Detection of a novel equine-like G3 rotavirus associated with acute gastroenteritis in Brazil


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INTRODUCTION

Diarrhoeal diseases represent a major worldwide cause of morbidity and mortality in children aged <5 years worldwide, and rotavirus (RV) still remains a very common cause of severe diarrhoea, leading to approximately 200 000 deaths each year (Das & Bhutta, 2016; Tate et al., 2016).

RVs belong to the Reoviridae family, Sedoreovirinae subfamily and Rotavirus genus (ICTV, 2015). The RV virion possesses a triple-layered, non-enveloped capsid of icosahedral symmetry surrounding a genome of 11 segments of dsRNA. Altogether, the RV genome encodes six structural proteins (VP1–VP4, VP6 and VP7) and five or six non-structural proteins (NSP1–NSP5/6), with the majority of segments being monocistronic, except for RNA segment 11 which encodes NSP5 and NSP6. In view of the segmented nature of the genome, genetic reassortment involving co-circulating strains of human and animal origins has shown to be a common event (Donato et al., 2014).

Based on the serological reactivity and genetic variability of VP6 gene, at least nine different groups (or species), RVA–RVI, have been identified, of which RVAs account for the majority of infections in humans (Matthijnssens et al., 2012; Mihalov-Kovács et al., 2015).

On the basis of whole RVA genome sequencing, Matthijnssens et al. (2008) have proposed a comprehensive classification system that assigns a specific genotype for each of 11 genes. Two major non-G and non-P RVA genogroup constellations are known to circulate among humans, including genogroup 1 [I1-R1-C1-M1-A1-N1-T1-E1-H1 (Wa-like constellation)] and genogroup 2 [I2-R2-C2-M2-A2-N2-T2-E2-H2 (DS-1]
like)]. Further minor RVA (AU-1-like constellation) comprises non-G/P genogroup 3, including I3-R3-C3-M3-A3-N3-T3-E3-H3 (Patton, 2012).

In general, a double nomenclature is used for designating RVA genotypes, based on the VP7- and VP4-type specificities, of which G1P[8], G2P[4], G3P[8], G4P[8], G6P[8] and G12P[8] account for over 80% of circulating strains globally. While G1P[8], G3P[8], G4P[8], G6P[8] and G12P[8] strains possess the Wa-like constellation and, in general, exhibit a long electropherotype, G2P[4] strains are assigned to the DS-1-like constellation and show a short RNA migration pattern (Guerra et al., 2015; Matthijnssens et al., 2009).

Currently, two major orally administered, live-attenuated RVA vaccines are licensed, differing in respect to their antigenic composition and immunization schedule. Rotarix, manufactured by GlaxoSmithKline, is a two-dose schedule monovalent vaccine derived from a G1P[8] strain; RotaTeq, developed by Merck, is a three-dose schedule pentavalent (G1, G2, G3, G4 and P[8]) vaccine. As of December 2015, at least 80 countries have introduced RVA vaccines into their universal immunization schedule, including Brazil where Rotarix was adopted into the public sector in 2006 (PATH, 2015). A number of recent post-licensure studies have shown that both vaccines are effective against severe RVA gastroenteritis, provide protection against a broad variety of RVA strains and significantly reduce the frequency of gastroenteritis-related hospitalizations and deaths throughout the world (Costa et al., 2016; Greenwood, 2014; Linhares & Justino, 2014).

In view of the segmented nature of the RVA genome, reassortment events involving different strains are common and recognized as one of the mechanisms governing the evolution of novel viruses. Besides enhancing genetic diversity, reassortment may give rise to interspecies transmission with subsequent adaptation to the human host (Maestri et al., 2012; Mascarenhas et al., 2007). Mostly in low-income settings, it is likely that close proximity between humans and animals favours the occurrence of interspecies transmission, leading to the emergence of RVA variants that may potentially challenge current RVA vaccination strategies (Parashar et al., 2016).

A number of studies on novel RVA circulating variants have been conducted to date. In this context, Komoto et al. (2015) have detected a G1P[8] strain in Thailand that exhibited a DS-1-like constellation with short electropherotype pattern. Cowley et al. (2016) and Komoto et al. (2016) have reported the occurrence of G3P[8] strains that exhibited a DS-1-like constellation with a short electrophoretic profile in Australia and Thailand, respectively.

G3 genotype is known as the third most common RVA genotype in humans, most frequently combined with P[8]-type specificity. Of note, G3 strains have been identified in a broad host range, including cats, cows, dogs, horses, pigs, rabbits, sheep, monkeys and bats, in association with several P types (Malik et al., 2016; Nemoto et al., 2015). Following introduction of RVA vaccines, G3 strains were detected worldwide in humans in combination with P[4], P[6], P[9] and P[8] (Cowley et al., 2016; Malasa et al., 2015; Medici et al., 2016; Soares et al., 2014; WHO, 2015).

This study aims to describe the genetic characteristics of a possible novel equine-like G3P[8] strain associated with childhood acute gastroenteritis that has emerged in 2016 across Northern Brazil.

RESULTS

In March and April 2016, a total of 48 faecal samples were collected in healthcare centres from Northern Brazil, of which 14 (29.1%) were RVA positive by ELISA. The analysis of RVA-RNA by PAGE in positive samples showed that short and long migration patterns were detected in ten (71.4%) and three (21.4%) of positive samples, respectively, and only one sample was negative by PAGE.

Of the samples tested by a semi-nested multiplex reverse transcription PCR (RT-PCR), ten (71.4%) and four (28.6%) were found to bear G3P[8] and G12P[8] genotype specificities, respectively. Notably, all G3P[8] RVA strains exhibited similar short electropherotypes, similar to those which are characteristic of DS-1-like strains (data not shown). With regard to vaccination status of G3P[8] RVA-positive patients, 60% (6/10) had received at least one dose of Rotarix.

G3P[8] samples were subjected to partial sequencing, and whole-genome phylogenetic analysis demonstrated that all strains exhibited an I2-R2-C2-M2-A1-N2-E2-H2 genotype background (Fig. 1), except for two samples (AM-16-36 and AM-16-61) that did not amplify the NSP1 gene. Phylogenetic analysis of structural VP7 gene yielded the highest nucleotide (nt) sequence similarities (99.1 to 99.7%) with cognate genes of human/eqine reassortant RVA strains that were detected in Japan (S13-45), Thailand (SKT-271), Australia (WAPC1740, D388) and Spain (SanSebastian98244072015). Furthermore, the Brazilian strains were closely related (89.8 to 90.3%) to an equine strain isolated in India (Erv 105). Of note, when compared to the VP7 gene of RotaTeq vaccine nt, similarities ranged from 79 to 79.6% (Fig. 2f). G3P[8] Brazilian strains were compared to G3P[8] Wa-like human strains and G3 equine strains with nt similarities ranging from 77.7 to 78.2% and 84.3 to 85.5%, respectively.

Analysis of the structural VP4 gene demonstrated that G3P[8] Brazilian strains grouped into lineage P[8]-3, and these were most closely related to human strains from Australia (WAPC1740, D388, WAPC2016), Thailand (SKT-281, SKT-289, SKT-41) and Philippines (TGO12-045), with nt sequence identities in the range of 99 to 99.5% (Fig. 2d).

Structural protein genes VP1, VP2, VP3 and VP6 exhibited the highest nt sequence identities with human samples reported in Japan, Thailand and Australia,
yielding nt sequence similarities ranging from 99.5 to 100% for VP1, 98.1 to 99.7% for VP2, 98.8 to 100% for VP3 and 97.5 to 99.8% for VP6, all DS-1-like genotype (Fig. 2a–c, e).

With regard to NSP genes encoding NSP1, NSP3, NSP4 and NSP5, the highest nt sequence identities were found with cognate genes of strains of human origin reported in Japan, Thailand and Australia with nt similarities of 98.9 to 99.9% for NSP1, 98.3 to 99.8% for NSP3, 98.5 to 99.6% for NSP4 and 98.9 to 99.4% for NSP5 (Fig. 3a, c–e). Interestingly, G3P[8] Brazilian strains carried a NSP2 genotype 1 (WA like) and were closely related to G1P[8] strains (Fig. 3b).

The amino acid (aa) sequences were further analysed for VP7 and VP4 and compared to Rotarix and RotaTeq vaccines, showing aa changes in antigenic regions described by Aoki et al. (2009) for VP7 (neutralizing epitopes 7-1a and 7-1b) and by Dormitzer et al. (2002, 2004) for VP8* (neutralizing epitopes 8-1 and 8-3) as demonstrated in Fig. 4.

**DISCUSSION**

Following the introduction of RVA vaccines into routine use, the long-term monitoring of circulating RVA strains has been strongly recommended throughout the world, in order to assess whether there is any correlation between vaccine introduction and strain ecology (Patel et al., 2012). Currently, molecular biology techniques including full-genome sequencing analyses have demonstrated a substantial genomic diversity of co-circulating RVA strains across several regions and at various times. Of interest in this context, there are the inter-genogroup reassembly events that occur commonly under natural conditions, which may give rise to strains bearing mixed genotype specificities from both human and animal viruses, and that could theoretically pose a challenge to vaccination strategies (Silva et al., 2015).

In this study, we have reported the occurrence of a novel DS-1-like G3P[8] RVA strain in Northern Brazil. These samples were recovered from diarrhoeic children native to Amazonas state, a touristic setting that receives travellers from many continents, mostly from Europe (46.1%) and North America (36.1%), followed by Asia (10%), South America (4.2%), Oceania (1.6%) and Africa (1.2%) based on 2014 data in (Amazonastur, 2014).

DS-1-like G3P[8] RVA Brazilian strains have displayed a short electropherotype, in sharp contrast to the long RNA profile usually exhibited by strains bearing these G and P specificities. Of note, short RNA patterns similar to ours were also found in Thailand by Komoto et al. (2016), who described the genetic and antigenic characterization of a novel human/equine reassortant, DS-1-like G3P[8] strain, in children with gastroenteritis.

G1P[8], G3P[8], G4P[8] and G9P[8] are common genotypes associated with Wa-like constellation, while G2P[4] genotype is associated more often with DS-1-like genotypes (Medici et al., 2016; Patton, 2012). Inter-genogroup reassortants were detected in Asia, being described as G1P[8]

<table>
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<tr>
<th>Genotype</th>
<th>VP7</th>
<th>VP4</th>
<th>VP1</th>
<th>VP2</th>
<th>VP3</th>
<th>VP6</th>
<th>NSP1</th>
<th>NSP2</th>
<th>NSP3</th>
<th>NSP4</th>
<th>NSP5</th>
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<tbody>
<tr>
<td>RVA/Human-tc/USA/Wa/1974/G1P[8]</td>
<td>G1</td>
<td>P[8]</td>
<td>R1</td>
<td>C1</td>
<td>M1</td>
<td>J1</td>
<td>A1</td>
<td>N1</td>
<td>T1</td>
<td>E1</td>
<td>H1</td>
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**Fig. 1.** Genomic constellation of prototypes and G3P[8] Brazilian RVA strains. Dark grey denotes Wa-like constellation, white denotes DS-1-like constellation and light grey denotes AU-1 constellation.
genotype with a DS-1-like constellation in studies conducted by Fujii et al. (2014), Komoto et al. (2015), Kuzuya et al. (2014) and Yamamoto et al. (2014), and more recently were reported as G3P[8] DS-1-like constellation strains by Komoto et al. (2016).

However, there have been occasional reports recently showing the emergence of unusual G3P[8] strains that appear to have evolved genetically through reassortment events between Wa and DS-1 genogroups, such as those isolated in Thailand and Australia (Cowley et al., 2016; Komoto et al., 2016). Of potential concern is the fact that DS-1-like G3P[8] strains have been detected in some geographical areas (e.g. Australia) in association with severe childhood RV gastroenteritis (Cowley et al., 2016; Komoto et al., 2016; Malasao et al., 2015).

Phylogenetic analysis of the VP7 gene of the Brazilian G3P[8] strains has demonstrated high nt sequence identities with a human–equine DS-1-like G3P[4] genotype circulating in Japan, as reported by Malasao et al. (2015). Of note, RVA strains bearing G3 genotype specificity have frequently been reported infecting horses across distinct geographical areas of the world (Garaicoechea et al., 2011; Gulati et al., 2007; Nemoto et al., 2015; Papp et al., 2013). More recently, there have been reports describing the emergence of equine-like reassortant G3P[8] strains causing infections in humans, from regions in Asia and Oceania (Cowley et al., 2016; Komoto et al., 2016; Malasao et al., 2015).

Using phylogenetic analysis of G3P[8] Brazilian DS-1-like strains, it was observed that an additional reassortment event in the NSP2 gene was detected, which has exhibited typical genotype 1, Wa-like constellation. This is in contrast with the findings of Cowley et al. (2016) and Komoto et al. (2016) in Australia and Thailand, respectively, who described G3P[8] strains with NSP2 gene typically assigned to genotype 2, DS-1-like constellation.

Of interest, the DS-1-like G3P[8] strains possessed aa sequence of the VP7 and VP4 proteins, which differed from...
those of the RotaTeq and Rotarix vaccines, although it cannot be concluded at present whether this observation may signify a potentially lower vaccine efficacy.

In neutralizing epitopes of VP7, aa changes were observed in 7-1a (T87S) and 7-1b (N213T, K238D, D242A) regions in comparison with the RotaTeq G3 strain. Zeller et al. (2012) reported aa changes when comparing G3 Belgium strains to G3 RotaTeq in 7-1b and 7-2, and Morozova et al. (2015) reported aa changes in the same regions (7-1b and 7-2), including 7-1a. It was observed that G3 human–equine strains showed specific aa changes in the 7-1b region.

Regarding neutralizing epitopes of VP4, Brazilian strains carried aa changes when compared to RotaTeq and Rotarix P[8] strains in the 8-1 (N150S, N/D196G) and 8-3 (N113D) regions; aa changes in this region were also demonstrated by Zeller et al. (2012) in G3 Belgium strains, with the exception of substitutions in the aa 150 position that were observed in P[8] Brazilian, Thai and Australian strains. Attempts to adapt the Brazilian G3P[8] RVA strains to growth in cell culture (MA104 cells) have so far been unsuccessful, and therefore cross-neutralization assays in vitro could not be carried out. This will be the aim of future work.

It is well known from several studies conducted throughout the world that genetic reassortment between RVA strains of human and animal origins represents a common event (Komoto et al., 2016; Malasao et al., 2015). Notably, in this context, Grant et al. (2011) have reported G3P[3] and G3P[9] RVA strains exhibiting a high degree of genetic relatedness with human–bovine–canine–feline RVA. In Northern Brazil, a number of studies have highlighted the occurrence of reassortant human–animal RVA strains denoting different origins such as human–porcine, human–bovine and human–feline RVAs (Maestri et al., 2012; Mascarenhas et al., 2007).

The detection of DS-1-like G3P[8] RVA strains in Brazil adds strong evidence to the possible ongoing spread of RVAs of the unusual genotype constellation across the globe. Finally, the detection of a novel human–equine reassortant RVA strain G3P[8] in Belém, Northern Brazil,
METHODS

Ethics statement. This study was conducted in the context of the official Brazilian Ministry of Health’s surveillance network for RVA; therefore, no ethical clearances were needed.

Clinical specimens. Faecal specimens were obtained during March and April 2016 from Brazilian children aged 3 to 63 months who were hospitalized for acute gastroenteritis. These specimens were obtained from two health care centres situated in the northern region of Brazil [Amazonas state (23 samples) and Tocantins state (25)]. An aliquot of each sample was collected and stored at 2 to 8 °C, subsequently being transported on ice to the Instituto Evandro Chagas in Belém, Brazil, a national RV reference laboratory of the Brazilian Ministry of Health, where samples were immediately stored at −20 °C until testing for the presence of RVA antigen.

The commercially available ELISA RIDASCREEN (R-Biopharm) was used to detect RVA antigen in stool samples according to the instructions of the manufacturer; each of the plates included a positive and a negative control. Virus dsRNA was extracted from the supernatant of a 10% stool sample using the guanidinium isothiocyanate–silica method, as described previously (Boom et al., 1990). The extracted dsRNA was further used for (i) PAGE analysis and (ii) full-genome sequence analysis. PAGE was carried out in Tris/glycine buffer, and the RVA electropherotype was visualized and classified following electrophoresis through vertical 5% acrylamide–bisacrylamide gels, followed by silver staining (Pereira et al., 1983).

RT-PCR. All ELISA RVA-positive samples were subjected to two-step RT-PCR. Briefly, the first round was performed with consensus primers Beg9/End9 and 4con3/4con2 to amplify VP7 and VP4 genes, respectively. Subsequently, G and P genotypes were determined in a second-round semi-nested type-specific multiplex PCR assay utilizing primers for G (G1, G2, G3, G4, G9 and G12) and P types (P[4], P[6], P[8] and P[9]), as reported previously (Gentsch et al., 1997; Matthijnssens et al., 1992; Gouvea et al., 1990). The remaining non-G and non-P structural genes, as well as the non-structural genes, were amplified according to procedures described previously (Both et al., 1984; Cunliffe et al., 1997; Matthijnssens et al., 2006; Varghese et al., 2006). Altogether, those included partial amplifications of genes VP1 (686 bp), VP2 (686 bp), VP3 (702 bp), VP6 (1356 bp),...

Fig. 4. Alignment of the deduced aa sequences inside antigenic epitopes of VP7 and VP8* proteins of G3P[8] Brazilian RVA strains analysed compared to RotaTeq and Rotarix vaccines strains. Grey colour denotes aa residue that occurred following changes in antigenic regions. Letters within squares denote aa changes in G3P[8] Brazilian strains.

<table>
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<tr>
<th>Neutralizing epitopes in VP7</th>
<th>7-1a</th>
<th>7-1b</th>
<th>7-2</th>
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<tr>
<th>Neutralizing epitopes in VP8*</th>
<th>8-1</th>
<th>8-2</th>
<th>8-3</th>
<th>8-4</th>
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NSP1 (1565 bp), NSP2 (1038 bp), NSP3 (1062 bp), NSP4 (738 bp) and NSP5 (664 bp).

**Nucleotide sequencing and phylogenetic analysis.** Sequencing of PCR amplicons for all genes was carried out utilizing the same primers as those used in the PCR assay and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer’s instructions. The sequences were collected from an automated ABI Prism 3130xl DNA sequencer (Applied Biosystems) and were assembled using the software CAP3, aligned with Mafft v.7.221, edited with Geneious v.8.1.7 and compared to sequences from other viruses isolated and available in GenBank (http://www.ncbi.nlm.nih.gov). Phylogenetic analyses were carried out using MEGA software program version 4.0.1 by the neighbour-joining method (Kimura, 1980). The statistical significance of the genetic relationships was estimated by bootstrap resampling analysis (2000 replications). Partial nt sequences determined in this study were deposited in the GenBank database detailed in Table S1 (available in the online Supplementary Material).

**ACKNOWLEDGEMENTS**

The authors would like to acknowledge the staff of the State Central Laboratories (LACENs) and the technical assistance given by the laboratory personnel at the Virology Section of the Instituto Evandro Chagas, Health Surveillance Secretariat, Brazilian Ministry of Health.

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