	S	.	S		R	.	N	T	S	D	I	S	.
RVA/Human-tc/USA/DS-1/1976/G2P[4]	A	N	S	D	.	.	E	N	.	D	N	.	N	.		.	D	.	N	.	N		R	.	N	T	S	D	I	S	.
G2P[4] Brazilian strains	.	N	S	N	.	.	E	N	.	D	T	M	N	.		.	D	.	.	.	S/N		R	.	N	T	S	D	I	S/A	.

	Antigenic residues in VP8* protein																						
	8-1										**8-2**		**8-3**									**8-4**	
	100	146	148	150	188	190	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]	D	S	S	N	S	S	N	L	N	N	E	R	N	P	V	D	S	S	N	D	N	N	T	N
RVA/Human-tc/USA/DS-1/1976/G2P[4]	.	G	.	G	.	N	D	.	.	.	.	.	S	Q	T	N	N	E	.	N	D	.	.	.
G2P[4] Brazilian strains: clusters a, b, and c	.	G	S/G	S	.	N	D	.	.	.	.	.	N/S	Q	T	N	N	E	.	S	D	.	.	D
G2P[4] Brazilian strains: cluster d	.	G	.	S	.	D	.	.	.	.	.	.	S	.	T	N	N	E	.	S	D	.	.	D

Fig. 3. Alignment of the deduced amino acid sequences inside antigenic epitopes of VP7 and VP8* proteins of Brazilian G2P[4] strains analyzed compared with reference DS-1, RV1 and RV5 G2 strains (VP7). Amino acid differences are indicated with a grey shadow.

Table 3
Genomic background of RV1, RV5, and G2P[4] Brazilian group A rotavirus (RVA) strains. Brazilian G2P[4] strains from vaccinated children are marked with an asterisk, and genotypes in italic were obtained with partial sequences.

| Strain	RVA Vaccines Genomic Background										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P[5]	G1	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P[5]	G2	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P[5]	G3	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P[5]	G4	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P[8]	G6	P[8]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	Brazilian G2P[4] Genomic Background										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-wt/BRA/MA19557/2011/G2P[4]*	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/SC19868/2011/G2P[4]*	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/SE19300/2010/G2P[4]	G2	P[4]	I2	*R2*	-	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/RJ17745/2010/G2P[4]*	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/ES16238/2009/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/RS15851/2008/G2P[4]	G2	P[4]	I2	*R2*	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/MA14286/2007/G2P[4]*	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/SE13801/2007/G2P[4]	G2	P[4]	I2	*R2*	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/RJ12225/2006/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/RS13086/2006/G2P[4]	G2	P[4]	I2	*R2*	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/MS11142/2005/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
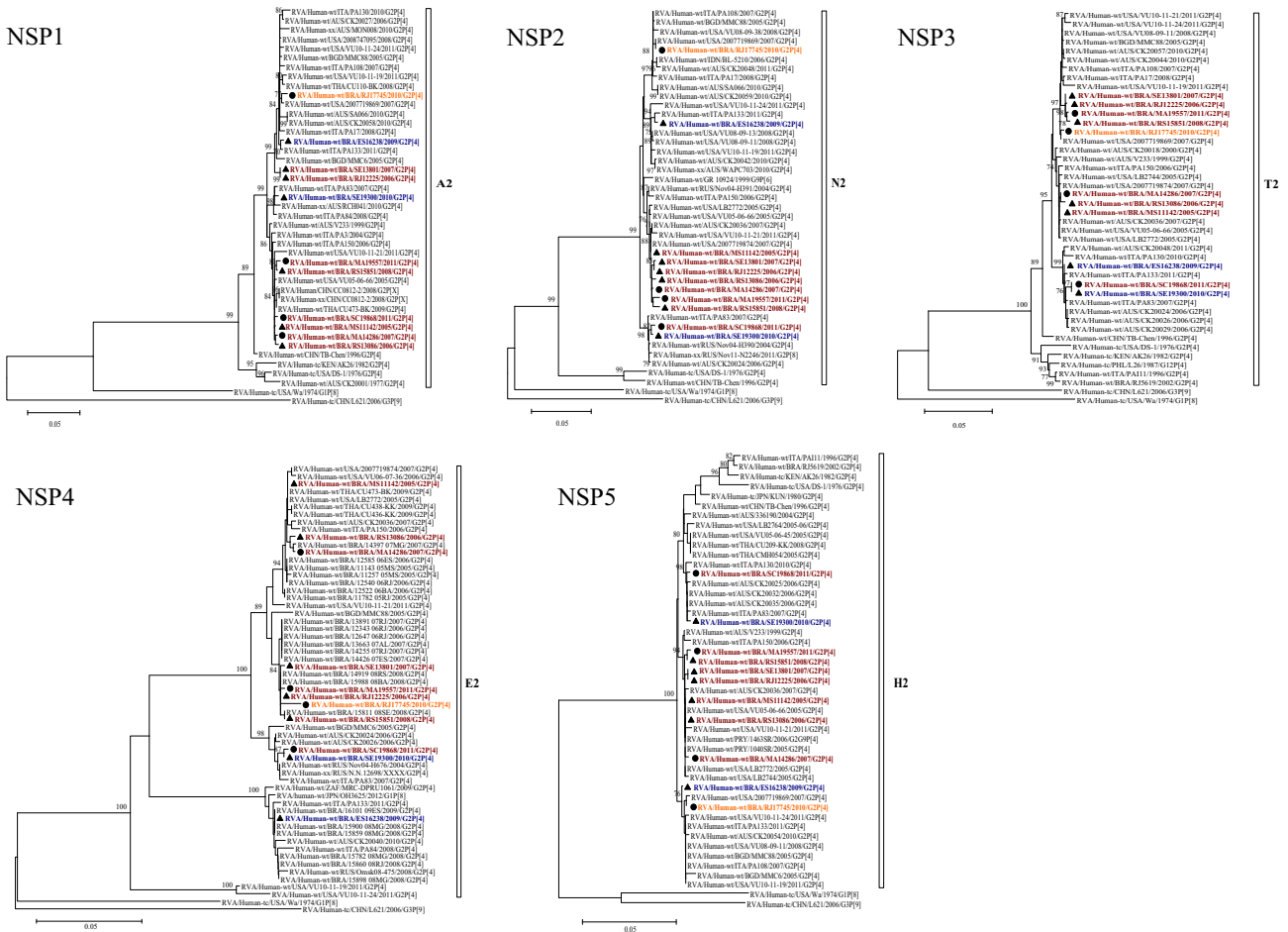
Fig. 4. Phylogenetic analyses based on NSP1-5 nucleotide sequences from Brazilian G2P[4] strains detected between 2005 and 2011, and group A rotavirus (RVA) strains available from GenBank. Brazilian G2P[4] strains analyzed in the current study are marked with a filled circle (vaccinated) and a filled triangle (non-vaccinated). Bootstrap values under 70% are not shown. Genotypes are shown at the right-hand side of each phylogenetic tree. Scale bars represent 0.05 nucleotide substitution per site.

in blue), and RVA/Human-wt/BRA/RJ17745/2010/G2P[4] (colored in orange), showed different phylogenetic pattern comparing with strains colored in red (Figs. 4 and 5).

Phylogenetic analysis based on NSP1-3, NSP5, VP1-4, and VP6 obtained sequences revealed that strain RVA/Human-wt/BRA/ES16238/2009/G2P[4] (colored in blue) was closely related with RVA/Human-wt/ITA/PA133/2011/G2P[4] strain (Figs. 4 and 5). Depending on the analyzed encoding gene, this strain showed closely phylogenetic relationship with other human G2P[4] strains. For the NSP1 and NSP5 encoding gene this Brazilian strain grouped together with RVA/Human-wt/BRA/RJ17745/2010/G2P[4]; for the NSP3, with RVA/Human-wt/BRA/SE19300/2010/G2P[4] and RVA/Human-wt/BRA/SC19868/2011/G2P[4]; and for VP4 with RVA/Human-wt/BRA/SE19300/2010/G2P[4] strain (Figs. 4 and 5). For the NSP4 encoding gene strain RVA/Human-wt/BRA/ES16238/2009/G2P[4] showed close phylogenetic relationship with previous Brazilian detected strains (2008–2010). Phylogenetic analysis based on VP3 showed that strain RVA/Human-wt/BRA/ES16238/2009/G2P[4] was closely related with RVA/Human-wt/ITA/PA133/2011/G2P[4] strain, strains detected in Bangladesh (2005), Thailand (2008), Australia (2010), USA (2011), and showed 97% of nucleotide identity with a caprine strain detected in 1999 (Fig. 5).

Strain RVA/Human-wt/BRA/RJ17745/2010/G2P[4] (colored in orange) was closely related to strain RVA/Human-wt/USA/2007719869/2007/G2P[4]. Depending on the encoding gene, closely related strains were human G2P[4] strains (Figs. 4 and 5).

For the NSP4 encoding gene this strain showed close phylogenetic relationship with previous Brazilian detected strains (2006–2008 and in 2010).

RVA Brazilian strains RVA/Human-wt/BRA/SE19300/2010/G2P[4] (colored in blue) and RVA/Human-wt/BRA/SC19868/2011/G2P[4] (colored in red) grouped together for the NSP2-5, VP3 and VP6 encoding genes (Figs. 4 and 5). For NSP1, VP2 and VP4, strain RVA/Human-wt/BRA/SC19868/2011/G2P[4] grouped together with other Brazilian strains (2005–2008, 2011) (colored in red) (Figs. 4 and 5). Phylogenetic analysis based on VP1 revealed that strain RVA/Human-wt/BRA/SC19868/2011/G2P[4] grouped together with human G2P[4] strains detected in Australia (2006) and Italy (2007), and a caprine G6P[1] strain detected in 1999, showing 97% of nucleotide identity (Fig. 5).

The VP2 sequence of strain RVA/Human-wt/BRA/SE19300/2010/G2P[4] could not be obtained, despite repeated attempts. Partial sequences of VP1 encoding gene were obtained for RVA/Human-wt/BRA/RS13086/2006/G2P[4], RVA/Human-wt/BRA/SE13801/2007/G2P[4], RVA/Human-wt/BRA/RS15851/2008/G2P[4], RVA/Human-wt/BRA/SE19300/2010/G2P[4] strains.

Analysis of VP1-4, VP6 and NSP1-5 sequences showed that G2P[4] Brazilian strains shared between 87.3–100%, and 93–100% of nucleotide and amino acid identity values, respectively (Table 2). Strain RVA/Human-wt/BRA/ES16238/2009/G2P[4] differed more than 10% comparing with other Brazilian strains analyzed in the current study for NSP4 and VP3 encoding genes (data not shown).
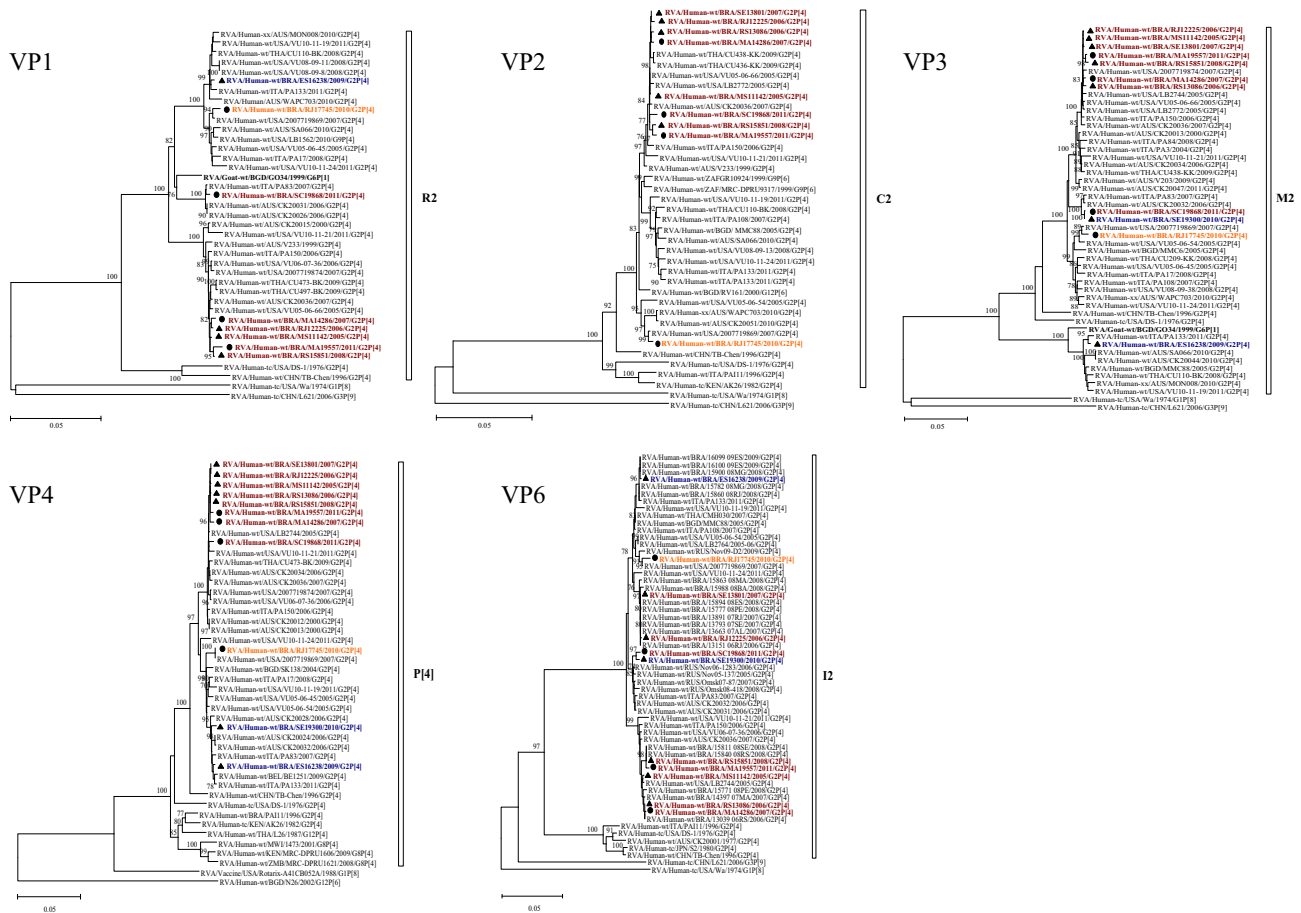
Fig. 5. Phylogenetic analyses based on VP1-4, and VP6 nucleotide sequences from Brazilian G2P[4] strains detected between 2005 and 2011, and group A rotavirus (RVA) strains available from GenBank. Brazilian G2P[4] strains analyzed in the current study are marked with a filled circle (vaccinated) and a filled triangle (non-vaccinated). Bootstrap values under 70% are not shown. Genotypes are shown at the right-hand side of each phylogenetic tree. Scale bars represent 0.05 nucleotide substitution per site.

4. Discussion

G2P[4] has been the most common genotype detected in Brazil after the onset of RV1 mass vaccination (Gurgel et al., 2007; de Oliveira et al., 2008; Nakagomi et al., 2008; Leite et al., 2008; Carvalho-Costa et al., 2009, 2005; da Silva Soares et al., 2014). The emergence of this genotype has motivated a great discussion and a relationship between this phenomenon and RV1 vaccination has been proposed suggesting that universal vaccination would have exerted selective pressure on circulating RVAs, selecting G2P[4] (Gurgel et al., 2007; Nakagomi et al., 2008; Matthijnssens et al., 2012).

The results obtained in the current study demonstrate that the detection rate of G2P[4] in RVA positive samples has been high in the years after the introduction of mass vaccination with RV1. With the exception of 2009 this rate has been above 50%, reaching 87% in 2010. G2P[4] has also been frequently detected in children vaccinated with RV1. The drop in the detection rate of G2P[4] in 2009 was associated with an overall reduction in detection rates of RVA in Brazil and with an increase in the relative detection of G1, G3 and G9, as well as P[6] in some regions of the country. In this context, the RVA season in 2009 was of low intensity. In this period, it was believed that the circulation of G2 could have gotten into the exhaustion phase (Carvalho-Costa et al., 2005), which was not confirmed in 2010 and 2011, when this genotype was again frequently detected. We examine in more detail the geographical location of G2P[4] positive samples, trying to identify a route of spread of this genotype during its process of emergence in Brazil. A very low rate of G2P[4]

detection between 2001 and 2004 was obtained from RVA-surveillance performed in RRRL-LVCA. In the present survey, we confirmed that G2P[4] was absent in the RVA positive samples studied in the first semester of 2005. In this year, the first cities in which G2P[4] was detected are located in Western Brazil. Throughout 2005, G2P[4] continued to be detected in this region and in Amazonian Brazil, being absent from the most populous Brazilian regions of the Atlantic coast. Only in December 2005, G2P[4] was detected in the main Brazilian cities in Eastern Brazil, specifically in Rio de Janeiro. Universal vaccination against RVA began in March 2006 and, starting this year, G2P[4] was detected early in all monitored regions, in a phase in which the cohort of vaccinated children was still very limited.

Analysis of the VP7 and VP8* encoding genes showed that Brazilian G2P[4] strains identified in vaccinated and unvaccinated children grouped together in the same genetic clusters, and revealed that contemporary G2P[4] strains (circulating from 2005 to 2011) belonged to distinct lineages from the reference strain DS-1 and the SC2-9 G2-reassortant strain of RV5, as previously reported (Fig. 2) (Doan et al., 2011; Gómez et al., 2005; Zeller et al., 2012; Mouna et al., 2013; Do et al., 2014; Donato et al., 2010; Giammanco et al., 2014). Comparison of the RV1 amino acid sequences inside previous described antigenic epitopes in VP7 and VP8*, with Brazilian G2P[4] strains revealed several amino acid differences as expected since G2 is rather divergent from G1 strains (Fig. 3). The same differences have been previously described in G2P[4] strains detected in other countries (Zeller et al., 2012; Mouna et al., 2013). In addition, most of the differences observed have been maintained in the Brazilian strains along the sampling

period and all of them were present before RV1 introduction. Regarding the G2 VP7 gene of RV5 strain, phylogenetic analysis showed that Brazilian G2P[4] strains did not share a close phylogenetic relationship with RV5 strain, and many amino acid differences were observed inside antigenic epitopes as previously observed in Belgium, Tunisia, and Australia (Figs. 2 and 3) (Zeller et al., 2012; Mouna et al., 2013; Donato et al., 2010). This data suggest that the same genetic variants of RVA genotype G2P[4] VP7 and VP8* proteins have been circulating in the population worldwide in the last decade, but whether amino acid differences observed inside antigenic epitopes resulted in a reduced vaccine efficacy remains to be investigated.

The analysis of the genomic background of G2P[4] strains identified throughout the process of implementation of mass immunization with RV1 may be useful to clarify its potential role on observed changes in the distribution of RVA genotypes which occurred between 2005 and 2011 in Brazil. The analysis of the genomic background of eleven Brazilian G2P[4] RVA strains analyzed in the current study revealed a complete DS-1-like background, and genetic variants were observed circulating in the Brazilian population (Figs. 4 and 5). Differently from what was recently observed by Dennis et al. (2014), phylogenetic analyses of G2P[4] Brazilian RVA strains based on NSP1-5, VP1-4, and VP6 showed different clustering patterns (Figs. 4 and 5) suggesting the occurrence of reassortment events among strains. This discrepancy with the results from Dennis et al. (2014) and results obtained in our study might be related with the fact that samples analyzed in the current study were detected in different Brazilian regions, sometimes geographically distant, and strains detected during a relatively long time period were analyzed (2005–2011). On the other hand, similar results regarding NSP4 and VP3 encoding gene were observed for Brazilian G2P[4] strains, these two genes were the most divergent among the eleven RVA genes (Figs. 4 and 5). With the exception of VP1 and VP3 encoding genes, G2P[4] Brazilian strains were all closely related to other human RVA strains. Strains RVA/Human-wt/BRA/SC19868/2011/G2P[4] (for VP1) and RVA/Human-wt/BRA/ES16238/2009/G2P[4] (for VP3) showed close phylogenetic relationship with RVA/Goat-tc/BGD/GO34/1999/G6P[1] animal strain (97% identity). Similar results were recently described for G2P[4] RVA strains detected in USA and Italy (Dennis et al., 2014; Giammanco et al., 2014). As happens in other countries, available data regarding animal RVA strains is still poor and make it difficult to study the origin of this kind of animal-like genome segment incorporation into human RVA strains.

In conclusion, our data suggest that the G2P[4] strains circulating in Brazil during implementation of mass immunization with RV1 are not separated into genetic clusters defined by pre and post vaccination periods, suggesting that implementation of RV1 vaccination might not influence the genetic diversity observed among analyzed strains. Furthermore, Brazilian G2P[4] strains are closely related with human G2P[4] strains circulating in other countries, some of which had not yet implemented universal vaccination against RVA. A possible hypothesis is that the introduction of RV1 vaccine into the NIP has helped to decrease the prevalence of predominant Wa-like strains facilitating the increase of the heterotypic (DS-1-like) RVA strain G2P[4] in the Brazilian population. In addition, the long period of low or no circulation of genotype G2P[4] in Brazil previous to RV1 introduction would have created favorable conditions for the accumulation of immunological susceptible individuals (Carvalho-Costa et al., 2009; Assis et al., 2013). These facts, together with the genetic diversity found in different geographical regions throughout the years before, and after the introduction of the vaccine might explained the high prevalence of genotype G2P[4] in Brazil since 2005.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.09.012.

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