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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