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1. Background

Group A rotaviruses (RV-A) are the main etiological agent of acute viral gastroenteritis in infants and young children worldwide.1,2 Rotavirus genus belongs to the Reoviridae family, and its genome consists of 11 double-stranded RNA (dsRNA) gene segments encoding six structural (VP) and six non-structural proteins (NSP). Based on the two genes that code for the outer capsid proteins, VP4 and VP7, a widely used binary classification system was established for RV-A, defined G (from VP7, glycoprotein) and P (from VP4, protease-cleaved protein), genotypes. To date, at least 23 G and 31 P genotypes were identified.3-8 However, a new classification system based on all RV-A genes was recently proposed.4,5 Five RV-A G genotypes (G1–G4 and G9) and two P genotypes (P[8] and P[4]) are prevalent worldwide.9 Since the mid-1990s there has been a global emergence of genotype G9.9 Very recent studies carried out in Brazil revealed the emergence and predominance of genotype G9 in that country just before the introduction of universal vaccination against RV-A.10-16

Currently, 6 different phylogenetic lineages and 11 sublineages within G9 RV-A have been described based on the genetic variability of the VP7 gene.17 In the same way, sequence and phylogenetic analysis of the VP4 gene has demonstrated genetic diversity within the P[8] genotype, and four lineages have been described.18-20

The emergence and predominance of genotype G9 in Brazil has been associated with the introduction of universal vaccination against RV-A.10-16

Background: Group A rotavirus (RV-A) genotype P[8]G9 has emerged as one of the leading causes of gastroenteritis in children worldwide and currently is recognized as one of the five most common genotypes detected in humans. High intragenotype diversity in G9 RV-A has been observed, and nowadays, based on the genetic variability of the VP7 gene, six different phylogenetic lineages and eleven sublineages were described.

Objectives: To study the degree of genetic variation and evolution of Brazilian P[8]G9 RV-A strains.

Study design: Phylogenetic analysis of 19 P[8]G9 RV-A strains isolated from 2004 to 2007 in five different Brazilian states was conducted using the NSP1, NSP3, NSP5, VP4 and VP7 genes. For the VP4 and VP7 genes, 3D protein structure predictions were generated to analyze the spatial distribution of amino acid substitutions observed in Brazilian strains.

Results: Based on the phylogenetic analyses, all Brazilian strains clustered within lineage G9-III and P[8]-3 for VP7 and VP4, respectively, and were classified as genotype A1, T1 and H1 for the NSP1, NSP3 and NSP5 genes, respectively. Interestingly, all the strains isolated in Acre State (Northern Brazil) formed a closely related cluster clearly separated from the other Brazilian and prototype strains with regard to the five genes studied. Unique amino acid substitutions were observed in Acre strains in comparison with the prototype and Brazilian strains.

Conclusion: Inclusion of Acre strains in the phylogenetic analysis revealed the presence of a novel genetic variant and demonstrated a diversification of P[8]G9 rotaviruses in Brazil.

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In order to gain insight into the genetic variability of Brazilian P[8]G9 strains, phylogenetic analysis of NSP1, NSP3, NSP5, VP4 and VP7 genes from strains isolated in five different Brazilian states was performed. The results of these studies revealed both a new P[8]G9 genetic variant exclusively composed of strains from the north of Brazil (Acre State) and a diversification of RV-A P[8]G9 in Brazil.

2. Study design

2.1. Fecal samples, viral RNA extraction and PCR amplification

A total of 19 diarrheic stool specimens were collected between 2004 and 2007 from children up to 5 years old hospitalized with acute diarrhea. The samples, which were obtained from children from Rio de Janeiro and Espirito Santo (South East), Bahia (Northeast), Rio Grande do Sul (South) and Acre (North) States, were genotyped as P[8]G9 as previously described. Sequences of the VP4, VP7, NSP1, NSP3 and NSP5 genes were obtained by using the same set of primers utilized in RT-PCR.

To obtain the complete coding sequence of the NSP1 and NSP3 genes, internal primers were used in the sequencing step. These were the set of primers designed by Shah et al. for NSP1, and another set of primers was designed to obtain the complete coding sequence of these genes (Table 1).

Sequences were aligned using the CLUSTAL W program. Once aligned, phylogenetic relationships between the strains were reconstructed using the MEGA v. 4.0 software package and the neighbor-joining method with the Kimura two-parameter as the model of nucleotide substitution.

The names and accession numbers of the Brazilian strains analyzed in this study are presented in Table 2.

2.2. Sequencing and phylogenetic analysis

DNA sequencing was performed with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit® and an ABI Prism 3730 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA) by Genomic Platform of DNA sequencing PDTIS/FIOCRUZ, as previously described. Sequences of the VP4, VP7, NSP1, NSP3 and NSP5 genes were obtained by using the same set of primers utilized in RT-PCR.

In order to gain insight into the genetic variability of Brazilian P[8]G9 strains, phylogenetic analysis of NSP1, NSP3, NSP5, VP4 and VP7 genes from strains isolated in five different Brazilian states was performed. The results of these studies revealed both a new P[8]G9 genetic variant exclusively composed of strains from the north of Brazil (Acre State) and a diversification of RV-A P[8]G9 in Brazil.

2.3. 3D protein structure prediction

The crystallographic structure of VP4 and VP7 proteins from P[8]G9 RV-A strains are currently unknown. In order to model 3D structures of both proteins, we employed the most approximate structures available. For these reasons, crystallography data of the VP4 protein of the human RV-A Wa strain (P[8]G1) was imported from Protein Data Bank (PDB, accession number 2dwr) by the use of an Interactive Server-side Molecule Image Generator (AISMIG). In the case of VP7, crystallography data of this protein from RRV strain (P[3]G3) was imported from the Protein Data Bank (PDB) under accession number 3FMG, using the PDB ProteinWorkshop 3.6 program from PDB (available at: www.rcsb.org/pdb).

### Table 1
Internal primers used in the sequencing step to obtain complete coding sequence of NSP1 and NSP3 genes of RV-A genotype P[8]G9 Brazilian strain.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Primer position (bp)</th>
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</thead>
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<tr>
<td>NSP3</td>
<td>NSP3F-Seq(^a)</td>
<td>5′-TACGGTGAWGARARRATGGA-3′</td>
<td>497–516</td>
</tr>
<tr>
<td></td>
<td>NSP3R-Seq(^a)</td>
<td>5′-TCAACTTCTCATYTTYCTWCAA-3′</td>
<td>501–522</td>
</tr>
<tr>
<td>NSP1</td>
<td>RC 5Beg 1(+)(^a)</td>
<td>5′-TCATCATTCTACTCATT-3′</td>
<td>402–423</td>
</tr>
<tr>
<td></td>
<td>RC 5End 1(-)(^a)</td>
<td>5′-AAACAAATTATACACTMCAC-3′</td>
<td>949–968</td>
</tr>
<tr>
<td></td>
<td>5 Beg 1 (+)(^a)</td>
<td>5′-AAA TGT AGA AAT GAA TGT ATG A-3′</td>
<td>402–423</td>
</tr>
<tr>
<td></td>
<td>5 End 1 (-)(^a)</td>
<td>5′-YGA KGT TAM TAA TTT GGT TT-3′</td>
<td>949–968</td>
</tr>
</tbody>
</table>

\(^a\) Set of primers designed in this study.
\(^b\) Set of primers designed by Shah et al.29

### Table 2
RV-A genotype P[8]G9 Brazilian strain analyzed in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Year of isolation</th>
<th>Brazilian state</th>
<th>Accession numbers</th>
</tr>
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<tr>
<td>rj8224/04</td>
<td>2004</td>
<td>Rio de Janeiro</td>
<td>FJ793949</td>
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<tr>
<td>rj11149/05</td>
<td>2005</td>
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<td>FJ793951</td>
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<td>rj11759/05</td>
<td>2005</td>
<td>Rio de Janeiro</td>
<td>FJ793956</td>
</tr>
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<td>rj11768/05</td>
<td>2005</td>
<td>Rio de Janeiro</td>
<td>FJ793957</td>
</tr>
<tr>
<td>rj11772/05</td>
<td>2005</td>
<td>Rio de Janeiro</td>
<td>FJ793958</td>
</tr>
<tr>
<td>rs11126/05</td>
<td>2005</td>
<td>Rio Grande do Sul</td>
<td>FJ793950</td>
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<td>ac11443/05</td>
<td>2005</td>
<td>Acre</td>
<td>FJ793952</td>
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<tr>
<td>ac11475/05</td>
<td>2005</td>
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<td>FJ793953</td>
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<tr>
<td>ac11547/05</td>
<td>2005</td>
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</tr>
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<td>ac11548/05</td>
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<td>Acre</td>
<td>FJ793955</td>
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<td>ac11822/06</td>
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<td>ac11823/06</td>
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<td>2006</td>
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<td>ba12537/06</td>
<td>2006</td>
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<td>ba13619/07</td>
<td>2007</td>
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<td>FJ793968</td>
</tr>
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</table>
3. Results


In order to gain insight into the degree of genetic variability in the P[8]G9 RV-A strains circulating in Brazil, the sequences of VP4, VP7, NSP1, NSP3 and NSP5 genes from 19 P[8] strains circulating in five different Brazilian states were analyzed through phylogenetic analysis (Figs. 1 and 2). The phylogenetic analysis showed that all Brazilian strains grouped together within the P[8]-3 lineage and G9-III lineage (within sublineage d) for the VP4 and VP7 genes, respectively (Fig. 1A and B).

The phylogenetic analysis of NSP1, NSP3 and NSP5 genes, showed that all the Brazilian samples analyzed were classified within the genotypes A1, T1 and H1, respectively (Fig. 2).

All strains clustered with very high bootstrap values. Inside the lineages (P[8]-3 and G9-III-d) and genotypes (A1, T1 and H1), all Brazilian strains were assigned to different clusters together with strains isolated elsewhere. Interestingly, based on the five genes analyzed a cluster composed exclusively of strains isolated in Acre, was identified. These results show that the strains isolated in Acre have a closer genetic relationship among themselves and a more distant genetic relationship with all other strains isolated in Brazil or elsewhere.


The nucleotide sequences of the NSP1, NSP3, NSP5, VP4 and VP7 genes from Brazilian P[8]G9 strains were aligned together with prototypes using CLUSTAL W. Once aligned, they were translated to amino acid sequences using the MEGA v. 4.0 program in order to identify amino acid substitutions in these proteins (supplementary material Fig. 1). In the RV-A P[8]G9 strains isolated in Acre, several amino acid substitutions were found in NSP1 and NSP3, while only one was found in NSP5. Interestingly, three amino acid substitutions in the VP7 gene, located at positions 44 (A → V), 46 (S → P) and 263 (V → I), were observed in all of the Acre strains.

Fig. 1. Neighbor-joining phylogenetic tree analysis of structural genes of P[8]G9 Rotaviruses isolated in Brazil. Bootstrap values (2000 replicates) are shown at the branch nodes, values lower than 75% are not shown. The scale bar at the bottom of the trees indicates distance. The strains in bold indicate the Brazilian P[8]G9 samples sequenced in this study. In (A) the VP4 phylogeny of P[8] strains is shown. In (B) the VP7 phylogeny of G9 strains is shown. Cluster of strains isolated in Acre (Northern Brazil) is highlighted in gray. In the VP4 phylogeny, selected prototype strain representing the four lineages of P[8] genotype are described by names, followed with country of isolation, P[ ] and G genotypes, and accession number of each strain. In the case of the VP7 phylogeny, the prototypes used were the same as described by Phan et al. for the new classification system of genotype G9 RV-A in lineages and sublineages.
Fig. 1. (Continued).
Fig. 2. Neighbor-joining phylogenetic tree analysis of non-structural genes of P[8] G9 Rotaviruses isolated in Brazil. The phylogeny obtained for NSP1, NSP3, and NSP5 are shown in (A), (B) and (C), respectively. Prototypes sequences used in the phylogenetic analysis were the same as described by Matthijnssens et al. (2008) for the new classification system for RV-A.
unit of VP4 also showed several substitutions at different positions in the protein.

3.3. Mapping of amino acid substitutions observed in VP4 and VP7 genes of RV-A P[8]G9 strains isolated in Acre in a 3D protein structure

The amino acid substitutions found in the VP4 proteins of the P[8]G9 strains isolated in Acre were mapped spatially onto the 3D protein structure of the VP8* sub-unit of VP4 from the human RV-A Wa prototype (Fig. 3) (PDB accession number 2dwr).32

Five neutralization epitopes were mapped in this sub-unit of the VP4 protein.35 As can be seen in Fig. 3, none of the substitutions found in the Acre strains occurred in any of the five neutralization epitopes previously described. Nevertheless, two of the mutations found in these strains map closely to neutralization domain V115-123G.

Fig. 2. (Continued).
In the case of VP7, four different major antigenic sites have been described for RV-A VP7. Interestingly, the 3D structure of VP7 revealed that the major antigenic sites seem to be distributed in layers in different regions of the VP7 protein. The mutation at position 263, exclusively found in strains isolated in Acre, maps spatially closely to major antigenic site B (Fig. 4).

4. Discussion

RV-As exploit all known mechanisms of genetic variation, including punctual mutation, rearrangement, reassortment, recombination and interspecies transmission. Most of the previous studies of RV-A evolution have focused on genetic variation within the major structural genes (VP4 and VP7). However, the degree of genetic variation and mode of evolution of non-structural genes such as NSP1, NSP3 and NSP5 are largely unknown despite the role of these genes in several important aspects of rotavirus biology. NSP1 inhibits type 1 interferon (IFN-1) expression by degrading the interferon regulatory factors IRF3, IRF5 and IRF7. NSP3 protein competes with PABP (Poly-A binding protein) for interaction with the eukaryotic translation initiation factor eIF4G1 thereby shutting off cellular protein synthesis. NSP5 is necessary for viroplasm formation (the intracellular inclusion body where rotavirus morphogenesis takes place). For these reasons, amino acid substitutions occurring in the coding sequences of these proteins may have important implications for RV-A infection and pathogenesis. Although no significant genetic variation was found in NSP3 and NSP5 genes in P[8]G9 RV-A strains isolated in Brazil and enrolled in these studies, specific substitutions were found in strains isolated in Acre with respect of other strains isolated elsewhere in Brazil (supplementary material Fig. 1). Where these substitutions may have a selective advantage for these viruses is currently unknown. Further studies will be needed to address the biological significance of these substitutions found in strains isolated in this region of Brazil.

Recent studies on origin and spread of the G9 RV-A genotype have revealed a high degree of genetic diversity within this genotype throughout the world. These studies have also shown a close similarity between human and porcine G9 RV-A strains and two different evolutionary mechanisms: (a) zoonoses; (b) con-
vergent evolution, were proposed. Several studies performed in Brazil have shown the involvement of RV-A from swine origin (particularly G5 and G9 genotypes) in outbreaks causing diarrhea in children. Although reassortment events among animal and human strains continue to be an important mechanism for RV-A evolution and emergence, other evolutionary mechanisms may account for the emergence of P[8]G9 genotype, as suggested by recent studies, and the results of this work. Importantly, further genetic analysis including VP6 and NSP4 proteins may provide important information about the origin and the potential zoonotic characteristics of this genotype. These two genes, as well as the other genes that constitute the complete RV-A genome will be analyzed in a near future, for this specific variant. This is in agreement with the new RV-A classification proposed recently by Matthijsens and collaborators (2008).

Phylogenetic analysis of P[8]G9 RV-A strains isolated in Brazil revealed the presence of a novel genetic variant comprised of strains isolated in the state of Acre (northern Brazil). The results demonstrate genetic variation in the five genes studies (Figs. 1 and 2). The Acre strains studied were isolated during outbreaks in 2005 and 2006, and the 2005 outbreak was responsible for 12,145 hospitalizations and 8 deaths in the Rio Branco municipality (Acre State) from May to October 2005. The RV-A genotype P[8]G9 was determined to be the etiologic agent of this outbreak. This was Brazil’s largest known outbreak of diarrhea mainly due to RV-A. These outbreaks taking place in the Acre State, occurring a high number of hospitalization and deaths. Recently, RV-A genotype G9 was associated with more-severe disease in Australia as well as in Latin American regions.

Analysis of the deduced amino acid sequence alignments revealed amino acid substitutions in all five of the analyzed genes that were unique to the seven Acre strains (supplementary material Fig. 1). It is possible that the substitutions allowed the new strains belonging to this novel genetic variant to infect a naïve population of children and cause two extensive consecutive outbreaks. The crystallographic structures of VP4 and VP7 proteins of P[8]G9 of RV-A strains are currently unknown. In this study, we employed the most approximate available structures for both proteins, obtained from RV-A strains belonging to other genotypes.

Our analysis identified amino acid substitutions in all the proteins studied, some of them outside the previously identified neutralization domains in the surface structural proteins of the virus. In the particular case of VP7, this protein is a principal target of protective antibodies. Removal of free calcium ions (Ca2+) dissociates VP7 trimers into monomers, releasing VP7 from the virion, and initiates penetration-inducing conformational changes in the other outer-layer protein, VP4. All Acre strains shared an amino acid substitution at position 263 (V → I) that is spatially very close to the major antigenic site B, and this could possibly modify the antigenicity of the corresponding region (Fig. 4). All the Acre strains and some others from the states of Rio de Janeiro and Bahia share amino acid substitutions at positions 91 (I → V) and 108 (V → I) in VP4 that
map closely to the neutralization domain V115–123G (Fig. 3). These mutations could affect viral infectivity and might be diagnostically important. More studies will be needed to determine the potential effects of the substitution changes identified in this work. Crystal structure determinations of VP4 and VP7 proteins from P[8]G9 strains will be important to obtain a definitive answer to the possible effects of the substitutions found in VP4 and VP7 proteins observed in this work. The potential effects that these identified substitutions may have in relation with virus interaction with the immune system also remains to be established.

Previous reports have attributed unusual epidemiological and clinical features to the novel G9 strains. In these reports, recently emerged G9 strains caused symptomatic infections in children up to 5 years old, in whom pre-existing immunity was expected to provide sufficient heterotypic protection against common genotypes. In addition, G9 strains have also caused disease in newborns, suggesting that acquired maternal antibodies did not neutralize these strains efficiently. Taking all these findings into account, it is possible that G9 RV-A has escaped the pre-existing immunity evoked by other strains, possibly because these populations were immunologically naive to G9 specificity.

Rotarix®, a live attenuated vaccine prepared from P[8]G1 human strain isolated from a child, was introduced into the Brazilian National Immunization Program in March 2006. In a recent study, the efficacy and safety of Rotarix® against severe RV-A gastroenteritis was evaluated in Latin American children, showing an efficacy of 80.5% against non-G1[P]8 genotypes (including P[8]G9 genotype). However, as the results of this work show, more studies will be needed in order to evaluate its efficacy against different lineages and genetic variants of P[8]G9 genotype circulating in Brazil.

Monitoring temporal changes in various genes may help us to understand the nature and pattern of RV-A evolution. This study revealed the presence of a novel genetic variant and the diversification of P[8]G9 RV-A in Brazil. WHO strongly recommends strain surveillance to monitor the strain diversity of circulating RV-A to detect possible strain replacement following the introduction of universal RV-A vaccination. For this reason, the potential impact on the effectiveness of RV-A vaccine of new genetic variants and
the possible antigenic changes that might occur are facts that must not be underestimated. Therefore, it will be important to monitor the abilities of the vaccines currently in use to provide heterotypic protection against diverging G9 strains.

Conflict of interest

The authors would like to declare that there is no financial or personal relationship with other people or organizations that could appropriately influence their work during the submission process.

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Appendix A. Supplementary data


References


