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Research paper

G1P[8] Rotavirus in children with severe diarrhea in the post-vaccine introduction era in Brazil: Evidence of reassortments and structural modifications of the antigenic VP7 and VP4 regions

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ABSTRACT

Worldwide rotaviruses A (RVA) are responsible for approximately 215,000 deaths annually among children aged < 5 years. RVA G1P[8] remains associated with > 50% of gastroenteritis cases in this age group. The aim of this study was to assess the genetic variability of G1P[8] strains detected in children with severe diarrhea in Belém, Pará, Brazil, during the post-rotavirus vaccine introduction era. Phylogenetic analysis clustered the VP4 and VP7 genes of 40 samples selected between 2009 and 2011 into lineages found to be different from the Rotarix® vaccine strain. A detailed investigation of their complete genotype constellations identified 2 reassortant viruses (5%), resulting from reassortments between the genogroups Wa-like and DS-1-like (G1-P[8]-I1-**R2**-C1-M1-A1-N1-**T2**-E1-H1) and Wa-like and AU-1-like (G1-P[8]-I1-**R3**-C1-M1-A1-N1-T1-E1-H1) genotype constellations. A comparison of the amino acid residues presents in the antigenic epitopes of VP7 and VP4, showed differences in the electrostatic charges distribution, between wild type Brazilian strains and the Rotarix® and RotaTeq® vaccine strains. These findings reflect the structural analyses of the antigenic regions of VP7 and VP4 of the RVA G1P[8] in children with gastroenteritis in Northern Brazil raising the hypothesis that structural modifications at these sites over time may account for the emergence of new strains that could possibly pose a challenge to current vaccines.

1. Introduction

Worldwide, group A rotaviruses (RVA) still exert a major role as diarrheal pathogens, leading to around 128,500 deaths per year, mainly among children younger than five years, both in developed and developing nations (Troeger et al., 2018).

RVA belongs to the *Reoviridae* family, *Rotavirus* genus, and possess a genome with 11 double-stranded RNA segments (dsRNA), encoding six structural proteins (VP1–4, VP6, VP7) and six non-structural (NSP1–5/6) (Estes and Greenberg, 2013).

To date, rotaviruses have been classified into nine species (A-I), and a newly proposed species, RVJ (Bányai et al., 2017). Moreover, RVA strains have a binary classification, which is based on the VP4 and the VP7 surface capsid proteins. VP4 is sensitive to **P**rotease and is cleaved by trypsin into two subunits, VP5* and VP8* (Yeager et al., 1990; Jayaram et al., 2004), whereas VP7 is a **G**lycoprotein. VP7 and VP4 are classified into G- and P-types, respectively.

The G/P-genotype combinations most commonly identified in humans around the world are: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] (Iturriza-Gómara et al., 2011; Santos and Hoshino, 2005; WHO, 2015).

The vast majority of RVA strains infecting humans are associated with the Wa-like, DS-1-like or AU-1-like genotype constellations. The Wa-like constellation (genogroup 1) contains the I1-R1-C1-M1-A1-N1-T1-E1-H1 genotypes; the DS-1-like constellation (genogroup 2) is constituted by I2-R2-C2-M2-A2-N2-T2-E2-H2 genotypes; and the AU-1-like

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constellation (genogroup 3) corresponds to I3-R3-C3-M3-A3-N3-T3-E3-H3 genotypes (Patton, 2012). Wa-like and DS1-like RVA strains are responsible for most of the infections in humans. In addition, genogroup AU-1-like strains have been less frequently identified causing infections in humans (Matthijnssens et al., 2008; Matthijnssens and Van Ranst, 2012).

Currently, two attenuated live oral rotavirus vaccines are available and, since 2009, have been recommended by the WHO to be used worldwide into national immunization programs: Rotarix® (GlaxoSmithKline biologicals, Rixensart, Belgium, containing a human attenuated G1P[8] RVA strain); and RotaTeq® (Merck & Co., Whitehouse Station, NJ, which contains 5 strains with the following genotypes: G1P[5], G2P[5], G3P[5], G4P[5] and G6P[8]) (Matthijnssens et al., 2010; WHO, 2009, 2013; 2015). In Brazil, Rotarix® was introduced for universal use into the public sector in March 2006. Although the G1 and P[8] specificities are present in both vaccine formulations, G1P[8] strains remain the most prevalent genotype in various parts of the world, being responsible for approximately 50% of RVA infections (Do et al., 2016).

It is postulated that the global predominance of G1P[8] strains throughout the world may be related to their broad antigenic and genetic heterogeneity, leading to a continuous reemergence of VP4 and VP7 protein variants and, consequently, new and distinct lineages. The occurrence of frequent reassortment events is likely to further facilitate the emergence of such typical potential genetic diversity of G1P[8] strains (Desselberger, 2014; Iturriza-Gómara et al., 2001; Matthijnssens et al., 2011; Phan et al., 2007; Zeller et al., 2017).

Studies carried out in Brazil with samples collected after Rotarix® introduction have shown the occurrence of reassortment events in G1P[8] strains, as well as modifications in the antigenic epitopes (da Silva et al., 2015; Rose et al., 2013). In this regard, Dormitzer et al. (2004), Mcdonald et al. (2009) and Zeller et al. (2012) have suggested that amino acid exchanges (aa) can confer a loss of recognition for specific epitopes, permitting the escape of these strains from vaccine-induced immunity.

The aim of the present study was to assess the genetic variability of the G1P[8] strains detected in children with severe diarrhea in Belém, Pará, Brazil, during the post-rotavirus vaccine introduction period.

2. Materials and methods

2.1. Preparation of samples

This study was approved by the ethics committee of the investigational center at Evandro Chagas Institute, Department of Health Surveillance, Brazilian Ministry of Health under number 44592915.1.0000.0019. Written informed consent was obtained from the parents/ guardians of all participating children prior to study entry as described by Justino et al. (2011).

A total of 160 samples previously characterized as G1P[8] RVA were selected from an earlier study involving children hospitalized for acute gastroenteritis in Belém, Pará (Guerra et al., 2015). Among these specimens, 40 were used in this study due to their appropriate availability, enough to characterize the 11 genes. Overall, samples were temporally distributed as follows: 2009 ($n = 12$), 2010 ($n = 15$), and 2011 ($n = 13$).

Fecal suspensions were prepared at 10% (v/v) in buffered Tris Ca^{++} 0.01 M, pH 7.2, with subsequent extraction of viral RNA as described by Boom et al. (1990). Polymerase chain reaction preceded by reverse transcription (RT-PCR) was carried out to partially amplify the following RVA genes: VP7 (1602 bp), VP6 (1356 bp), VP4 (875 bp), VP3 (702 bp), VP2 (686 bp), VP1 (686 bp), NSP5 (667 bp), NSP4 (738 bp), NSP3 (1078 bp), NSP2 (1059 bp) and NSP1 (1590 bp), using primers described in Table 1.

After the cDNA was obtained, the second step was started, which consisted of PCR amplification in the following conditions: 1 cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 42 °C for 30 s and 72 °C for 1 min, ending with a 72 °C cycle for 10 min of final extension for all genes.

2.2. Sequencing reaction and phylogenetic analysis

RT-PCR products were further purified, using the QIAQuick PCR purification kit (QIAGEN) according to manufacturer's protocol, and subjected to sequencing reaction according to the procedure described by Big Dye Terminator® v3.1 (Applied Biosystems), with the same primers used in RT-PCR.

The sequences were obtained from an automated ABI Prism 3130xl DNA sequencer (Applied Biosystems) and assembled using CAP3 software (Huang and Madan, 1999). Using BLAST analysis (Altschul et al., 2009), closely related reference strains available in GenBank were selected for further evaluation. The program MAFFT v. 7.221 was used to align and calculate the nt and aa identities (Katoh, 2013). The sequence edition was performed by the Bioinformatics Suite *Geneious* v. 8.1.7 (Kearse et al., 2012).

Phylogenetic analysis was carried out in three steps. First, utilizing the IQ-TREE program (Nguyen et al., 2014) in order to choose the most adequate evolutionary model for Maximum likelihood analysis. Then, phylogenetic reconstruction by FastTree software v.2.1.9 (Price et al., 2010), aiming to execute the non-parametric reliability test with 1000 replications applying bootstrap methods. Finally, the FigTree software v.1.4.2 (Rambaut and Drummond, 2010) was used for the generated phylogenetic tree edition. The sequences obtained in this study were submitted to the GenBank database under the following accession numbers MF161541 to MF161980 of all genes (available in Supplementary Material).

2.3. Modelling of proteins

The homology modelling of proteins was performed using three-dimensional protein structures, with an initial search for selection of templates. For this purpose, the Protein Database was used (http://www/rcsb.org/pdb), applying VP4 and VP7 RVA proteins sequences to achieve an initial parameter for the current study. The templates selected for the analyzed genes were: 3FMG and 2DWR, respectively. The Modeller 9.15 software (Sali and Blundell, 1993) was utilized to construct the three-dimensional models of both Rotarix® and RotaTeq® vaccine sequences under analysis, and the models generated were subsequently validated using PROCHECK (Laskowski et al., 1998) and VERIFY3D (Eisenberg et al., 1997), for evaluation of stereochemical quality parameters. Visualization and production of images were performed by PYMOL software (Delano, 2002).

3. Results

3.1. Genomic constellation of G1P[8] RVA

The genotype constellation of 38 RVA strains was identified as Wa-like (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1), whereas two RVA strains appeared to be reassortant, involving gene segments VP1 and NSP3. RVA strain 2A1456 possessed a AU-1-like VP1 genotype, resulting in the following genotype constellation G1-P[8]-I1-**R3**-C1-M1-A1-N1-T1-E1-H1. Furthermore, RVA strain 2A1498 strain, showed DS-1-like VP1 and NSP3 genes, resulting in the G1-P[8]-I1-**R2**-C1-M1-A1-N1-**T2**-E1-H1 genotype constellation.

3.2. Phylogeny and characterization of lineages

The sequence analysis of RVA genes from selected samples showed that the Wa-like genotype constellation was associated with the formation of more than one clade, as shown in Fig. 1 and supplementary material, it can be observed that the samples of unvaccinated children

Table 1
Description of primers used in the amplification and sequencing reactions of RVA.

Primer	Sequence	Gene	Amplicon (bp)	Reference
VP1F	5′ GGC TAT TAA AGC TGT ACA ATG GG 3′	VP1	686	Varghese et al. (2006)
VP1R	5′ TAA TCC TCA TGA GAA AAC ACT GAC 3′			
VP2F	5′ GGC TAT TAA AGG GCT CAA TGG CG 3′	VP2	686	
VP2R	5′ CTT CAT CTT GAA ATA TAG CAA TCA C 3′			
VP3F	5′ GGC TAT TAA AGC AGT CCA GTA G 3′	VP3	702	
VP3R	5′GTA AAC ATA GAT TCA TTA CGC GGA CC 3′			
4con3F	5′ TGG CTT CGC CAT TTT ATA GAC A 3′	VP4	875	Gentsch et al. (1992)
4con2R	5′ ATT TCG GAC CAT TTA TAA CC 3′			
VP6F	5′ GGC TTTT AAA CGA AGT CTT 3′	VP6	1356	Matthijnssens et al. (2006)
VP6R	5′ GGT CAC ATC CTC TCA CTA CA 3′			
Beg9f	5′ GGC TTT AAA AGA GAG AAT TTC CGT CTG G 3′	VP7	1602	Gouvea et al. (1990)
END9R	5′ GGT CAC ATC ATA CAA TTC TAA TCT AAG 3′			
NSP1F	5′ GGG CTT TTT GAA AAG TC 3′	NSP1	1590	Matthijnssens et al. (2006)
NSP1R	5′ GGT CAC ATT TTA TGC TGC CTA 3′			
NSP2f	5′ GGC TTT TAA AGC GTC TCA G 3′	NSP2	1059	
NSP2R	5′ GGT CAC ATA AGC GCT TTC 3′			
NSP3F	5′ GGC TTT TAA TGC TTT TCA GTG 3′	NSP3	1078	
NSP3R	5′ ACA TAA CGC CCC CTA TAG C 3′			
JRG30F	5′ GGC TTT TAA AAG TTC TGT T 3′	NSP4	738	Cunliffe et al. (1997)
JRG31R	5′ ACC ATT CCT TCCATT AAC 3′			
NSP5F	5′ GGC TTT TAA AGC GCT ACA G 3′	NSP5	667	Matthijnssens et al. (2006)
NSP5R	5′ GGT CAC AAA ACG GGA GT 3′			

grouped with those from children who received at least one vaccine dose. Gene analysis of samples from the present investigation revealed that sequences grouped with prototype strains collected in several countries, as Paraguai and Japan, and with prototype brazilian samples from states of Sergipe, Pernambuco and Mato Grosso do Sul. For some genes, two groups were formed (VP1–3, VP6, NSP1–5), although some clusters present distinct viral groups, evidencing reassortment between genes. For the VP2, VP3, VP6, NSP1, NPS2, NSP4 and NSP5 genes only the Wa-like genogroup was detected (See phylogeny tree in Supplementary Material). For the genes VP2, VP3, NSP1, NSP2, NSP4 and NSP5 the specimens all specimens corresponded to C1, M1, A1, N1, E1 and H1 genotypes, respectively.

With regards to the VP7 gene, all samples clustered into lineage G1-I, whereas the Rotarix® vaccine strain (JN849114) grouped into lineage G1-II (Fig. 1A). Nucleotide (nt) identities ranged from 95.0% to 99.9% (92.6%–100% on the aa level) among the studied samples, 91.7% to 93.6% (90.2% to 94. 9% on the aa level) when compared to the Rotarix® vaccine strain, and from 87.6% to 90.9% (78.6%–83.3% on the aa level) when compared to RotaTeq® vaccine strain (Table 2).

For the VP4 gene segment (Fig. 1B), all samples clustered into lineage P[8]-3, with nt identities ranging from 94.3% to 100% (90.4%–100% aa identify), 88.7% to 91.1% (aa similarity 88.1% to 92.4%) when compared with the Rotarix® vaccine strain (JN849113) that belongs to lineage P[8]-1, and from 90.8% to 93% (90.5%–94.2% on the aa level) when compared to RotaTeq® vaccine strain (Table 2). Nucleotide and amino acidic identities related to the remaining gene segments are described at Table 2.

For the VP1 gene, three genotypes were detected (Fig. 1C). Genotype R1 formed two distinct clusters and comprised most of the circulating strains. Two strains detected in 2010 grouped in different genogroups: 2A1456 belonged to the DS-1-like R2 genogroup, and grouped to samples from Paraguai, G12P[9] genotype, and one sample from Japan, G3P[9] genotype. The strain 2A1498 was found in the Au-1-like R3 genogroup, and clustered with samples from Paraguai, Belgium, Australia, Canada and Brazil, all of which correspondent to G2P[4] genotype. With regards to the NSP3 gene, phylogenetic analysis demonstrated that the 2A1498 strain belonged to the T2 genotype with high nt and aa (96.6% and 96.2%, respectively) similarity with strains detected in Australia. The other samples belonged to the T1 genotype (Fig. 1D).

3.3. Modelling of the structural proteins VP4 and VP7

The analysis of VP4 protein antigenic sites showed differences when comparing our G1P[8] strains to the RotaTeq® and Rotarix® vaccine strains. These differences were observed in relation to the electrostatic charge of the VP8* subunit: S146G/A, S190 N (Rotarix® only), A192V, N195D and N196G (antigenic site 8–1) N113D, S125 N (Rotarix® only), S131R/K (Rotarix® only), N132K, D133N, N136D (Rotarix® only) (antigenic site 8–3) as shown in the Fig. 2. The antigenic epitopes that presented alterations are exposed on the surface of the protein and are represented in Fig. 3.

The changes in the electrostatic charge of VP4 protein showed that aa 113, 195 and 196 presented lower positivity, whereas aa 131, 132, 133 and 136 presented higher electrical positivity. No electrostatic differences were identified when comparing 125, 146 and 192 aa residues; in addition, a neutral charge was identified at the site 190. Residues 136, 146, 190 are known as neutralization escape sites by monoclonal antibodies and aa modifications were observed at these residues when compared with the Rotarix® and RotaTeq® vaccine strains.

Concerning toVP7 gene, when comparing our Brazilian strains with vaccine strains, we demonstrated 8 aa differences at the sites N94S, T87A/L, D97E (RotaTeq® only), S147 N (RotaTeq® only), D130A/C and K291R (antigenic site 7-1a); D213N and N238K (antigenic site 7-1b) and in the sites S123N and M217T (antigenic site 7–2) (Fig. 4). The sites 94 and 213 presented higher negativity, while 87, 123, 130 and 217 showed a lower negativity, and residues 238 and 291 demonstrated a higher electrical positivity.

The mapping of the differences in the surface representation of VP7 protein antigenic epitopes are shown in Fig. 5. It was detected that few strains showed aa modification in sites that are considered escape neutralization residues (87, 238, 213).

4. Discussion

Several studies have shown that the G1P[8] RVA genotype remains a largely predominant strain even after the worldwide growing introduction of RVA vaccines into the national immunization schedules (da Silva et al., 2015; Zeller et al., 2017). This warrants continuous monitoring of circulating RVA strains, preferably in conjunction with a full-genome analysis, which allows the identification of their complete genotype constellation (da Silva et al., 2015; Rose et al., 2013; WHO

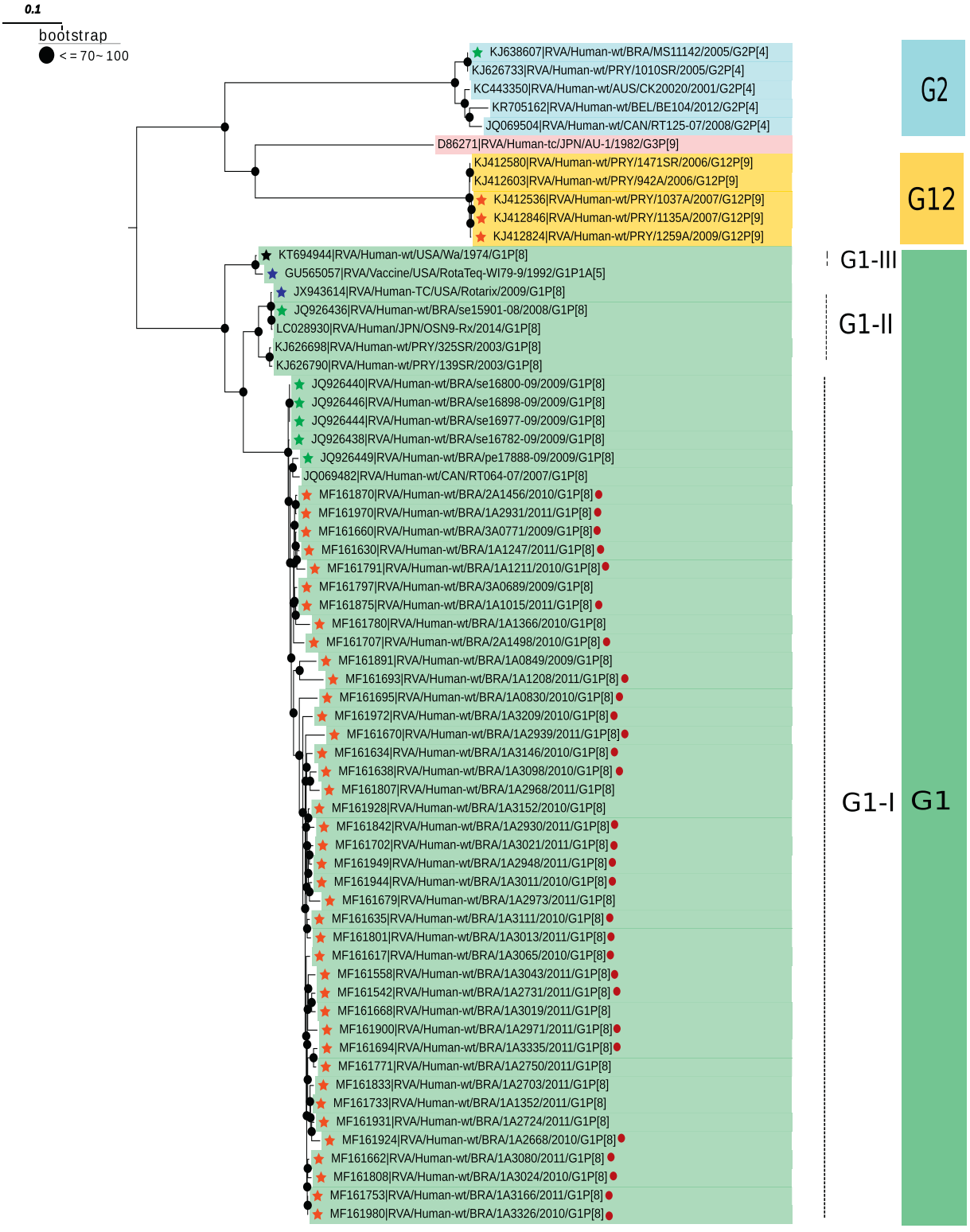

Fig. 1. Phylogenetic analysis based on the nucleotide sequence of the genes that codify for the structural proteins VP7 (A), VP4 (B), VP1 (C), NSP3 (D) involving 40 RVA samples of G1P[8] type. The bootstrap values (1000 pseudo-replications) are indicated on the tree nodes with the black dots, for values higher than 70. The samples of the present study are represented by red stars, Brazilian samples from Genbank by green stars, vaccine samples are illustrated by a blue star and Wa-like strain is indicated by a black star. Samples labeled with dark red circles represent children who had at least one dose of Rotarix® vaccine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2015; Zeller et al., 2017).

Genes VP2, VP3, NSP1, NSP2, NSP4 and NSP5 of our specimens grouped with those of samples from Sergipe (SE16977–09, SE16800–09 and SE16782–09), which were originated from vaccinated children hospitalized presenting three or more diarrhea episodes (Rose et al., 2013). For these genes, distinct clusters were formed, even though not always by the same viral group, evidencing their genetical and likely evolutionary distinction. Studies that included this genogrouping

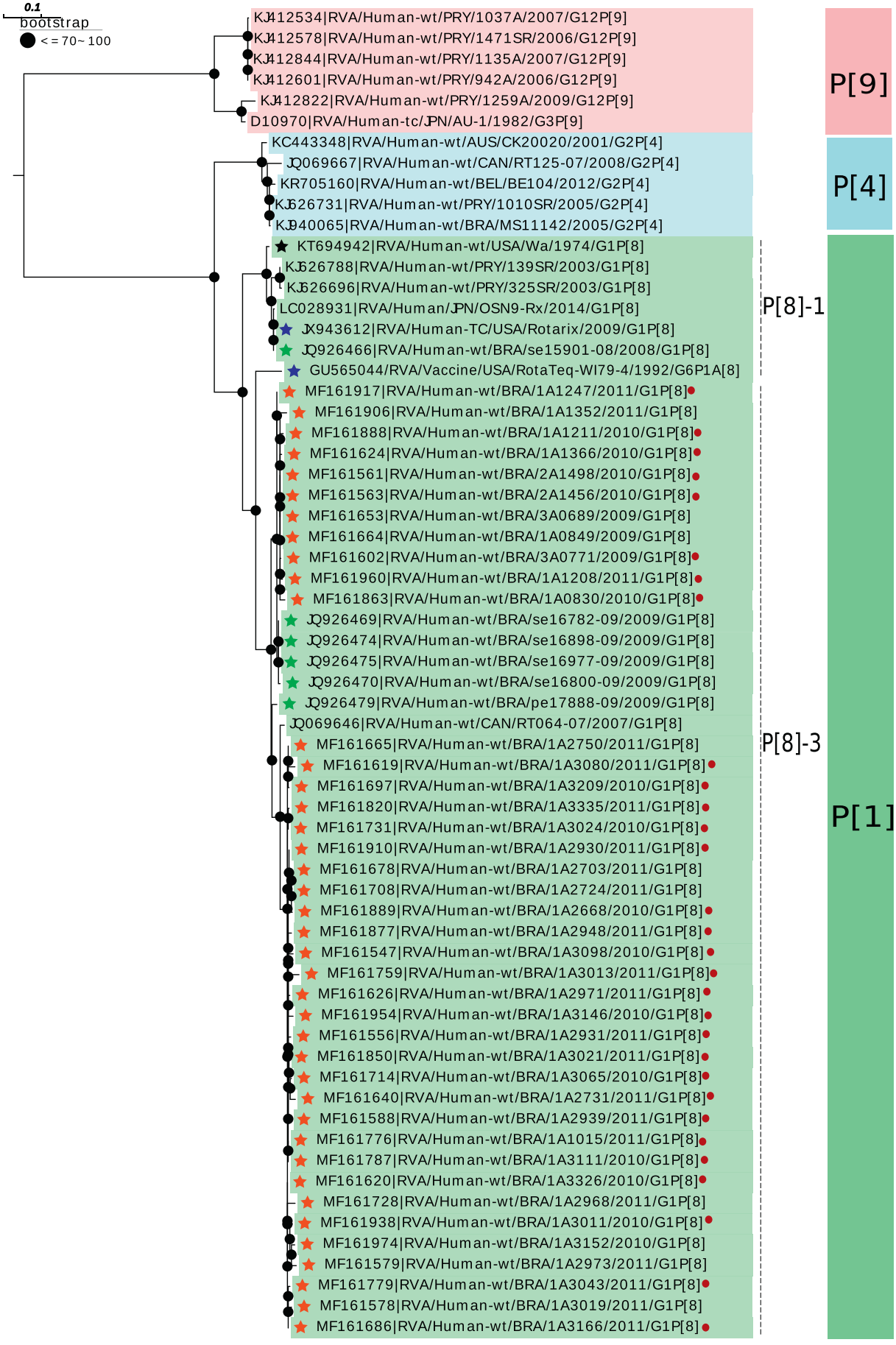

Fig. 1. (*continued*)

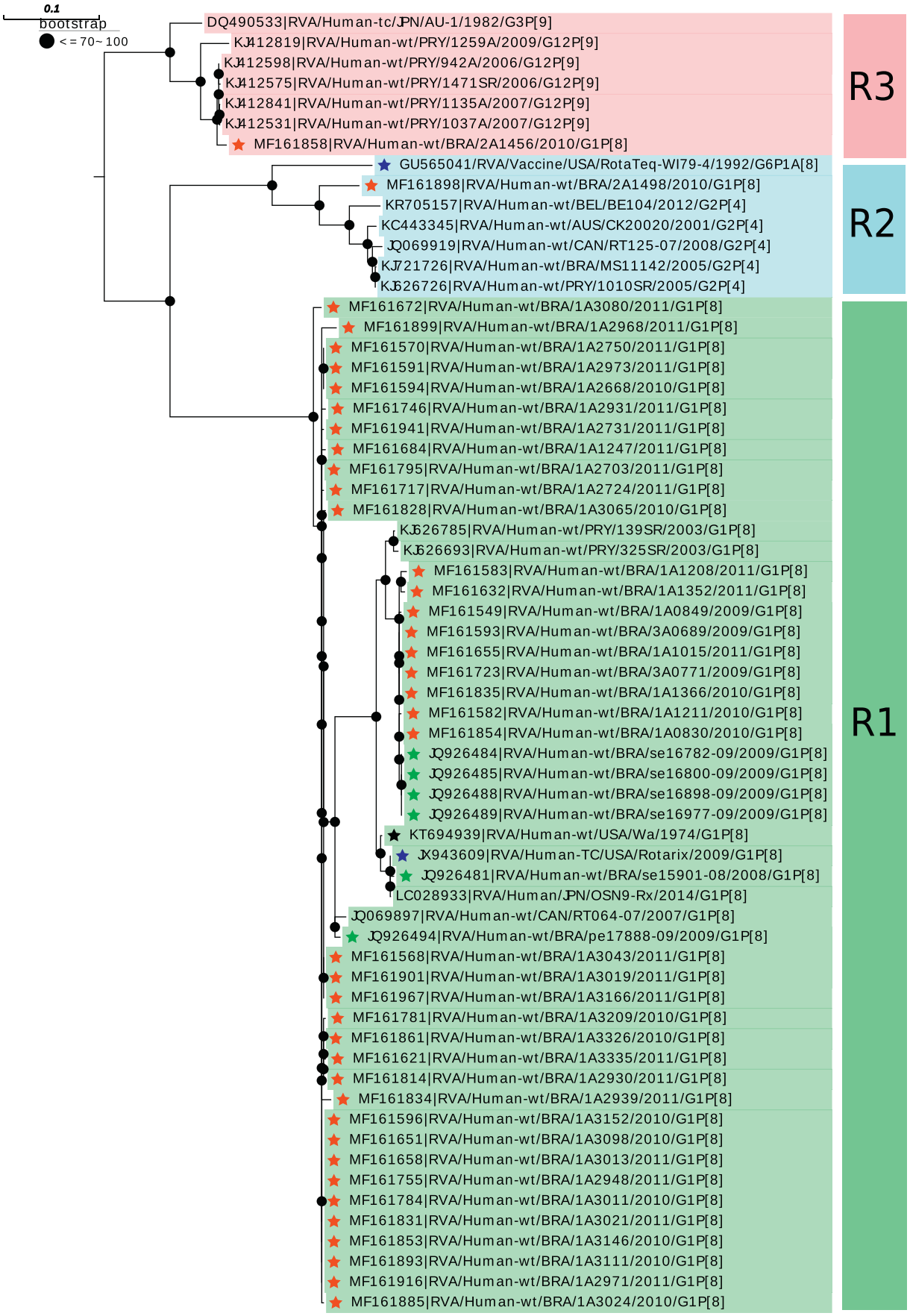

Fig. 1. (*continued*)

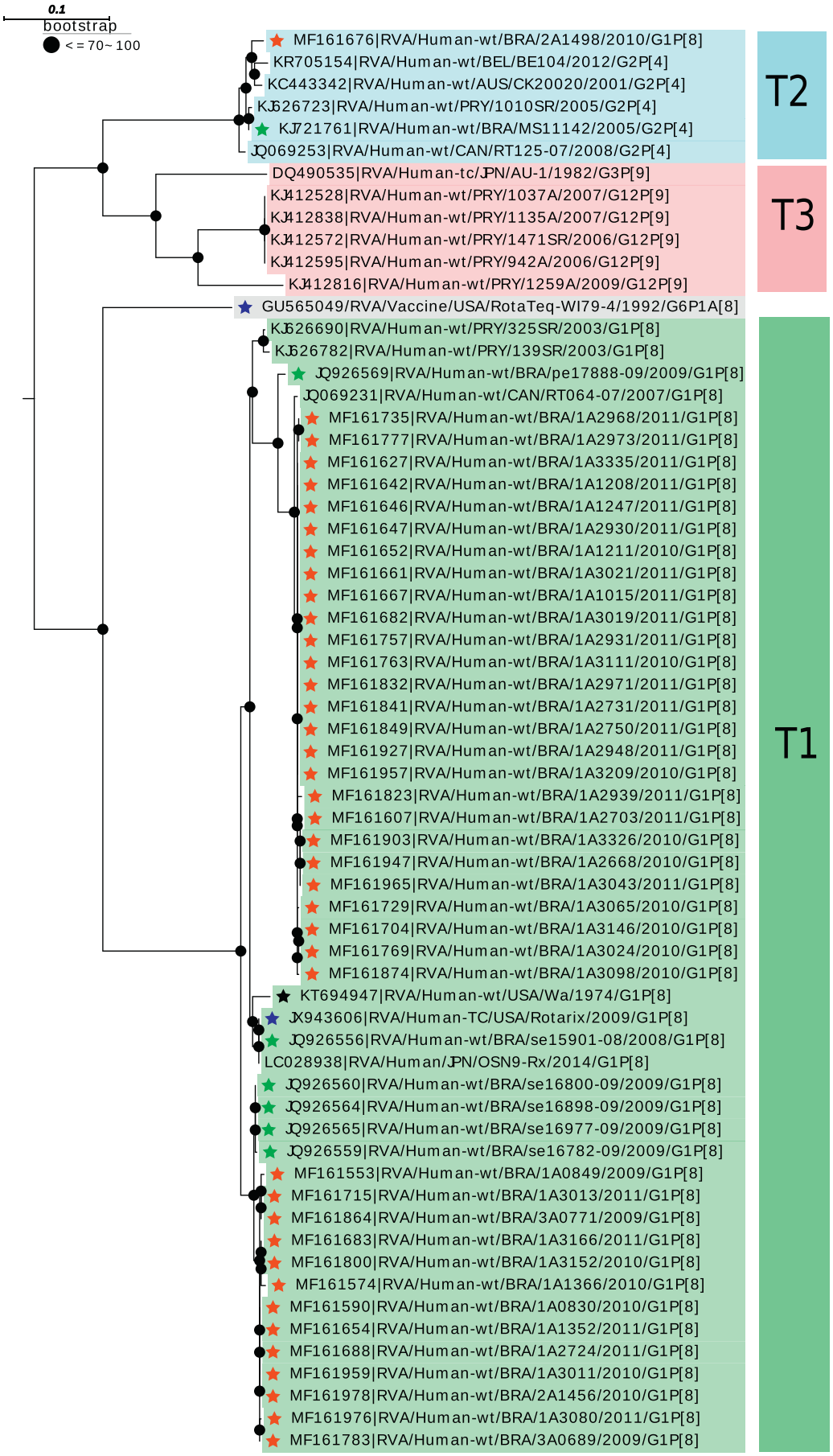

Fig. 1. (*continued*)

Table 2
Nucleotide and amino acidic similarities between RotaTeq® and Rotarix® vaccine strains and G1P[8] samples of the study.

		VP7	VP4	VP1	VP2	VP3	VP6	NSP1	NSP2	NSP3	NSP4	NSP5/6
G1P[8] strains[a]	nt	95.5–99.8	99.2–94.8	74.7–99.8	96.5–99.8	85.3–99.5	95.1–99.8	97.2–100	86–100	71.5–99.8	84.5–100	96.2–98.8
	aa	91.2–98.9	92.6–98.1	85.2–99.5	92.4–99.5	84.1–98.6	96–99.5	93.2–100	90–100	80.9–99.7	82–100	95.3–97.4
Rotarix®	nt	91.2–99.4	88.7–90.5	73.1–96.8	87.2–91	87.3–96.4	86.6–88.3	80.8–81.2	85.2–88.2	71–97.5	86.2–97.9	90.7–93.3
	aa	82.5–86.1	87.4–92.2	85.8–97.6	88.5–96.7	86.8–98.4	94.4–96	63.1–67.3	89.8–95.5	80.5–98	85.3–97.7	90.4–94.2
RotaTeq®	nt	87.6–90.9	90.8–93	71.6–85.2	69.6–78.3	87.3–96.4	71.8–75.5	45.9–52.2	76.6–81.3	69.2–78.1	69.5–76.5	85.5–87.1
	aa	78.6–83.3	90.5–94.2	86.8–95.8	84.5–95	86.8–97.5	88.2–91.9	38.2–34.4	83.5–88.6	80.1–86.6	73.5–84.6	89.2–92

[a] Mean nucleotide and amino acidic similarities of all strais from this study.

system were performed to prove the existence of inter-genogroup reassortment between human RVA genogroups (Heiman et al., 2008).

In the current study, only G1-I lineage was identified, differing from Rotarix® and RotaTeq®, that belong to lineage II. This observation is comparable to the reported by da Silva et al. (2015), who demonstrated that genotypes G1-I and G1-II were present in Brazil in 1990, 2000 and 2010, evidencing the fact that these two lineages have circulated before and after Rotarix® vaccine implementation. This reinforces the existence of phylogenetic differences that potentially tend to become more pronounced over time between the strains under comparison (Zeller et al., 2012). RVA strains belonging to lineage I were also found to be predominant in South Korea from 2004 to 2006, as reported by Le et al. (2010), and in Brazil, as reported by da Silva et al. (2013). Several studies on G1 strains have shown that G1 types may differ over time and according to the geographical region (Arora et al., 2009; Trinh et al., 2007).

Regarding to VP4-type specificity, P[8] strains clustered into lineage 3, as based on sub lineages defined by Espínola et al. (2008) and Arora and Chitambar (2011). da Silva et al. (2013) have reported the occurrence of lineage P[8]-3 for the first time in Brazil in combination with the G5 genotype, in samples from Rio de Janeiro. Since then, P[8]-3 was further found in specimens collected in pre- and post-vaccine period, from hospitalized children with acute gastroenteritis from other regions of Brazil, thus highlighting its continuous circulation in this country (Dulgheroff et al., 2016; Almeida et al., 2017).

Phylogenetic analysis of G1P[8] RVA Brazilian strains showed that most of them possess a Wa-like genotype constellation. These findings corroborate those of previous investigations conducted in Bangladesh (Rahman et al., 2010), China (Shintani et al., 2012), India (Arora and Chitambar, 2011), South Korea (Le et al., 2010) and Brazil (Rose et al., 2013).

Notably, analysis of the VP1 and NSP3 genes showed reassortment events. The data from our study are in agreement with the findings of Fujii et al. (2014) and Komoto et al. (2016), who observed this inter-genogroup reassortment involving Wa and DS-1-like constellations when analyzed G1P[8] RVA strains of children with diarrhea during the post-vaccine introduction period. These events in NSP3 gene were also observed in studies in Brazil by Rose et al. (2013) and da Silva et al. (2015), in which samples presented reassortments in T3 genotype.

Balatsos et al. (2012) have emphasized the importance of comparing

VP4

	8-1											8-2		8-3									8-4		
	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	136	87	88	89
G1P[8] (A41CB052A) of Rotarix®	D	S	S	N	S	S	A	N	L	N	N	E	R	N	P	V	D	S	S	N	D	N	N	T	N
G6P[8] (WI79-4) of RoTateq®	D	S	S	N	S	N	A	N	L	N	D	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A1015/2009/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3111/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3021/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2930/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3326/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3024/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3166/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2750/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3209/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2668/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2703/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2724/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2971/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3065/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2939/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3146/2010/G1P[8]	D	A	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2948/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3335/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2931/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3043/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3019/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3011/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3098/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3152/2010/G1P[8]	D	A	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2973/2011/G1P[8]	D	A	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2731/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A2968/2011/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3080/2010/G1P[8]	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A0830/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A0849/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/2A1456/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/2A1498/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/3A0689/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/3A0771/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A1208/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A1366/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A1247/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A1211/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A1352/2009/G1P[8]	D	G	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/BRA/1A3013/2011/G1P[8]	-	G	S	N	S	N	V	N	L	N	G	E	R	D	P	V	D	N	K	K	N	D	-	-	-

Fig. 2. Alignment of amino acidic residues of VP4 antigenic sites between Rotarix® and RotaTeq® vaccines and study strains. Different antigenic sites at both vaccine strains are shown in blue. Different antigenic sites at Rotarix® vaccine strain only are shown in green. Neutralization epitopes are shown in red numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

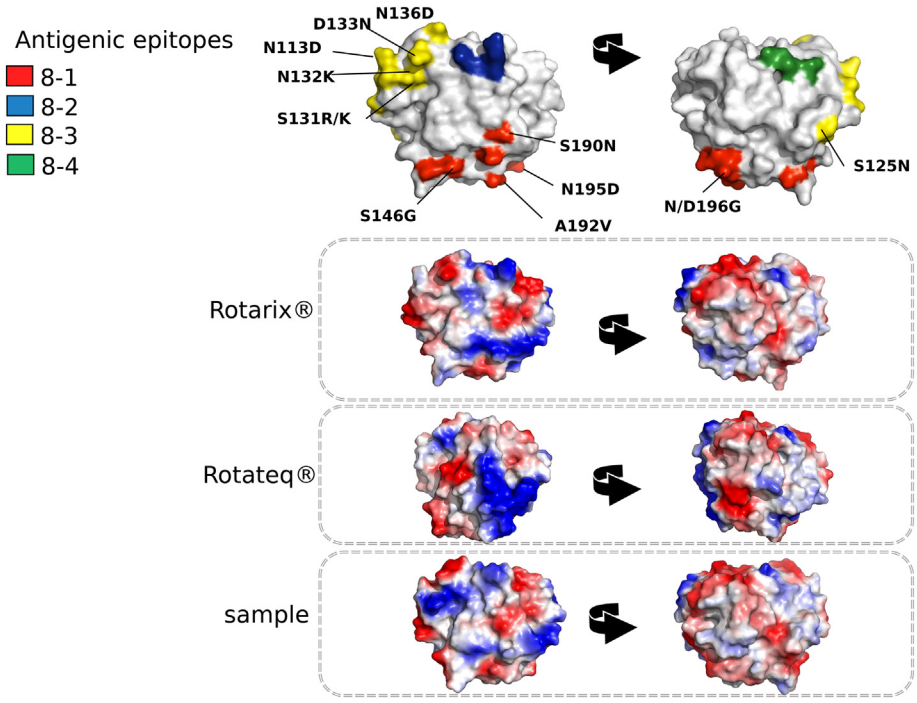

Fig. 3. Representation of VP8* protein nucleus surface. Antigenic epitopes are in red (8–1), blue (8–2), yellow (8–3) and green (8–4). Electrostatic changes in the surface protein are shown in comparison between Rotarix® and RotaTeq® vaccines and strains from the current study. Electronegative and electropositive charges are represented in red and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the protein structures to detect surface alterations generated by the electrical charge exchanges. The alteration of the electrical charge might promote modifications concerning to protein-protein interactions (Via et al., 2000). In our study, a number of changes involving aa present in the antigenic sites were detected, what became evident when comparing the VP4 and VP7 proteins of the circulating strains with proteins analogous proteins from Rotarix® and RotaTeq®.

The modelling of VP7 protein showed modification that may lead to higher changes related to charge when compared to Rotarix® and RotaTeq® VP7 protein. Our data are corroborated by findings from

VP7

	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
G1P[8] (A41CB052A) of Rotarix®	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
G1P[5] (WI79-9) of RoTateq®	T	T	N	G	D	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T		K	D	Q	S	L	S	M	N	G
RVA/Human-wt/BRA/1A0830/2009/G1P[8]	A	T	S	G	E	W	K	D	Q	N	V	V	A	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A1015/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2931/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/2A1456/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/3A0771/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A1247/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/3A0689/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A1211/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/2A1498/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A1352/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2703/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2724/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	N	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3326/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3080/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3166/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3111/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3065/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2731/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3019/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3152/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3024/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3146/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2930/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3011/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2948/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3021/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3013/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3043/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3098/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2750/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3335/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2668/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	N	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2971/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2968/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A3209/2010/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2973/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A1366/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A2939/2011/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A0849/2009/G1P[8]	L	T	S	G	E	W	K	D	Q	N	V	V	D	R		Q	N	V	D	K	T		K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BRA/1A1208/2009/G1P[8]	L	T	S	G	E	W	K	D	Q	N	V	V	C	R		Q	N	V	D	N	T		K	D	Q	N	L	S	T	N	G

Fig. 4. Alignment of amino acidic residues of VP7 antigenic sites between Rotarix® and RotaTeq® vaccines and study strains. Different antigenic sites at both vaccine strains are shown in blue. Different antigenic sites at RotaTeq® vaccine strain only are shown in green. The neutralization epitopes are shown in red numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

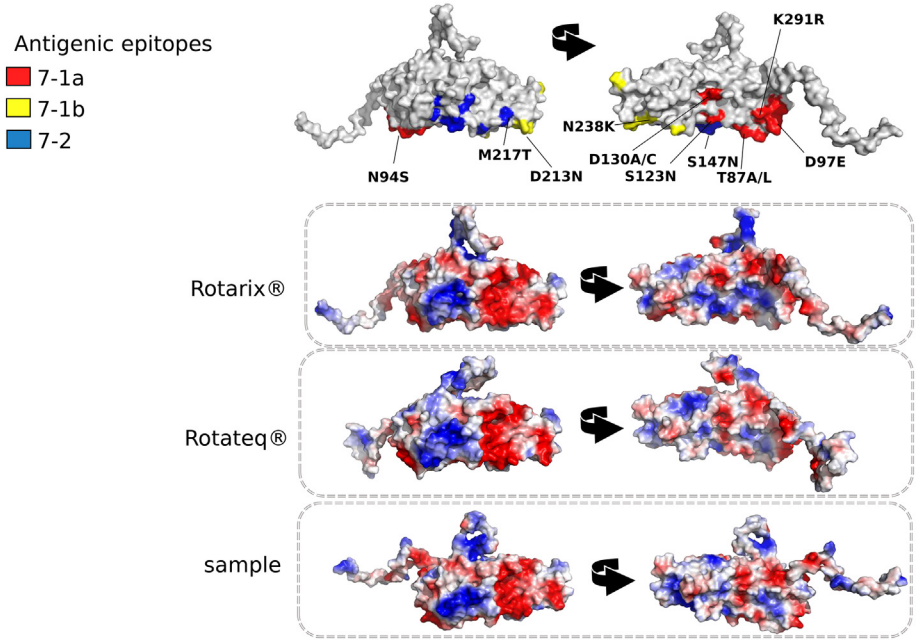

Fig. 5. Representation of VP7 protein nucleus surface. Antigenic epitopes are in red (7-1a), yellow (7-1b) and blue (7–2). Electrostatic changes in the surface protein are shown in comparison between Rotarix® and RotaTeq® vaccines and strains from the current study. Electronegative and electropositive charges are represented in red and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Phan et al. (2007) and Zeller et al. (2012), who reported antigenic alterations in similar regions; they were also able to identify substitutions involving residues 7-1a, 7-1b and 7–2 that are related to the loss of recognition of specific epitopes by G1 monoclonal antibodies (Mcdonald et al., 2009; Zeller et al., 2012). This event highlights the inherent broad genetic variability of G1 strains that infect humans. About VP4 gene, alterations in sites 8–1 and 8–3 were observed, similarly to those already described by Mouna et al. (2013) and Zeller et al. (2012).

Understanding the structural modifications of VP4 and VP7 binding proteins is highly significant in attempting to elucidate how such variations may lead to an efficient binding to cellular receptors, increasing infectivity levels. RVA binds to the cell via the type A HBGA receptors expressed in erythrocytes, epithelial cells and mucous membranes (Liu et al., 2013). Although these receptors are usually required, some evidences indicate that they are not essential for some human and animal RV strains (Fukudome et al., 1989; Keljo and Smith, 1988; Rolsma et al., 1994). VP4 protein is encoded by the fourth RV gene and, according to Ludert et al. (1996), may bind to the cell with or without sialic acid, both in vivo and in vitro. Investigations concerning about HBGA correlate the amplitude and epidemiology of virus-host interaction and demonstrate the importance of the VP4/VP8 * protein during RVA infection and pathogenesis. Therefore, structural changes may enhance virus interactions with the host cell and impair new vaccine strategies, since VP4 protein is present in both licensed vaccines (Huang et al., 2012; Liu et al., 2013).

Crystallographic studies performed by Hu et al. (2012) demonstrated that HBGA type A binds to human VP8* protein at the same binding site than sialic acid in animal VP8*. These findings suggest that even slight alterations within the same structure may enable the receptor switch.

Because of its localization at virus' outer capsid, VP7 protein is often exposed to selective pressure, what stimulates the development of viral immune evasion mechanisms as amino acidic hypervariability. This event may confer some survival advantage to the virus, leading it to escape from the host's immunological system action (Parbhoo et al., 2016). Elucidating studies showing how virus interaction with the host cells occur may support the understanding about possible vaccine failures.

Previous studies that have identified aa exchanges in VP4 and VP7 protein residues are scarce, especially in terms of their electrostatic charge changes. Therefore, our study dealt with the structural analyses of antigenic regions involving VP7 and VP4 regions of G1P[8] RVA in children with severe diarrhea in Northern Brazil.

Despite the significant reduction of RVA gastroenteritis cases after rotavirus vaccines implantation, studies on the genetic variability and the antigenic characteristics of G1P[8] RVA are worth to be performed, aiming to detect the emergence of novel variants that may have an impact on current vaccination strategies (Zeller et al., 2012; Arnold, 2018). Furthermore, the search for potential modifications in the protein structure may improve our knowledge of antigenic alterations that are able to give rise to potential immune escape mutants, thus posing a potential challenge to vaccination.

Finally, findings from our study are limited to samples obtained during the country's post-rotavirus vaccine introduction, so that a comparison with samples from the pre-rotavirus vaccine introduction era could not be made. A crucial aspect in this context is to keep continuously monitoring the circulation of rotavirus genotypes in our setting, extending the routine G- and P-typing assessment towards a broader, full-genomic characterization of prevailing strains. This would allow a more precise and representative identification of new emerging strains that might potentially pose a challenge to the currently adopted vaccination strategies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.02.009.

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