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Phylogenetic analysis of G1P[6] group A rotavirus strains detected in Northeast Brazilian children fully vaccinated with Rotarix™

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ABSTRACT

In 2009 the World Health Organization recommended the use of group A rotavirus (RVA) vaccines in all national immunization programs (NIPs) in order to control severe RVA gastroenteritis disease. In Brazil, Rotarix™ was introduced in the NIP in March 2006, and a significant reduction in mortality rates among children ⩽5 years old was observed, especially in the Northern and Northeastern Brazil. In the current study the 11 gene segments of six Brazilian G1P[6] RVA strains, isolated in 2009 and 2010 from vaccinated children, were analyzed in order to investigate if the genetic composition of these strains might help to elucidate why they were able to cause acute gastroenteritis in vaccinated children. All six Brazilian RVA strains revealed a complete Wa-like genotype constellation: G1-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1. Phylogenetic analysis showed that all six strains were nearly identical and showed a close genetic relationship with contemporary typical human Wa-like RVA strains. These results suggests that the fact that these strains were able to cause acute gastroenteritis in vaccinated children is likely not due to the genetic background of the strains, but rather to other factors such as host relating factors, co-infecting pathogens or vaccine efficacy. P[6] RVA strains are detected rather occasionally in humans in most regions of the world, except for South Asia and Sub-Saharan Africa. However, recently two studies conducted in Brazil showed the circulation of G12P[6] and G2P[6]. This is the first report on the detection and complete genome analyses of G1P[6] RVA strains in Brazil. Surveillance studies will be crucial to further investigate the prevalence of this genotype in the Brazilian population, and the efficacy of current licensed vaccines, which do not contain the P[6] genotype.

1. Introduction

Group A Rotaviruses (RVAs) belongs to the family *Reoviridae* and possesses a segmented double-stranded RNA genome composed of 11 segments encoding five (or six) nonstructural proteins (NSPs) and six structural proteins (VPs) (Estes and Kapikian, 2007). Two viral surface proteins, VP7 and VP4, are used to classify RVA strains into G- (Glycolyslated) and P-types (Protease sensitive), respectively. An extended classification system for RVA strains based on all the 11 gene segments was developed by the Rotavirus Classification Working Group (RCWG). This system defines the following genotypes: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, based on nucleotide similarities cut off values for the VP7, VP4, VP6, VP1-3, NSP1-5/6 encoding genome segments, respectively. Currently, 27 G-, 37 P-, 17 I-, 9 R-, 9C-, 8 M-, 16 A-, 10 N-, 12 T-, 15 E-, and 11 H-genotypes have been described (Guo et al., 2012; Matthijnssens et al., 2011; Papp et al., 2012; Trojnar et al., 2012). Worldwide the majority of human RVA strains 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) (Heiman et al., 2008; Matthijnssens et al., 2008; McDonald et al., 2009; Matthijnssens and Van Ranst, 2012). Until now, only one Brazilian RVA strain, RVA/Human-wt/BRA/IAL28/1992/G5P[8], has been completely characterized to date, and possesses a Wa-like genome constellation (Heiman et al., 2008).

Annually RVA gastroenteritis accounts for approximately one third of the total diarrheal deaths worldwide (Black et al., 2010). In developing countries improving sanitary conditions and access to a safe water supply alone will not be sufficient to prevent RVA

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gastroenteritis. Malnourished children with poor health are more vulnerable to serious infections causing acute diarrhea and suffer multiple episodes of acute diarrhea every year (United Nations Children's Fund (UNICEF)/World Health Organization (WHO), 2009). Therefore, vaccination is considered the best alternative among public health measures to reduce and prevent the global burden caused by RVA infections. In Brazil, the monovalent (G1P[8]) Rotarix™ vaccine (GlaxoSmithKline, Rixensart, Belgium) was introduced in the NIP in March, 2006, and vaccine coverage have been estimated at 83% in 2010 (Centers for Disease Control, 2011). Recent studies have shown a significant reduction in morbidity and mortality rates among children younger than 5 years old, especially in the Northern and Northeastern regions of Brazil where the rates of mortality are higher than in other Brazilian regions (do Carmo et al., 2011; Carvalho-Costa et al., 2011).

RVA strains bearing the P[6] genotype have been detected in combination with a wide range of G-genotypes (G1-G6, G8-G12, and G25) worldwide, and in combination with both Wa-like and DS-1 like genotype constellations (Kirkwood et al., 1999; Linhares et al., 2002; Rahman et al., 2007a; Heiman et al., 2008; Esona et al., 2010; Mwenda et al., 2010; Rippinger et al., 2010; Le et al., 2011). In industrialized countries P[6] RVA strains are rarely detected in humans. However, human P[6] RVA strains have been described as one of the most prevalent genotypes in South Asia and Sub-Saharan Africa (Ramani, 2007; Armah et al., 2010; Todd et al., 2010). In Brazil, the P[6] genotype has been sporadically detected in association with G1–5, G8, and G9, in four out of five Brazilian regions: North, Northeast, Southeast, and West Central (Santos et al., 1994; Timenetsky et al., 1994; Leite et al., 1996; Araujo et al., 2001; Linhares et al., 2002; Mascarenhas et al., 2002, 2006, 2007; Volotao et al., 2006; Gurgel et al., 2008; Leite et al., 2008; Carvalho-Costa et al., 2011; Soares et al., 2012).

A limited number of human P[6] RVA strains have been completely characterized to date, none of them in combination with G1 (Rahman et al., 2007b; Heiman et al., 2008; Matthijnssens et al., 2006, 2008; Pietsch and Liebert, 2009; Rippinger et al., 2010; Wang et al., 2010; Jere et al., 2011; Than et al., 2011; Heylen et al., 2012; Zeller et al., 2012a). The main objective of the current study was to analyze six G1P[6] RVA strains isolated from vaccinated children that were hospitalized with acute gastroenteritis in Northeastern, Brazil, in order to investigate if the genetic composition of these strains might help to understand why these strains were able to cause acute gastroenteritis despite the fact that these children were vaccinated.

2. Materials and methods

2.1. Clinical samples

Six stool samples collected from hospitalized children with acute gastroenteritis vaccinated with two doses of Rotarix™ were collected from northeastern Brazilian states: Bahia (RVA/Human-wt/BRA/BA17290/2009/G1P[6]), Ceará (RVA/Human-wt/BRA/CE17436/2010/G1P[6]), Alagoas (RVA/Human-wt/BRA/AL18874/2010/G1P[6]), and Pernambuco (RVA/Human-wt/BRA/PE18948/2010/G1P[6]; RVA/Human-wt/BRA/PE18949/2010/G1P[6] and RVA/Human-wt/BRA/PE18963/2010/G1P[6]) (Table 1).

2.2. Nucleic acid extraction and RT-PCR

Nucleic acid was extracted from 200 μl of 10% fecal suspensions by the glass powder method described by Boom et al. (1990), including the following modifications: 200 μl of 10% of fecal suspensions were added to 500 μl of L6 buffer, vortexed for 5 s and kept at room temperature for 5 min. Subsequently, 7.5 μl of silica solution was added and the tubes were placed in an orbital shaker for 20 min. After centrifugation at 16,000*g* for 30 s the supernatant was discarded and the silica pellet was washed with 500 μl of L2 buffer, 500 μl of 70% ethanol, and 500 μl of acetone. After each wash, the sample was centrifuged for 30 s at 16,000*g*, and the supernatants were discarded. Tubes were dried at 56 °C for 15 min. The pellet was dissolved in 60 μl of RNAse-DNAse free water, vortexed, and incubated for 15 min at 56 °C with the lid closed. Afterwards, the tube was vortexed and centrifuged at 16,000*g* for 3 min. Finally, the nucleic acid-containing supernatant was recovered in a new tube and stored at −20 °C.

Amplification of the VP6 and VP7 gene segments were performed using the SuperScript™ III One-Step RT-PCR System with the Platinum™ Taq DNA Polymerase Kit (Invitrogen™, Brazil) using the following temperature profile: 55 °C for 1 h, 94 °C for 5 min, 40 cycles of 94 °C/1 min, 55 °C/1 min, and 72 °C/3 min, with a final extension of 72 °C for 7 min. To amplify the NSP1–3 and NSP5 genes the SuperScript™ III One-Step RT-PCR System with the Platinum™ Taq DNA Polymerase Kit (Invitrogen™, Brazil) was used as described in previous studies (Nakagomi and Kaga, 1995; Matthijnssens et al., 2006). Reverse transcription (RT) for the NSP4, VP1-3, and VP4 (VP8*) gene segments were performed with the High Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems, Brazil) according to the manufacturer's instructions. The PCR protocol used to amplify VP8* was described by Gentsch et al. (1992), with modifications from Gómez et al. (2010). For NSP4 the protocol described by Gómez et al. (2011) was used. The VP1 gene segment was partially amplified according to the protocol of Varghese and colleagues (2006), and the PCR for the amplification of VP2 and VP3 was carried out as follows: 94 °C for 2 min, 40 cycles of amplification (30 s at 94 °C, 30 s at 50 °C, and 1.5 min at 72 °C, with a final extension of 7 min at 72 °C. All primers used in the current study, and the lengths of the obtained fragments are shown in Supplementary material Table 1.

2.3. Sequencing and phylogenetic analyses

Sequencing was performed with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit™ and an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Instituto de Tecnologia em Imunobiológicos (Bio-Manguinhos), FIOCRUZ. The same set of primers used in the RT-PCR was used for

Table 1
Available patient information for G1P[6] rotavirus A strains analyzed in this study, including: Brazilian states were the samples were collected; date of birth; age of the patient at the time of sample collection; vaccine doses administration dates.

Sample	Brazilian State	Date of birth	Age	1st Rotarix™ Dose	2nd Rotarix™ Dose	Sample collection date
RVA/Human-wt/BRA/BA17290/2009/G1P[6]	Bahia	–	2 years			30-Sep-2009
RVA/Human-wt/BRA/CE17436/2010/G1P[6]	Ceará	April 4, 2006	3 years and 9 months	6-May-2006	4-Aug-2006	14-Jan-2010
RVA/Human-wt/BRA/AL18874/2010/G1P[6]	Alagoas	January 30, 2009	1 year and 6 months	4-Jan-2009	1-Jun-2009	26-Aug-2010
RVA/Human-wt/BRA/PE18948/2010/G1P[6]	Pernambuco	March 13, 2007	3 years and 6 months	15-May-2007	17-Jul-2007	5-Oct-2010
RVA/Human-wt/BRA/PE18949/2010/G1P[6]	Pernambuco	February 1, 2007	3 years and 9 months	4-Mar-2007	2-Jul-2007	5-Oct-2010
RVA/Human-wt/BRA/PE18963/2010/G1P[6]	Pernambuco	September 18, 2009	1 year	19-Nov-2009	10-Feb-2010	5-Oct-2010

sequencing, plus two internal primers for the NSP3 encoding gene (Tort et al., 2010). All reactions were repeated at least twice for the accuracy of the study.

Sequences obtained in the current study were deposited in GenBank database under the following accession numbers: JN869248–JN869289; JX455052–JX455075.

Multiple sequence alignments were carried out using the ClustalW program (Thompson et al., 1994). Phylogenetic analyses were performed 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).

3. Results

3.1. Genome constellation of Brazilian G1P[6] RVA strains

Analysis of the 11 RVA genome segments revealed that all six Brazilian RVA samples detected from vaccinated children possessed the Wa-like genotype, and showed the following genotype constellation: G1-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1. The same genotype constellation has been previously described for human P[6] strains in combination with G3, G4, G11, and G12 (Table 2). The six analyzed strains were nearly identical across their entire genome both at the nucleotide and amino acid level, with identity values ranging from: 99.2–100% and 98.6–100%, respectively.

3.2. Phylogenetic and sequence analyses of VP8*(VP4) and VP7

Phylogenetic analysis based on the VP8* (VP4) encoding gene showed a close genetic relationship between the Brazilian P[6] strains and a G2P[6] RVA strain detected in the USA in 2006 (RVA/Human-wt/USA/06-242/2006/G2P[6]) and four recently described P[6] strains that were found in combination with G1 or G6 and were detected in Burkina Faso (RVA/Human-wt/BFA/267-BF/2010/G1P[6]; RVA/Human-wt/BFA/225-BF/2010/G1P[6]; RVA/Human-wt/BFA/259-BF/2010/G1P[6]; RVA/Human-wt/BFA/281-BF/2010/G1G6P[6]) inside P[6]-I lineage (Fig. 1A). Nucleotide and amino acid identity values among these strains and Brazilian G1P[6] strains ranged between 98.0–98.7% and 97.4–98.5%, respectively. Previously detected P[6] strains in combination with G2, G4 and G9 in Brazil also belonged to P[6]-I lineage but clustered separately. Seventeen amino acid differences were observed in previous described antigenic sites when compared with the Rotarix™ vaccine strain, which belongs to the P[8] genotype (Supplementary material Table 2).

Phylogenetic analysis revealed a close genetic relationship between the VP7 gene segments of G1P[6] and G1P[8] RVA strains detected worldwide between 2006 and 2010, all clustering in lineage G1-I (Fig. 1B). The most closely related strains were G1P[8] strains from USA (RVA/Human-wt/USA/2007719739/2007/G1P[8]), Belgium (RVA/Human-wt/BEL/BE00039/2008/G1P[8]) and Nicaragua (RVA/Human-wt/NCA/28 J/2010/G1P[8]), showing 98.9–99.1%–99.2–100% of nucleotide and amino acid identity values, respectively. Other Brazilian G1P[8] samples from the same geographical region (Northeast Brazil), detected from vaccinated children in Sergipe and Pernambuco in 2009 also clustered relatively close to the G1P[6] Brazilian strains (97.7–98.6%–98.1–99.6%, nucleotide and amino acid identity values, respectively). The VP7 gene of Rotarix™ grouped in lineage G1-II together with Brazilian samples detected between 1998 and 2005, and revealed 93.4–93.6% and 94.2–94.5% of nucleotide and amino acid identity. Analysis of the amino acid sequences, revealed that the Brazilian G1P[6] strains carried four amino acid substitutions inside previous described antigenic sites when compared with Rotarix™, at positions: N94S, S123N, K291R and M217T (Supplementary material Table 3).

3.3. Phylogenetic analyses of the NSP1–5 genome segments

Phylogenetic analysis based on NSP1–4 nucleotide sequences, shown that all six Brazilian G1P[6] strains belonged to a monophyletic cluster, showing a close genetic relationship with contemporary typical human Wa-like RVA strains detected worldwide (Australia, Bangladesh, Belgium, Brazil, India, Nicaragua, South Africa and USA) belonging to different G- (G1, G9, G11, G12) and P-genotypes (P[4], P[6], P[8], P[25]) (Fig. 2).

The most closely related RVA strains for NSP1 were: two G12 strains from South Africa, RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6] (Fig. 2A), with identity values ranging from 98.9–99.1% and 98.5–98.9% for nucleotide and amino acid sequences, respectively; for NSP2, two G1P[8] strains from Australia(RVA/Human-wt/AUS/CK00001/2004/G1P[8] and RVA/Human-wt/AUS/CK00009/2004/

Table 2
Genotype constellation comparison. The six Brazilian G1P[6] RVA strains analyzed in the current study are represented by RVA/Human-wt/BRA/BA17290/2009/G1P[6] strain. Green and red indicate Wa-like and DS-1-like gene segments, respectively. The P[6] VP4 genotype is colored yellow, and blue is used to indicate a gene segment of porcine origin. Brazilian strains are shown in bold.

	Strain	Origin	Genotypes										
			VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Wa-like	RVA/Human-tc/USA/Wa/1974/G1P[8]	Human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/USA/Rotarix-A41CB052A/1988/G1P[8]	Human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BGD/Dhaka12/2003/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/ZAF/3176WC/2009/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BGD/Matlab13/2003/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T2	E1	H1
	RVA/Human-tc/KOR/CAU195/200X/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/DEU/GER172/2008/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/ECU/EC2184/200x/G11P[6]	Human	G11	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-tc/GBR/ST3/1975/G4P2A[6]	Human	G4	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-tc/AUS/RV3/1977/G3P[6]	Human	G3	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BRA/BA17290/2009/G1P[6]	Human	G1	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
DS-1-like	RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	Human	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/BEL/F01322/2009/G3P[6]	Human	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/COD/DRC86/2003/G8P[6]	Human	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/BGD/N26/2002/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N1	T2	E6	H2
	RVA/Human-wt/BGD/RV161/2000/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E1	H2
	RVA/Human-wt/BGD/RV176/2000/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2
	RVA/Human-wt/USA/06-242/2006/G2P[6]	Human	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2

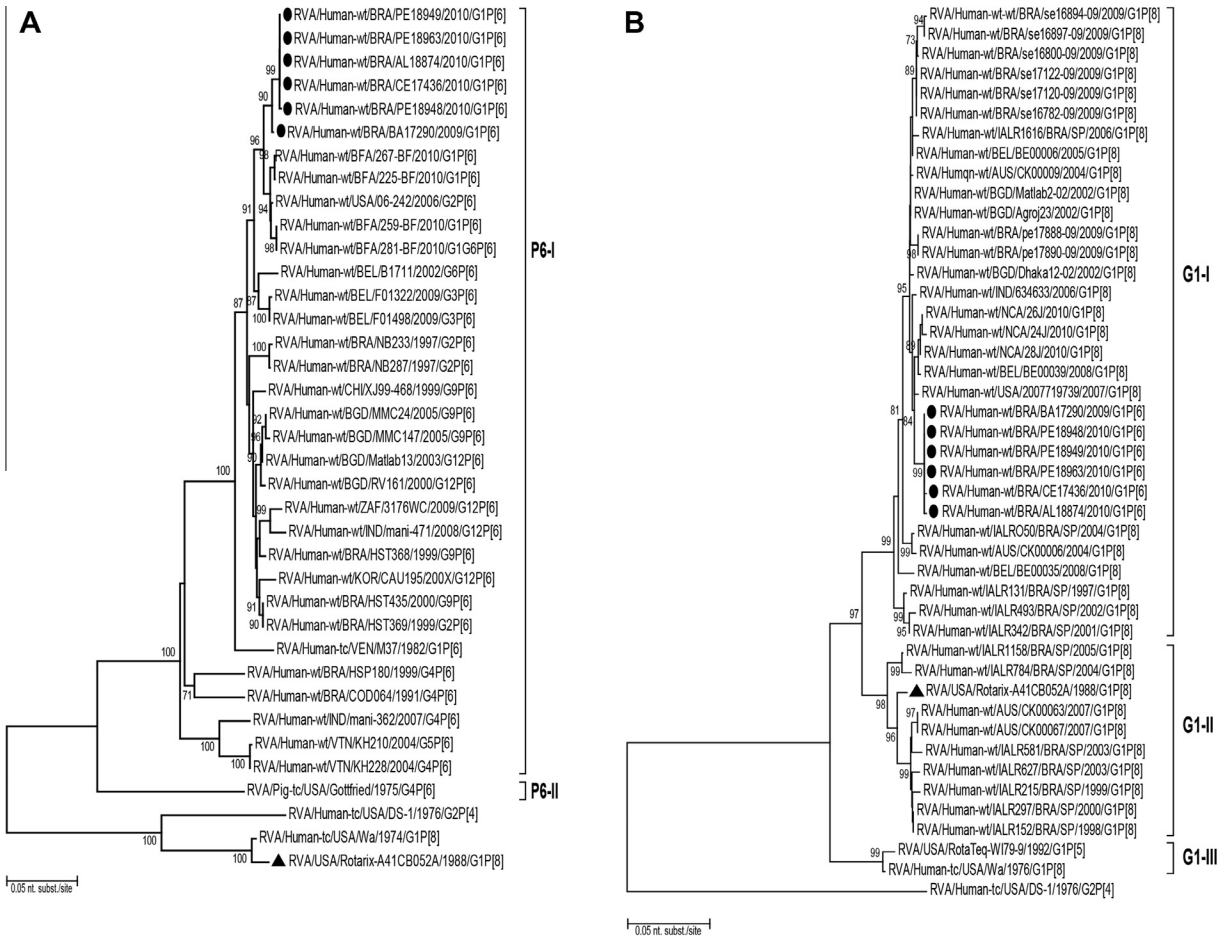

Fig. 1. Phylogenetic analysis based on the VP8* (A) and VP7 (B) gene nucleotide sequences of Brazilian rotavirus A G1P[6] strains, and sequences from the GenBank database. Numbers at the nodes indicate Bootstrap values, 2000 re-sampled datasets; only values above 70% are shown. The scale bar at the bottom represents 0.05 substitutions per nucleotide position (nt.subst./site). Brazilian G1P[6] strains are marked with a filled circle. Rotarix™ vaccine strain is marked with a filled triangle.

G1P[8]) and two from Bangladesh (RVA/Human-wt/BGD/Matlab36/2002/G11P[8] and RVA/Human-xx/BGD/MMC71/2005/G1P[8]), shared the highest identity values ranging from 98.6–98.8% at the nucleotide and 99–99.3% at the amino acid level (Fig. 2B); for NSP3, three G9P[8] strains from Rio de Janeiro (Southeast, Brazil), RVA/Human-wt/BRA/rj1528/1998/G9P[8], RVA/Human-wt/BRA/rj1527/1998/G9P[8] and RVA/Human-wt/BRA/rj1538/1998/G9P[8] (Fig. 2C), identity values ranged from 99.4–99.5% on the nucleotide level, and on the amino acid level strains were 100% identical; and for NSP4: two strains from the USA detected in 2007–2008, RVA/Human-wt/USA/2007719739/2007/G1P[8] and RVA/Human-wt/USA/VU08–09-39/2008/G12P[8] (Fig. 2D), showing 99.0–99.4% identity for nucleotide sequences and they were 100% identical in the amino acid level.

In the case of NSP5, phylogenetic analysis showed that the Brazilian G1P[6] strains clustered together with two G1P[8] Brazilian strains, also from vaccinated children, detected in Pernambuco, Northeastern, in 2009 (RVA/Human-wt/BRA/pe17888/2009/G1P[8] and RVA/Human-wt/BRA/pe17890/2009/G1P[8]), and two samples from South Africa (Fig. 2E). Identity values among Brazilian G1P[6] strains and G1P[8] RVA strains from Pernambuco ranged from 99.8–100% and 99.4–100% on the nt and aa level, respectively. Two strains from South Africa (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) also clustered nearby and showed 99.6–99.8% and 99.4–100% nucleotide and amino acid identity values comparing with the Brazilian G1P[6] strains.

3.4. Phylogenetic analyses of the VP1–3 and VP6 genome segments

As observed for the NSP coding genes, the VP1–3 and VP6 encoding gene segments of the G1P[6] Brazilian strain showed a close genetic relationship with Wa-like strains detected worldwide (Argentina, Australia, Bangladesh, Belgium, Brazil, India, Nicaragua, South Africa, Thailand and USA) possessing the G1, G9, G11, G12 and P[4], P[6], P[8], P[25] genotypes (Fig. 3).

For the VP1 and VP2 encoding genes, Brazilian samples grouped in a monophyletic cluster and showed the closest genetic relationship with strains from Australia (VP1: RVA/Human-wt/AUS/CK00095/2010/G1P[8]; VP2: RVA/Human-wt/AUS/CK00006/2004/G1P[8]) and USA (VP1: RVA/Human-wt/USA/VU06-07-21/2006/G3P[8]; VP2: RVA/Human-wt/USA/2007719739/2007/G1P[8]) (Fig. 3A and B). Nucleotide and amino acid identity among Brazilian strains and closely related strains was 98.7–99.1%–100%, and 95.6–99.2%–98.6–99.6%, for VP1 and VP2, respectively. The Brazilian G1P[6] strains also clustered in a monophyletic cluster when analyzing the VP3 encoding gene (Fig. 3C). The most closely related strain was RVA/Human-wt/AUS/CK00006/2004/G1P[8], showing 98.6–98.7% and 99.0–99.3% of nucleotide and amino acid identity values, respectively. The VP6 nucleotide sequences of the Brazilian G1P[6] strains formed a monophyletic cluster (Fig. 3D). The most closely related strains were one strain from Australia (RVA/Human-wt/AUS/CK00088/2009/G1P[8]) and one strain from India (RVA/Human-xx/IND/ISO94/2005/G9PX), showing 99.1–99.5% and 99.4–99.7% of nucleotide and amino acid identity values, respectively, compared with Brazilian G1P[6] strains.

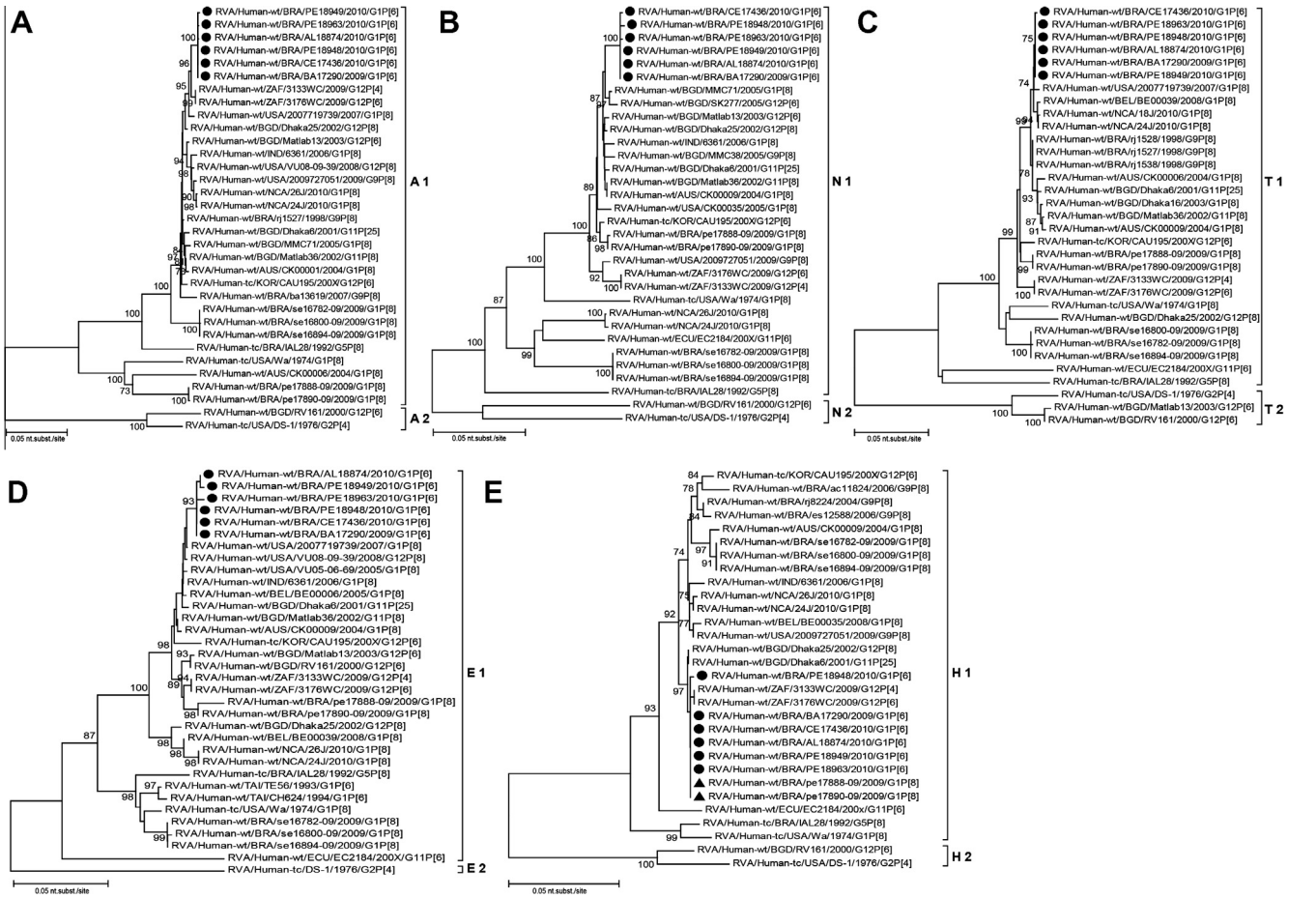

Fig. 2. Phylogenetic analysis based on NSP1 (A), NSP2 (B), NSP3 (C), NSP4 (D) and NSP5 (E) nucleotide sequences of Brazilian rotavirus A G1P[6] strains, and sequences from the GenBank database. Numbers at the nodes indicate Bootstrap values, 2000 re-sampled datasets; only values above 70% are shown. The scale bar at the bottom represents 0.05 substitutions per nucleotide position (nt.subst./site). Brazilian G1P[6] strains are marked with a filled circle.

4. Discussion

In this study, six G1P[6] RVA strains have been identified from children vaccinated with two doses of Rotarix™ vaccine in the Northeastern region of Brazil. Current licensed RVA vaccines target common antigens (G1–G4 and P[8]) considered important in eliciting neutralizing antibody responses. Studies regarding the efficacy of the Rotarix™ vaccine against circulating genotypes did not include P[6] strains, since this genotype was not a prevalent P-genotype during the clinical trials. Recently, two studies conducted in Brazil showed the circulation of P[6] strains: (a) Fumian and colleagues (2011) revealed the circulation of P[6] genotypes in Rio de Janeiro after introduction of the Rotarix™ vaccine, accounting for 25% of the P-genotypes detected from sewage influent samples, in combination with genotype G2; (b) Soares and colleagues (2012) detected G12P[6] RVA strains in the Northern region. Unfortunately, only partial NSP4 sequences are available from the P[6] detected in Rio de Janeiro, and only VP7 sequences are available from the G12P[6] strains detected in the Northern region. Together these results suggests that in recent years the P[6] genotype circulated in some parts of Brazil in combination with at least three different G-genotypes (G1, G2 and G12). Further studies should be conducted in order to investigate the prevalence of P[6] genotype in Brazil.

Results obtained in the current study showed that the VP8* sequences from P[6] Brazilian strains were closely related to African strains, as well as with one G2 strain from the USA (Heylen et al., 2012; Nordgren et al., 2012) (Fig. 1A). These strains all shared an amino acid change at position N135S, when compared with Rotarix™ strain, that was not present in other P[6] samples analyzed (Supplementary material Table 2). In total, seventeen amino acid changes were observed in the VP8* protein antigenic regions among Brazilian P[6] strains and the Rotarix™ vaccine strain (Supplementary material Table 2), showing that they are highly diverse as previously observed by Zeller et al. (2012b). Analysis of the VP7 encoding gene showed that the most recently detected G1 strains in Brazil belonged to a distinct G1 lineage (G1-I) when comparing with the Rotarix™ vaccine strain (genotype G1-II) (Fig. 1B). Four amino acid substitutions inside previous described antigenic sites were observed when compared Brazilian strains with the Rotarix™ vaccine at positions N94S, S123N, M217T, and K291R (Supplementary material Table 3). The fact that these substitutions were observed in almost all G1-I strains may suggests that they have been positive selected over time and might be related to some kind of advantage regarding viral fitness. It has been previously suggested that G1-I strains that accumulated mutations in these amino acid residues, could evolve over time into a variant that may be able to (partially) escape the immune response elicited by the vaccine, potentially causing a vaccine breakthrough (Maranhao et al., 2012). It is important to emphasize that the samples analyzed in the current study did not correspond to an outbreak.

All six G1P[6] Brazilian strains analyzed in this study possessed a Wa-like genotype constellation (Table 2), and showed a close genetic relationship among each other and with contemporary RVA strains from all over the world, with different G- and P-genotype combinations, and also for the other RVA genes (Figs. 1A and B, 2

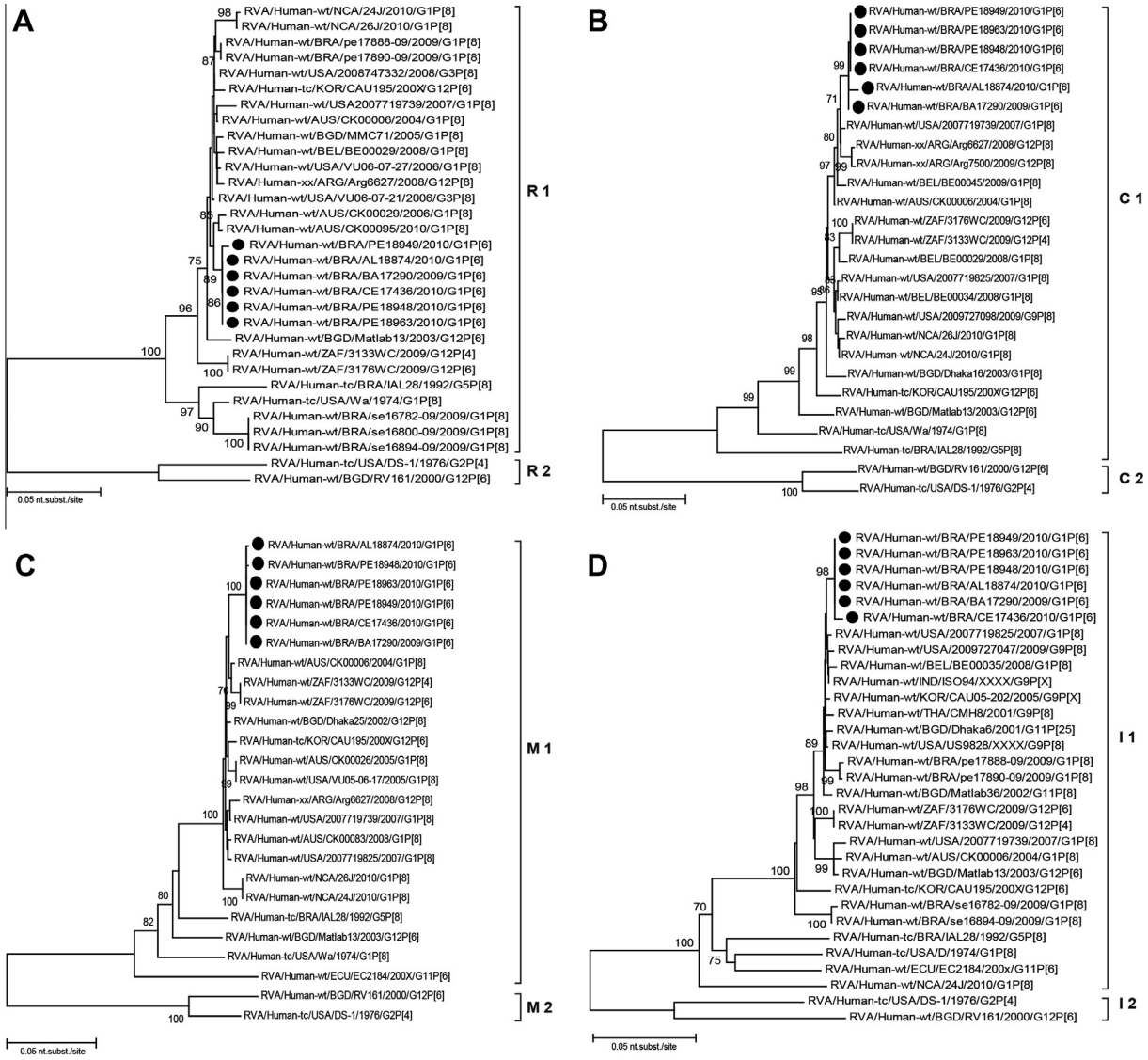

Fig. 3. Phylogenetic analysis based on VP1 (A), VP2 (B), VP3 (C) and VP6 (D) nucleotide sequences of Brazilian rotavirus A G1P[6] strains, and sequences from the GenBank database. Numbers at the nodes indicate Bootstrap values, 2000 re-sampled datasets; only values above 70% are shown. The scale bar at the bottom represents 0.05 substitutions per nucleotide position (nt.subst./site). Brazilian G1P[6] strains are marked with a filled circle.

and 3). Phylogenetic analysis of the NSP5 encoding gene suggests the occurrence of reassortment events between P[8] and P[6] co-circulating strains in Brazil (Fig. 2E). The fact that these strains just shared 1 out of the 11 RVA encoding genes could suggest that the P[6] strains analyzed in the current study were imported from elsewhere or that they are related to yet unknown RVA strains circulating in Brazil.

In addition, Brazilian G1P[6] strains analyzed cannot be considered as heterotypic strains when compared with Rotarix™ strain since they possess a Wa-like genome constellation and only the VP8* (VP4) encoding gene belonged to a different P-genotype (Matthijnssens and Van Ranst, 2012). These results suggests that most likely the reason of the breakthrough cases was not due to the genetic background of the analyzed strains, but rather due to other factors. Whereas VP4 and VP7 play an important role by stimulating the production of neutralizing antibodies, other factors such as high titers of rotavirus-specific antibodies in breast milk and levels of RV-specific IgA antibodies in the gut lumen have been described as possibly interfering with the efficacy of orally administered RVA vaccines. However further studies are needed to be carried out for a better understanding of the mechanisms associated to RVA protection (Desselberger and Huppertz, 2011).

The six RVA genomes reported in this paper represents the first report of 11 RVA genome segments from G1P[6] strains. As previously mentioned, to date only one strain from Brazil has been completely characterized (Heiman et al., 2008). Sequence data from all 11 RVA genome segments from circulating strains may help to assess the impact of RVA vaccines in strain diversity and evolution, as well as in identifying RVA strains that might challenge the efficacy of currently licensed vaccines (Leite et al., 2008; Carvalho-Costa et al., 2011; Yen et al., 2011).

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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.2013.03.028.

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