Molecular Analysis of VP4, VP7, and NSP4 Genes of P[6]G2 Rotavirus Genotype Strains Recovered From Neonates Admitted to Hospital in Belém, Brazil

Joana D’Arc P. Mascarenhas,1 Alexandre C. Linhares,1 Amanda Patrícia G. Bayma,1 Jackson C. Lima,1 Maísa S. Sousa,1 Irene T. Araújo,2 Marcos B. Heinemann,2 Rosa Helena P. Gusmão,3 Yvone B. Gabbay,1 and José Paulo G. Leite2
1Virology Section, Instituto Evandro Chagas, Secretaria de Vigilância em Saúde, Ministério da Saúde, Ananindeua, Brazil
2Department of Virology, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
3University of Pará State, Belém, Brazil

This investigation describes the molecular characterization of P[6]G2 rotavirus strains from hospitalized neonates with community-acquired diarrhea (CAD), nosocomial diarrhea (ND), and asymptomatic nosocomial infection (ANI) in Belém, Brazil. Twenty-six rotavirus strains with P[6]G2 genotype were sequenced to genes coding for VP4, VP7, and NSP4 proteins. Phylogenetic analysis of the VP4 gene, including prototype strains RV3, ST3, M37, and U1205, showed that local P[6]G2 strains clustered forming a distinct lineage (bootstrap of 99%). Brazilian P[6]G2 strains had the highest homology (ranging from 96.0%–98.3%) with the African strain GR1107, G4P[6]. Phylogenetic tree for VP7 gene was constructed including old and new G2 African strains SA3958GR/97, SA356PT/96, SA514GR/87, SA4476PT/97, BF3676/99, GH1803/99, and representative strains of G1, G3, G4, G5, G8, and G9 genotypes. The Brazilian P[6]G2 samples fell into a distinct group (bootstrap value of 97%) and showed homology rates ranging from 92.1% to 93.5% with P[6]G2 African strains BF3676/99, GH1803/99, and SA3958GR/97. Nucleotide sequence analysis of the NSP4 gene, including human prototype strains S2, KUN, DS-1, RV5, RV3 and ST3, and animal prototype OSU, showed that all neonatal isolates fell into genotype A and clustered with a bootstrap value of 100%, with in-group similarities ranging from 99.3% to 100%. In this study no significant differences in nucleotide sequences of the VP4, VP7, and NSP4 genes could be observed when comparing diarrheic (CAD and ND) and non-diarrheic (ANI) babies. Monitoring of rotavirus strains in hospital environments is of particular importance, since it is claimed currently that an efficacious rotavirus vaccine, when available for routine use, will determine an impact on hospital-acquired rotavirus disease. J. Med. Virol. 78:281–289, 2006. © 2005 Wiley-Liss, Inc.

KEY WORDS: unusual P[6]G2 genotype; neonatal rotavirus strains; VP4 gene; VP7 gene; NSP4 gene

INTRODUCTION

Group A rotaviruses are the most common cause of acute viral diarrhea in humans and animals throughout the world [Kapikian et al., 2001]. Rotaviruses are a member of the Reoviridae family. Complete virus particles have a triple-layered protein capsid surrounding a genome consisting of 11 segments of double-stranded RNA (dsRNA), which encode six structural (VP1-VP4, VP6, and VP7) and six non-structural (NSP1-NSP6) proteins [Kapikian et al., 2001]. Group A rotaviruses have been classified into three genogroups based on hybridization with probes prepared from Wa, DS-1, and AU-1 rotavirus strains. Members of the same genogroup share a high degree of genetic relatedness but significantly less homology with members of other genogroups [Nakagomi and Nakagomi, 1993]. Serotype designations are currently based on independent neutralization determinants on the outer capsid proteins VP7 (G serotype) and VP4 (P serotype) [Estes, 2001]. On the basis of the neutralizing antibodies

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and the oligonucleotide sequence, at least 15 G serotypes and 25 P genotypes, have been identified for group A rotaviruses [Estes, 2001; Kapikian et al., 2001; Rahman et al., 2005].

Studies conducted in hospital nurseries around the world have suggested that a single rotavirus strain can persist over long periods in neonatal wards [Rodger et al., 1981; Perez-Schael et al., 1984; Steele et al., 1992; Pager et al., 2000]. Furthermore, the apparent stability of rotavirus neonatal strains seems to differ from that of rotaviruses circulating in the community; the latter strains usually showing a higher genomic variability [Rodger et al., 1981; Steele et al., 1992].

Rotavirus strains infecting neonates were originally associated with an avirulent P[6] genotype recovered from asymptomatic subjects excreting rotavirus strains bearing G1, G2, G3, and G4-type specificities [Hoshino et al., 1985; Flores et al., 1986; Gorziglia et al., 1988]. However, Steele et al. [1995], Pager et al. [2000], and Cunliffe et al. [2002] have reported the detection of P[6] genotype associated with G4 and G8 rotavirus strains causing symptomatic and asymptomatic infections in African neonates.

This same P[6] genotype was detected in children suffering from acute diarrhea associated with serotypes G1, G2, G3, G8, and G9 all over Africa [Adah et al., 2001; Salu et al., 2003; Steele and Ivanoff, 2003; Page and Steele, 2004a,b].

The non-structural protein NSP4, encoded by gene segment 10, is a glycoprotein anchoring in the membrane of the endoplasmic reticulum [Estes, 2001]. This protein has been studied extensively due to its role in viral morphogenesis and its potential enterotoxicogenic property [Tian et al., 1995; Ball et al., 1996; Estes, 2001]. The NSP4 gene has been classified into three groups (A, B, and C). Group A has been genetically classified into at least five genotypes comprising the following strains: A (KUN), B (Wa), C (AU-1), D (EW), and E (avian-like) [Ciarlet et al., 2000; Ito et al., 2001; Mori et al., 2002]. NSP4, particularly a 22-amino acid (aa) synthetic peptide corresponding to residues 114–135, has been shown to trigger a signal transduction pathway that increases the intracellular calcium levels inducing diarrhea in young mice [Tian et al., 1995; Ball et al., 1996]. The “toxigenic” region seems to be extended from residues 114–135, has been shown to trigger a signal transduction pathway that increases the intracellular calcium levels inducing diarrhea in young mice [Tian et al., 1995; Ball et al., 1996]. The “toxigenic” region seems to be extended from residues 114–135, has been shown to trigger a signal transduction pathway that increases the intracellular calcium levels inducing diarrhea in young mice [Tian et al., 1995; Ball et al., 1996]. The “toxigenic” region seems to be extended from residues 114–135, has been shown to trigger a signal transduction pathway that increases the intracellular calcium levels inducing diarrhea in young mice [Tian et al., 1995; Ball et al., 1996].

Studies conducted in Belém, Brazil reported the first nosocomial transmission of P[6]G2 rotavirus strains among symptomatic and asymptomatic neonates [Linhares et al., 2002]. In addition, P[6] genotype specificity was found in association with G1, G2, G4, and G5 genotypes among children with diarrhea with ages ranging from 9 to 24 months who participated in a trial with the Rhesus-human reassortant tetravalent rotavirus vaccine (RRV-TV) during 1990–1992 in Belém, Brazil [Mascarenhas et al., 2002].

The aim of the present study was to determine the genetic diversity among P[6]G2 rotavirus strains recovered from Brazilian neonates with either asymptomatic or symptomatic rotavirus infection, based on phylogenetic analysis of sequence of genes coding for VP4, VP7, and NSP4 proteins.

MATERIALS AND METHODS

Patients and Specimens

Surveillance for rotavirus infection was conducted from May 1996 to May 1998 in neonatal care unit wards at a public hospital in Belém, Brazil. A total of 614 stool samples were obtained from 437 premature/sick newborn babies (NB) with ages from 1 to 28 days who developed community-acquired diarrhea (CAD), nosocomial diarrhea (ND), and asymptomatic nosocomial infection (ANI). All samples were screened for the presence of Group A rotavirus antigen using monoclonal antibodies by a commercially available enzyme-linked immnosorbent assay (ELISA) kit (Dakopatts, Denmark) according to the manufacturer’s instructions. Overall, 51 (11.7%) hospitalized neonates were excreting rotavirus, 42 (82.3%) had ANI, 5 had ND, and 4 babies were found to have CAD, as reported by Linhares et al. [2002]. Fifty (98%) and 1 (2%) rotavirus strains displayed short and long electropherotypes, respectively. In this study 26 rotavirus-positive samples having P[6]G2 specificity were recovered from ANI (n = 18), ND (n = 5), and CAD (n = 3) patients, and were sequenced including those displaying short genomic profile and being available in sufficient amount.

RT-PCR Amplification

Rotavirus dsRNA was extracted from fecal suspensions using silica powder glass extraction as described previously by Boom et al. [1990]. The purified viral dsRNA was denatured at 98 °C for 7 min and then used as a template for the reverse transcription PCR (RT-PCR). The reverse transcription of dsRNA was carried out with SuperScript™ (Invitrogen, Carlsbad, CA) and PCR amplification was performed with Taq DNA polymerase recombinant (Invitrogen). The full-length 738 bp gene, encoding the NSP4 protein, was amplified using primers and PCR methods described by Cunliffe et al. [1997]. The Nested-PCR primers and conditions for the amplification of a 244 bp fragment for VP7 gene, and a 267 bp fragment for VP4 gene were previously described by Linhares et al. [2002]. All PCR products (NSP4, VP4, and VP7) were purified by using QIAquick® PCR purification kit (Qiagen, Hilden, Germany) or QIAquick® Gel Extraction kit (Qiagen).

DNA Sequencing and Phylogenetic Analysis

The purified PCR products from asymptomatic and symptomatic neonates were automatically sequenced by using the con3 forward and P6 reverse primers (VP4 gene) described by Gentsch et al. [1992], 9con1 forward, and G2 reverse primers (VP7 gene) reported by Das et al. [1994], and jrg30 forward and jrg31 reverse primers for NSP4 gene [Cunliffe et al., 1997]. Sequencing was
carried out by the dideoxynucleotide chain terminator method on an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, CA), using the ABI Prism Big Dye Terminator cycle sequencing Ready Reaction Kit (Applied Biosystems). The products were further purified by ethanol precipitation and resuspended in formamide. The sequences obtained for the VP4, VP7, and NSP4 genes were aligned and edited using a BioEdit Sequence Alignment Editor (version 6.0). Phylogenetic analysis was performed using MEGA package (versions 2.0 and 3.0). Distances between sequences were analyzed using the neighbor-joining algorithm based on the Kimura two parameters distance estimator method for nucleotide [Kimura, 1980]. The second method was maximum parsimony with heuristic estimative method for nucleotide. The algorithm based on the Kimura two parameters distance estimative method for nucleotide [Kimura, 1980]. The second method was maximum parsimony with heuristic or branch-and-bound search. Bootstrap resampling (over 1,000 replicates) was performed for neighbor-joining analysis of sequences obtained of genes VP4, VP7, and NSP4.

Prototype strains for VP4, VP7, and NSP4 genes analysis were obtained from GenBank at the National Center for Biotechnology Information, USA (www.ncbi.nlm.nih.gov), by conducting Blast and nucleotide search.

Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported in this paper were submitted to GenBank with accession numbers (VP4 gene) DQ070447–DQ070472; (VP7 gene) DQ070421–DQ070446; and (NSP4 gene) DQ070395–DQ070420.

RESULTS

Characterization of Rotavirus Strains by PAGE

PAGE analysis of strains with P[6]G2 specificity from neonates with CAD and ND has evidenced a strong strain identity, since all rotavirus strains displayed the same electropherotype (Fig. 1).

Amplification of the VP4, VP7, and NSP4 Genes

Twenty six amplicons were obtained based on the amplification of the NSP4 protein [Cunliffe et al., 1997] and VP7 and VP4 genes, as described previously by Linhares et al. [2002].

Sequence Analysis of VP4, VP7, and NSP4 Genes

Twenty six rotavirus strains with P[6]G2 genotype-specificity were sequenced and analyzed to assess genetic diversity among neonates with CAD (3 strains), ND (5 strains), and ANI (18 strains). Table I shows results concerning the 26 P[6]G2 rotavirus strains, when comparing the nucleotide homologies of the three groups studied (ANI, CAD, and ND), according to age and gender of neonates. The homologies ranged from 99.9% to 100% (VP4 gene), 99.2%–99.4% (VP7 gene), and 99.8%–99.9% (NSP4 gene).

Phylogenetic analysis showed that the local P[6] strains clustered forming a distinct lineage (bootstrap of 99%), including prototype strains RV3, ST3, M37, and U2105 (Fig. 2a). When only local strains were analyzed, they clustered into a lineage with a 92% bootstrap, which has been classified as lineage 2A by Banyai et al. [2004]. Collectively, the Brazilian P[6]G2 strains demonstrated the highest homology (ranging from 90.6%–98.3%) with the African strain GR1107, typed as P[6]G4. The porcine strain Gottfried (lineage 2B), the human strains BP1338 (lineage IV) and BP1227 (lineage V), and the outgroup strain AU-1 (lineage P3) were included in this analysis showing nucleotide divergence rates of 22.6%, 14.5%, 17.4%, and 53.2%, respectively. The divergence observed between Brazilian strains (lineage 2A and GR1107, included in this study, was 3.4%. Compared to Brazilian P[6] from neonates, the samples BP1227 and Gottfried, lineages V and 2B, respectively, diverged 18.2%.


Comparison of nucleotide sequences of the NSP4 genes from 26 P[6]G2 neonatal rotavirus strains were made including human prototype strains S2, KUN, DS-1, RV5, RV3, and ST3, and animal prototype OSU (Fig. 2c). All rotavirus neonatal samples from Belém clustered with bootstrap value of 100%, showing in-group similarities ranging from 99.3% to 100%. These isolates fell into a major group (bootstrap of 100%) designated genotype A by Lee et al. [2000], which contains human prototype strains S2, KUN, DS-1, and RV5. The diversities between genotypes A and B, B and C, and A and C were of 21.0%, 22.4%, and 25.1%, respectively. The mean genetic diversity in genotypes A and B were of 1.7% and 5.8%, respectively. The prototype AU-1 represents the NSP4 genotype C and was included in this tree as an out group.

DISCUSSION

Several reports have documented the association of neonatal P[6] genotype with different G serotypes such as G8, G9, G4, and G1 in Guinea-Bissau, during 1996 and 1997 [Fischer et al., 2000], in Malawi, during 1997 and 1998 [Cunliffe et al., 1999], in Africa, during 1996 and 1999 [Pager et al., 2000; Steele and Ivanoff, 2003], and in the United States, during 1996 to...

The Brazilian P[6]G2 strains are similar to those reported by Adah et al. [2001], Salu et al. [2003], and Page and Steele, [2004a], who conducted studies involving children with acute diarrhea in Nigeria, Ghana, Burkino Faso, and South Africa, respectively. In these studies, P[6]G2 strains typically shared subgroup I, VP7 serotype G2, and short RNA electropherotype.

### TABLE I. Comparison Between Homologies of VP4, VP7, and NSP4 Genes from the Diarrheic (CAD and ND) and Non-Diarrheic (ANI) Neonates, According to Age and Gender in Belém, Brazil

<table>
<thead>
<tr>
<th>Genes</th>
<th>CAD × ANI (%)</th>
<th>CAD × ND (%)</th>
<th>ND × ANI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP7</td>
<td>99.2</td>
<td>99.3</td>
<td>99.4</td>
</tr>
<tr>
<td>VP4</td>
<td>99.9</td>
<td>100.0</td>
<td>99.9</td>
</tr>
<tr>
<td>NSP4</td>
<td>99.9</td>
<td>99.8</td>
<td>99.9</td>
</tr>
</tbody>
</table>

Age and gender range in days. CAD, 16–28 days (75% female and 25% male); ANI, 8–22 days (61% female and 39% male); ND, 12–28 days (20% female and 80% male); +, mean percentage homology was calculated by MEGA package (version 3.0).
from M37-like, AU19-like, and Gottfried-like strains. In this study, all strains which formed a distinct lineage into a major group clustered with lineages M37-like, as statistically supported by bootstrap of 99%.

Phylogenetic analysis of the VP7 gene of G2 strains from Belém revealed a separated branch clustered together with “new” lineages IIb, IIc, and IId described by Page and Steele [2004b]. The strains from “old” lineage I, included in

this analysis, were more divergent as compared to neonatal strains (Fig. 2b). The P[6]G2 strains from Belém clustered with the Africa P[6]G2 strains included in this analysis, as supported by a bootstrap of 100%.

The human rotaviruses NSP4 genotype A has been associated usually with SGI specificity, whereas NSP4 genotype B with SGII specificity [Cunliffe et al., 1997; Kirkwood and Palombo, 1997]. Lee et al. [2000] have
described G1P[6] rotavirus strains recovered from neonates that clustered into genotype B. NSP4 genotype A was largely predominant among neonates in nurseries in Belém, Brazil, being associated with P[6]G2 specificity. The fact that all local strains had a 100% homology, regardless of being recovered from CAD, ND, or ANI group suggests that NSP4 gene may not be the sole determinant for rotavirus virulence.

Several studies have been conducted worldwide with asymptomatic and symptomatic rotavirus infections in neonatal wards, and the relationship between neonates and attenuated rotavirus strains are highlighted by these studies [Rodger et al., 1981; Perez-Schael et al., 1984; Steele et al., 1992; Steele and Sears, 1996]. In the local study, when comparing strains from neonates who developed ANI, CAD, and ND, higher homologies can be seen between VP4, VP7, and NSP4 genes.

The rotavirus strains infecting hospitalized neonates are in general different from community strains infecting children at home [Steele et al., 1995; Kilgore et al., 1996]. Unlike this assessment, Linhares et al. [2002] have suggested that P[6]G2 neonatal strains may have been introduced into the hospital’s NCU from the external community, as suggested by the identical short electrophoretic profiles yielded from PAGE. This was confirmed in this study since two cases of P[6]G2 rotavirus-related CAD (NB-140 and NB-142) were admitted to the ward around 01/1997, and the first ND (NB-148) case occurred on 02/1997. Strains from three cases were identical in their nucleotide and amino acid sequences (data, not shown).

It has been suggested that neonatal host factors may account for the adaptation of a strain to the intestine, leading it to become less virulent [Hoshino et al., 1985]. Furthermore, rotavirus antibodies of maternal origin were claimed to have a variable effect in protecting neonates against different strains of rotavirus [Snodgrass et al., 1977; Snodgrass and Wells, 1978; Sheridan et al., 1984; Offit and Clark, 1985]. This seems evident, as reported by Linhares et al. [2002], since symptomatic and asymptomatic P[6]G2 infection were noted among breast-fed neonates.

Five of eight neonates who developed CAD or ND received maternal milk only, of whom three presented with mild/moderate symptoms and two with severe symptoms. It is noticeable that among the three neonates who received breastmilk plus formula, one developed mild/moderate illness and the remainder had severe symptoms.

Overall, no significant differences in nucleotide sequences of the VP4, VP7, and NSP4 genes could be observed when comparing diarrheic (CAD and ND) and non-diarrheic (ANI) babies.

Monitoring of rotavirus strains in hospital environments is of particular importance, since it is currently claimed that an efficacious rotavirus vaccine, when available for large-scale use, will determine a significant impact in hospital-acquired rotavirus disease [Fischer et al., 2004].

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Analysis of VP4, VP7, and NSP4 Genes from Neonatal Rotavirus Strains


