Molecular analysis of the NSP4 and VP6 genes of rotavirus strains recovered from hospitalized children in Rio de Janeiro, Brazil

Irene Trigueiros Araújo,1 Marcos Bryan Heinemann,1 Joana D’Arc P. Mascarenhas,2 Rosane M. Santos Assis,1 Alexandre Madi Fialho1 and José Paulo G. Leite1

Correspondence
José Paulo G. Leite
jpgleite@ioc.fiocruz.br
1Department of Virology, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
2Virology Section, Instituto Evandro Chagas, Secretaria de Vigilância em Saúde, Ministério da Saúde, Ananindeua, Brazil

Received 16 June 2006
Accepted 9 February 2007

Group A rotaviruses are the main cause of acute gastroenteritis in children throughout the world. The two outer capsid proteins, VP4 and VP7, define the P and G genotypes, respectively. Rotaviruses with P[8]G1, P[4]G2, P[8]G3 and P[8]G4 genotypes are predominant in infecting humans and the G9 genotype is emerging in most continents as the fifth most common G type worldwide. The inner capsid protein VP6 is responsible for subgroup (SG) specificities, allowing classification of rotaviruses into SG I, SG II, SG I+II and SG non-I-non-II. The non-structural protein 4 (NSP4) encoded by segment 10 has a role in viral morphogenesis and five genetic groups have been described, NSP4 genotypes A–E. The aim of this investigation was to characterize the NSP4 and VP6 genes of rotavirus strains recovered from hospitalized children. Thirty rotavirus strains were submitted to RT-PCR followed by sequencing and phylogenetic analysis. Among the different G and P genotype combinations, two distinct genetic groups could be recognized for the NSP4 gene. Twenty-eight clustered with NSP4 genotype B. The two P[4]G2 strains fell into NSP4 genotype A and clustered distinctly, with a 100 % bootstrap value. The strains distinguished within a group were closely related to each other at the nucleotide and amino acid levels. A phylogenetic tree was constructed for the VP6 gene including the human strains RMC100, E210, Wa, US1205 and 1076, and the animal strains Gott, NCDV, SA-11, FI-14 and EW. This is the first report on Brazilian rotavirus strains describing NSP4 genotype A strains associated with VP6 SG I, and NSP4 genotype B strains associated with VP6 SG II.

INTRODUCTION

Viruses of the genus Rotavirus (family Reoviridae) have a genome comprising 11 segments of dsRNA, encoding six structural proteins (VP1–6) and six non-structural proteins (NSP1–6). Rotavirus genotypes are defined on the basis of genes that encode its two outer capsid proteins, VP4 and VP7, responsible for the double classification of rotaviruses into 27 P genotypes and 16 G genotypes, respectively (Kapikian et al., 2001; Rahman et al., 2005; Gulati et al., 2006; Martella et al., 2006; Khamrin et al., 2007). Seven groups (A–G) and two major subgroups (SG I and SG II) are also classified according to VP6 protein antigenicity, which is the middle capsid protein (Estes, 2001). Many epidemiological studies have used subgrouping enzyme immunoassays, and most of the human isolates fall into SG II (Arista et al., 1990; Iturriza-Gómez et al., 2001) whilst animal isolates fall into SG I (Tang et al., 1997).

The non-structural protein NSP4 is encoded by segment 10 and serves as an intracellular receptor on the membrane of the endoplasmic reticulum for double-layered particles (DLPs) and interacts with viral capsid proteins (Taylor & Bellamy, 2003). In addition, NSP4 has been found to have an enterotoxin-like activity, originally mapped between aa 114 and 135. Modifications in the toxigenic activity and virulence of rotavirus have been associated with amino acid changes in this region (Ball et al., 1996; Zhang et al., 1998). However, many studies have demonstrated that NSP4 is not a pathogenic determinant in rotavirus infection, whether recovered from children with or without diarrhoea, and it appears to be well conserved across all genotypes (Lee et al., 2000; Lin & Tian, 2003). The rotavirus A NSP4 gene has been sequenced, and five distinct genetic groups (genotypes), A–E, have been classified. Genotypes A, B and C, or
KUN, Wa and AU-1, have been detected in humans, while genotypes D and E, or EW and avian-like, have been detected in animals (Mori et al., 2002; Lin & Tian, 2003). Each NSP4 genotype appears to segregate according to the rotavirus host species (Ciarlet et al., 2000). The aim of the present work was to determine the genetic diversity among rotavirus strains recovered from hospitalized children with different G and P genotypes, based on sequencing and phylogenetic analysis of the genes encoding NSP4 and VP6.

**METHODS**

**Faecal specimens.** A total of 30 samples were collected from hospitalized children with acute diarrhoea in the city of Rio de Janeiro, Brazil. Four of these samples were collected in 1986, 1987, 1988 and 1990, respectively, and the remaining 26 were collected between 2001 and 2004. Stool suspensions of approximately 10% (w/v) were prepared in 0.01 M Tris/HCl (pH 7.2) with Ca\(^{2+}\) and stored at -20 °C. All 30 samples were identified as rotavirus positive using an enzyme immunoassay for rotavirus and adenovirus antigen detection (Pereira et al., 1985) and by PAGE and silver staining of dsRNA segments, as described previously (Leite et al., 1996).

**RNA extraction and PCR amplification.** Viral dsRNA was extracted using the glass powder method (Boom et al., 1990) and amplified by RT-PCR. The G and P types of these strains were determined as described previously (Araújo et al., 2001). The full-length 738 bp gene encoding the NSP4 protein was amplified by RT-PCR using the primers and conditions described by Cunliffe et al. (1997). For the amplification of a 379 bp fragment of the VP6 gene, the primers and conditions were as described by Iturriza-Gomara et al. (2002).

**Sequencing.** The NSP4 and VP6 PCR products were purified using a QIAquick PCR purification kit (Qiagen). DNA sequencing was performed by the dideoxynucleotide chain-termination method, using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). The primers for sequencing were the same as those used for PCR amplification.

**Phylogenetic analysis.** The VP6 and NSP4 sequences obtained were aligned and compared with VP6 and NSP4 sequences of human and animal rotaviruses available in GenBank. VP6 SGs and NSP4 genotypes were determined by phylogenetic analysis using the neighbour-joining method and the Tajima–Nei distance matrix (Tajima & Nei, 1984) in the MEGA analytical package (version 2.0).

**RESULTS**

Thirty rotavirus strains with diverse VP4 and VP7 genotypes were analysed by sequencing of the NSP4 and VP6 genes following RT-PCR.

All rotavirus strains employed in this study were compared with those of various animal and human prototypes (available from GenBank) in the phylogenetic analysis.
Comparison of nucleotide sequences of the NSP4 gene included human strains Wa, ST3, RMC321, DS-1, KUN and S2, and animal strains OSU and 10733. The NSP4 phylogenetic tree in Fig. 1 showed that 28 of the strains clustered into a major group (bootstrap of 99%) designated genotype B, which also contained the human strains Wa, ST3 and RMC321 and the porcine strain OSU. The remaining two strains clustered with a 100% bootstrap value with human strains DS-1, KUN and S2 and bovine strain 10733, designated genotype A. Within genotype B, the four samples selected from years 1986–1988 and 1990 differed more in their NSP4 gene compared with the other strains and clustered distinctly, showing in-group similarities ranging from 80 to 99%. Alignment of the NSP4 amino acid sequences (Fig. 2) showed some significant amino acid changes in NSP4 between genotypes A and B in the interspecies variable domain (aa 131–141; Fig. 2, marked in a box) and in the cytoplasmic domain of the protein within the DLP-binding region (aa 156–175; Fig. 2, indicated by diamonds). The enterotoxin domain (aa 114–135) is also indicated (Fig. 2, asterisks).

The VP6 nucleotide sequences were compared and 28 strains were determined to be SG II based on phylogenetic analysis with the human WA, E210 and RMC100 strains and the porcine Gott strain (bootstrap value of 68%) (Fig. 3). The two remaining strains were determined as SG I, showing a distinct cluster with the bovine NCDV, simian SA-11 and human US1205 and 1076 strains (bootstrap value of 73%). The representative equine FI-14 and murine EW strains were included in the analysis for SG I+II and SG non-I-non-II determination, respectively. The four samples analysed from years 1986–1988 and 1990 were distinguishable within SG II and clustered separately from the 2001–2004 strains, showing in-group similarities ranging from 93 to 97%.

![Fig. 2.](image-url)

Multiple alignment of the deduced amino acid sequence of the NSP4 protein of 14 human rotavirus strains with that of human prototype strains grouped in genotypes A, B and C. Dots indicate identity to the Wa/Hu strain.
Among the rotaviruses, NSP4 segregates into five major genotypes (A–E) (Lin & Tian, 2003). In this study, 30 rotavirus strains were sequenced and two genetic groups of the NSP4 gene could be recognized. Twenty-eight rotavirus strains representing the P_{8}G1, P_{8}G9, P_{4}G2 and P_{8}G5 genotypes were more closely related to NSP4 genotype B comprising human reference strains Wa and ST3 (bootstrap of 95 %). Two P_{4}G2 rotavirus strains were closely related to NSP4 genotype A, comprising human reference strains S2, KUN and DS-1 (bootstrap value of 100 %). NSP4 proteins of the Wa-like and KUN-like rotavirus strain genotypes comprise species-specific sequences allowing rotavirus strains to cluster generally to their species of origin by phylogenetic analysis (Ciarlet et al., 2000). NSP4 genotype C was represented by the AU-1 prototype strain included in the analysis and clearly differed from the rest, falling as an outgroup. Diversity within NSP4 genotypes among human species may be important, as NSP4 seems to play a role in immunity and protection (Ball et al., 2005). The role in immunity of the two genotypes identified in our study has not been defined and it is still unknown whether it is important for NSP4 to be included in rotavirus vaccination strategies. The genetic groups of the NSP4 gene observed in our study were in accordance with studies describing G2 and non-G2 rotavirus strains correlated with NSP4 genotypes A and B, respectively. In Taiwan, rotavirus strains recovered from children with or without diarrhea were P_{4}G2 genotype, closely related to the S2 strain, and P_{8}G1, P_{8}G3, P_{8}G4 and P_{6}G1 genotypes, closely related to the Wa and ST3 strains (Lee et al., 2000). Mascarenhas et al. (2006) described P_{6}G2 rotavirus strains from neonates in Belém, Brazil, having 100 % homology correlation with NSP4 genotype A. The NSP4 analysis of the four rotavirus strains from years 1986–1988 and 1990 showed that these strains clustered together, forming a separate group within NSP4 genotype B, and were most closely related to the ST3 strain. In addition, the majority of rotavirus strains collected between 2001 and 2004 appeared to cluster separately for each year, except for strains rj9473/04/P_{8}G9, rj6906/03/P_{8}G9, rj4956/01/P_{8}G9, rj7150/03/P_{8}G1 and rj6904/03/P_{8}G1, as shown in Fig. 1. This may indicate that the rotavirus NSP4 protein mutates via accumulation of single point substitutions, as no pattern of dispersion among strains was observed over
the years. Amplification of a 379 bp region of the VP6 gene that defines the SG-specific epitopes allowed the molecular subgrouping of all 30 rotavirus strains in our study. Sequence analysis revealed that strains with SG I specificity were associated with NSP4 genotype A and strains with SG II specificity were associated with NSP4 genotype B, as described by Iturriza-Gómar et al. (2003). Previous molecular characterization of this VP6 region has described only two subgroups, SG I and SG II, occurring in human rotaviruses (Iturriza-Gómar et al., 2002). Therefore, it could be assumed that the diversity of NSP4 genes among human rotavirus strains is restricted to two of the five NSP4 genotypes described. The linkage observed between NSP4 genotypes and VP6 subgrouping may occur as a result of NSP4 working as a receptor for VP6 in the course of virus maturation. During this process, recent DLPs are internalized into the lumen of the endoplasmic reticulum, mediated by direct association between the NSP4 and VP6 proteins (Estes, 2001). Comparison of complete deduced amino acid sequences of the NSP4 protein showed that most divergence among NSP4 genotypes was observed in the cytoplasmic domain of NSP4, where interactions with VP6 occur. However, it was noted that the interspecies variable domain possessed the highest diversity, observed at aa 131, 133 and 136–141, where the NSP4 protein is more distinguished among genotypes. Zhang et al. (1998) described amino acid substitutions in the variable region of porcine rotaviruses (Gottfried strain) that were associated with altered virulence in mice. The DLP-binding region (aa 156–175) contained five amino acid substitutions between NSP4 genotypes A and B at aa 157, 161, 167, 169 and 174. These significant changes may be important in identifying the existence of linkages between rotavirus gene segments.

REFERENCES


