Detection of a neonatal human rotavirus strain with VP4 and NSP4 genes of porcine origin

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A human rotavirus strain (NB-150) was detected in stool samples from a neonate hospitalized for mild/moderate community-acquired diarrhoea. This baby lived in the outskirts of Belém, Brazil, under poor sanitation conditions. The NB-150 strain displayed a typical long electrophoretic pattern with 11 gene segments. It had two VP7 alleles, G1 and G4, and belonged to VP6 subgroup II. A close relatedness with human rotaviruses was shown for VP7 alleles: G1 (96.9–100 % similarity at the amino acid level) and G4 (97.1–100 % similarity at the amino acid level). As for VP6, 95.1–97.5 % similarity at the amino acid level was noted. VP8* and NSP4 genes showed a close relatedness with those of porcine rotavirus strains, as follows: VP8* (95.0 % similarity at the amino acid level) and NSP4 (93.7–96.0 % similarity at the amino acid level). This is believed to be the first report in Brazil of a rotavirus infection involving a strain with G1 and G4 alleles, with VP8* and NSP4 genes of porcine origin. These findings strongly suggest the occurrence of interspecies transmission.

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INTRODUCTION

Rotaviruses are worldwide enteric pathogens of a number of animal species including humans, cattle and pigs. In humans, rotavirus diarrhoea results in significant morbidity and mortality, especially in developing countries (Kapikian *et al.*, 2001; Parashar *et al.*, 2006). The rotavirus genome consists of 11 segments of double-stranded RNA (dsRNA), which encode 12 viral proteins: 6 structural proteins and 6 non-structural proteins (Estes, 2001). The segmented nature of the rotavirus genome provides a unique mechanism for the generation of genetic diversity through genetic reassortment that is likely to occur during *in vivo* and *in vitro* mixed infections (Ramig, 1997).

The intermediate capsid protein VP6 bears subgroup (SG) specificities that allow classification of group A rotaviruses

into SGI, SGII, both SGI and II, or neither SG, as based on the reactivity with SG-specific monoclonal antibodies (Estes, 2001). A long RNA electropherotype (e-type) and SGI combination is typical of animal rotaviruses, while long e-type in conjunction with SGII (G1, G4, P[8], P[6]) and short e-type plus SGI G2P[4] strains are commonly found in human rotaviruses (Kapikian *et al.*, 2001). Rotaviruses are in general typed by their two outer capsid proteins, VP4 (P-type) and VP7 (G-type), and certain types predominate in natural infections of specific animal species (Gentsch *et al.*, 1996, 2005).

The rotavirus non-structural protein NSP4 is encoded by gene segment 10, and represents an intracellular receptor that mediates the acquisition of a transient membrane envelope as subviral particles bud into the endoplasmic reticulum. This protein has been studied extensively due to its role in viral morphogenesis and its potential enterotoxigenic property (Tian *et al.*, 1995; Ball *et al.*, 1996; Estes, 2001). NSP4 proteins from group A rotaviruses have been genetically classified into at least five (A–E) genotypes: A (KUN prototype), B (Wa), C (AU-1), D (WE) and E (avian-like) (Horie *et al.*, 1997; Kirkwood & Palombo, 1997; Ciarlet *et al.*, 2000). In this report we describe the characterization

Abbreviations: EIA, enzyme immunoassay; e-type, RNA electropherotype; SG, subgroup.

The GenBank/EMBL/DDBJ accession numbers for the partial sequences of the neonatal rotavirus isolates are DQ299878 (VP6), DQ299877 (VP4), DQ299879 (VP7 G1), DQ299880 (VP7 G4) and DQ299876 (NSP4).

of a neonatal rotavirus strain as based on the analysis of the VP8* trypsin-cleavage fragment of the VP4, VP6, VP7 and NSP4 genes.

METHODS

Specimens. The neonatal strain NB-150 was collected during a hospital-based survey for rotavirus infection conducted from May 1996 to May 1998 in Belém, northern Brazil. This strain was obtained in the context of molecular epidemiological studies involving rotavirus strains infecting hospitalized neonates with community-acquired diarrhoea, nosocomial diarrhoea and asymptomatic nosocomial infection (Linhares *et al.*, 2002). Of the 51 neonates excreting rotavirus, 50 (98%) were P[6]G2 and displayed a short e-type profile. Of these, 26 were partially sequenced for VP4, VP7 and NSP4 genes; all strains were characterized as genotype G2 and NSP4 genotype A (Mascarenhas *et al.*, 2006). The only exception was strain NB-150, recovered from a neonate with community-acquired diarrhoea, which showed long e-type, and G1 and G4 type specificities.

G-serotyping and subgrouping. G-serotyping and subgrouping were performed by enzyme immunoassay (EIA) using monoclonal antibodies specific for G1, G2, G3 and G4 types, and SGI and II, as described by Taniguchi *et al.* (1987) and Greenberg *et al.* (1983), respectively.

RNA extraction and typing by PCR. Viral dsRNA was extracted from faecal samples using silica powder glass (Boom *et al.*, 1990), with modifications as described by Araújo *et al.* (2001). PAGE analysis was carried out in Tris-glycine buffer as previously described by Pereira *et al.* (1983). Multiplex PCR for G and P typing was made using a pool that consisted of G1-, G2-, G3-, G4- and G9- (Das *et al.*, 1994), and P[8]-, P[4]-, P[6]- and P[9]-specific primers (Gentsch *et al.*, 1992). For amplification of VP6 and NSP4 genes, RT-PCR was carried out using VP6-F/VP6-R (Iturriza-Gómara *et al.*, 2002) and Jrg30/Jrg31 (Cunliffe *et al.*, 1997) to amplify 379 and 738 bp, respectively. All PCR products (VP8*, VP6, VP7 and NSP4) were purified by using a QIAquick PCR purification kit (Qiagen).

Cloning, sequencing and phylogenetic analysis. The partial length of the cDNA fragments of the 876 (VP8*), 904 (VP7) and 738 bp (NSP4) were cloned using a plasmidial-bacterial system. Purified amplicons were first ligated into a cloning pGEM-T Easy vector (Invitrogen) at the *lacZ* α -peptide gene in order to obtain recombinant DNA. Plasmids with the inserted viral cDNA were then transformed and amplified in *Escherichia coli* competent bacteria (JM109 lineage) and the plasmids were recovered using the S.N.A.P. plasmid miniprep kit (Invitrogen). The nucleotide sequences of the cloned cDNA were determined by the dideoxy chain-termination method using the sequencing ABI PRISM dye terminator kit (Applied Biosystems) and resolved in an ABI 377 DNA sequencer. Universal primers designed on the basis of plasmid promoter region T7 and SP6 were used to sequence the recombinant DNA in both directions. Portions of the specific regions obtained from sequencing were as follows: VP8* gene (nt 11–887), VP7 gene (nt 37–941) and NSP4 gene (nt 1–738). The VP6 amplicon (379 bp, corresponding to nt 724–1103 of the coding region) was directly sequenced with the same primers used in PCR. The sequence alignment and phylogenetic analysis were done using the BioEdit editor (version 7.0) and MEGA package (version 3.1) using the neighbour-joining algorithm based on the Kimura two-parameters (Kimura, 1980) distance estimative method for nucleotide, providing statistical support with bootstrapping over 2000 replicates. For analysis, prototype strains for VP4, VP6, VP7 and NSP4 genes were obtained from GenBank at the National Center for Biotechnology Information, USA (<http://www.ncbi.nlm.nih.gov>).

RESULTS

Characterization of e-type, serotype/genotype and SG

The NB-150 strain displayed a long e-type clearly showing 11 gene segments. NB-150 was found to have G1 and G4 specificities by EIA and PCR typing. This strain has shown to be SGII by EIA and PCR. NB-150 also demonstrated P[6] VP4 genotype and NSP4 genotype B of porcine origins.

Analysis of cDNA cloning and sequencing

The sequences from two clones of the VP8* gene and of two clones of the NSP4 gene were determined and assembled. The sequences of seven clones from the VP7 gene were also obtained revealing mixed G1 and G4 genotypes.

VP6 analysis

Sequence analysis of the VP6 gene confirmed results from both EIA and PCR genotyping assigning NB-150 strain to genogroup II, and therefore belonging to SGII (data not shown). The NB-150 strain denoted a close relatedness to two SGII human rotaviruses, Wa and RV3, showing 95.1–97.5% amino acid identity, suggesting that neonatal NB-150 strain has a higher relatedness to SGII human strains than to porcine SGII (Table 1, Fig. 1). Specific amino acids at positions 305 (N), 310 (Q), 315 (Q), 339 (N) and 342 (L) all predictive of human SGII specificity were conserved (data not shown).

VP8* analysis

The analysis of the deduced amino acid sequence of the VP8* subunit of VP4 (282 aa), corresponding to aa 1–282 of the coding region of the gene, was constructed including representative lineages following what has been outlined by Martella *et al.* (2006) with human and porcine strains. The NB-150 strain fell into lineage I, but did not cluster specifically with any of the sublineages (Ia, Ib, Ic and Id) proposed by these authors. In fact, NB-150 strain has shown a highest similarity to porcine sublineage Ib (95.0%). In contrast, the sequence of P[6]G2 of the NB-244 strain, which co-circulates along with the P[6]G1/G4 of the NB-150 strain, fell into human sublineage Ia as described by Martella *et al.* (2006) (Table 1, Fig. 2).

VP7 analysis

The nucleotide sequences of the NB-150 strain showed two alleles for the VP7 gene, G1 and G4. The analysis of the G1 allele was done using a sequence of 261 aa of 22 G1 human strains isolated from several countries, including representative lineages I to IV, as reported by Jin *et al.* (1996). Two porcine strains were also included in the analysis. The NB-150 strain fell in lineage I (bootstrap value of 79%), showing a close relatedness to human rotavirus strains T73, B7402, Chi-45 and 2TT, with 96.9–100% similarity. In contrast, strains Brz-5 and Brz-6, isolated from diarrhoeic children who

Table 1. Percentage amino acid sequence similarities of VP6, VP8N, VP7 and NSP4 of neonatal rotavirus strain NB-150 as compared with several rotavirus strains

The highest identity is given in bold.

Host species	Strain	Subgroup (VP6)	Genotype (VP4/VP7)	NSP4 genotype	Percentage identity of NB-150 with:				
					VP6	VP8*	VP7		NSP4
							G1	G4	
Human	RV3	II	P[6], G3	B	97.5	91.8			90.2
Human	Wa	II	P[8], G1	B	95.1		94.2		92.0
Human	US1205	I	P[6], G9	A	91.1	91.8			
Human	RMC321	I	P[19], G9	B	89.5				96.0
Porcine	A2	N	P[7], G9	B					93.7
Porcine	A253	I	P[7], G11	B					95.4
Porcine	Gottfried	II	P[6], G4	B	91.1	83.6		93.0	
Porcine	OSU	I	P[7], G5	B	88.7				94.8
Human	CH61	N	P[6], G1	B					93.1
Simian	SA11	I	P[2], G3	A	92.7				
Porcine	221/04-13	I	P[6], G3	B		95.0			
Human	M37	II	P[6], G1	B		92.1			
Human	NB244	N	P[6], G2	A		88.8			
Human	ST3	II	P[6], G4	B		90.0		94.2	89.1
Porcine	134/04-10	I	P[6], G3	B		89.3			
Porcine	51-04	I	P[6], G4	B		85.9			
Human	2TT	N	N, G1	N			100.0		
Human	Chi-45	N	N, G1	N			98.0		
Human	B74-02	N	P[8], G1	N			97.7		
Human	T73	N	N, G1	N			96.9		
Human	Jp417	N	N, G1	N			95.7		
Human	Oh-40	N	N, G1	N			95.0		
Human	Isr-56	N	N, G1	N			94.2		
Human	Brz-5	N	N, G1	N			93.1		
Human	Brz-6	N	N, G1	N			93.1		
Porcine	C95	I	P[7], G1	N			90.8		
Human	R195	N	N, G4	N				100.0	
Human	Arg1662	N	P[8], G4	N				97.1	
Human	Arg958	N	P[8], G4	N				96.7	
Human	Arg1620	N	P[8], G4	N				92.2	
Human	Odélia	N	P[8], G4	B				94.6	
Human	PV5249	N	P[8], G4	N				95.9	
Porcine	ArgP28	N	P[6], G4	N				80.0	
Human	KUN	I	P[4], G2	A					83.4
Human	DS1	I	P[4], G2	A					83.4
Human	NB-140	N	P[6], G2	A					83.4

N, Not available.

participated in a rotavirus vaccine trial in Belém, Brazil (Linhares *et al.*, 1996), fell into lineage III (Table 1, Fig. 3). The divergence rates observed between NB-150 strain (lineage I) and prototype and strains from lineage III, Wa and Brz5 and Brz6 was of 5.8 and 6.9 %, respectively (data not shown).

A phylogenetic tree for allele G4 was constructed using a sequence of 735 nt, including the prototype ST3 strain and

representative lineages assessed by Bok *et al.* (2002) and Arista *et al.* (2005). NB-150 strain fell into sublineage 1c (Fig. 4), closely related to G4 human rotavirus strains Arg1662 from Argentina (Bok *et al.*, 2002), and R195 from Rio de Janeiro, Brazil (Volotão *et al.*, 2006). The strains Arg1662 and R195 showed 97.1–100 % aa identity when comparing to NB-150 strain (Table 1). The divergence between NB-150 and ST3 was 5.8 %.

A phylogenetic analysis of NSP4 protein was performed targeting 1 to 537 nt of the coding region of gene 10, and included NSP4 genotypes A, B, C and D. NB-150 strain was

The NB-150 strain showed potential N-linked glycosylation sites located at aa 8 and 18, two cysteine (C) residues conserved at aa 63 and 71 positions, a common aspartic acid (D) at position 19, proline (P) residues at positions 28, 34, 52, 138, 165 and 168 and a glutamine (Q) residue at position 97 (Estes, 2001) (data not shown).

P[6] rotavirus strains are usually associated with asymptomatic infection (Steele *et al.*, 1995; Pager *et al.*, 2000; Cunliffe *et al.*, 2002). P[6] genotype, associated with G1 and/or G4 genotypes, has also been detected in children with diarrhoea (Araújo *et al.*, 2002; Bányai *et al.*, 2004; Leite *et al.*, 1996; Mascarenhas *et al.*, 2002; Ramachandran *et al.*, 1996; Santos & Hoshino, 2005; Steele & Ivanoff, 2003; Timenetsky *et al.*, 1994). A mixed G1 and G4 infection in a diarrhoeic child from Belém, Brazil, has been reported previously using monoclonal antibodies only (Mascarenhas *et al.*, 1996). To our knowledge, neonatal NB-150 strain constitutes a unique finding in Brazil, since this strain has G1 and G4 alleles of human origin, and VP8* and NSP4 genes of porcine origin.

Fig. 3. Phylogenetic analysis of the NB-150 deduced amino acid sequence using the neighbour-joining method indicating its genetic relationship with VP7, allele G1.

On the other hand, studies conducted worldwide have demonstrated that G1 plus G4 rotavirus infection is uncommon. The frequencies of mixed infection by G1 and G4 serotypes are 3.2 and 16.1 % in studies conducted in Australia and Cairo, respectively, on the basis of monoclonal antibodies (Bishop *et al.*, 1991; Rawdan *et al.*, 1997). In Ireland, O'Mahony *et al.* (1999) detected cases of rotavirus infection by G1/G4P[8] in 4 % of the children based on reverse transcriptase PCR-based genotyping methods. Data on nucleic acid sequences were not available from these studies.

The close relationship between P[6] strains recovered from animals and humans has been characterized by Martella *et al.* (2006). These authors have detected porcine P[6] strains closely related to P[6]-I and P[6]-V human strains and distantly related to the porcine strain Gottfried, P[6]-II. The present study shows that the NB-150 strain clusters with P[6]-I human and porcine strains but it is more related to porcine strains than to human strains. Accordingly, the VP8* of strain NB-150 is likely of porcine origin, although being another sublineage. In contrast, NB-244 strain, recovered from a neonate with asymptomatic nosocomial infection associated with P[6]G2 and that co-circulated along with the NB-150 strain and G1/G4P[6]-Ib strain, fell

into human sublineage Ia as shown in Fig. 2. Such findings raise the hypothesis of an interspecies transmission involving humans and pigs. Evidence for genetic reassortment between human and animal rotaviruses has been reported particularly in cows and pigs (Martella *et al.*, 2006; Santos & Hoshino, 2005; Bányaí *et al.*, 2003; Iturriza-Gómara *et al.*, 2003).

The G1 allele of NB-150 strain fell into lineage I and was most closely related to 2TT strain (100 % amino acid similarity). In contrast, it had 94.2 % amino acid similarity when compared with prototype Wa or Brz-5 and Brz-6 strains; these two latter strains were recovered from children (placebo recipients) who participated in a rotavirus vaccine trial in Belém, Brazil, and were classified as belonging to lineage III (Jin *et al.*, 1996). NB-150 strain, however, showed divergence of 5.8 and 6.9 %, respectively, when compared to prototype Wa and Brz5, and to Brz6 strains from lineage III.

G4 rotavirus serotype had previously been divided into two subtypes, namely subtype A (ST3-like) and subtype B (VA 70-like) (Gerna *et al.*, 1988; Green *et al.*, 1992). The sublineage Ic G4 viruses were first detected in Argentina in 1998 by Bok *et al.* (2002), followed by Uruguay in 1999 (Berois *et al.*, 2003) and in Rio de Janeiro, Brazil, in 2000

Fig. 4. Phylogenetic analysis of the NB-150 deduced nucleotide sequence using the neighbour-joining method indicating its genetic relationship with VP7, allele G4.

(Volotão *et al.*, 2006). The NB-150 strain currently has data going back to 1997 (Linhares *et al.*, 2002) and falls into lineage 1c. Of note, the divergence observed between the NB-150 strain and the ST3 prototype was 5.4 %.

In considering that a mixed G1 and G4 infection has occurred, it seems likely that e-types of both viruses have fully overlapped, as 11 segments only could be identified. Although G1 and G4 serotype specificities may raise the hypothesis of a single rotavirus strain bearing a dual VP7 specificity, such an event seems very unlikely, as based on previous studies in Brazil (Genstsch *et al.*, 2005; Santos & Hoshino, 2005) and other countries, where mixed rotavirus infections are common events (Fischer *et al.*, 2005; Rahman *et al.*, 2005).

The potential role of NSP4 in virulence and in the understanding of diversity within NSP4 genotypes among species may be of importance, as NSP4 seems to play a role in immunity and possibly in protection (Ball *et al.*, 2005). Sequence analyses indicate that NSP4 genotype A does not

have shown strains of porcine origin, mostly G5 and G9 serotypes, causing diarrhoea in children, and suggesting a frequent exchange of genetic material among co-circulating human and animal rotavirus strains (Alfieri *et al.*, 1996; Leite *et al.*, 1996; Timenetsky *et al.*, 1997; Gouvea & Santos, 1999; Mascarenhas *et al.*, 2002; Santos *et al.*, 2005). It seems likely that NB-150 strain was a result of transmission from pigs to humans, since there are only a few genes of porcine origin in human-derived rotavirus strain and not vice versa. Of note in this context, it has been reported that pigs are in general more susceptible to human rotaviruses than any other species, suggesting that the products of at least some of the genes should be determinants of the host range restriction inherent to mammalian group A rotaviruses (Rao *et al.*, 1995; Santos *et al.*, 1999). Genetic and phylogenetic analyses of structural and non-structural genes have indicated a consistent pattern of evolutionary relationships between certain animal and human viruses, as based on comparisons of VP6, NSP1, NSP3, NSP4 and NSP5 (Dunn *et al.*, 1994; Cunliffe *et al.*, 1997; Fujiwara & Nakagomi, 1997; Horie *et al.*, 1997; Kirkwood & Palombo, 1997; Iturriza-Gómara *et al.*, 2003). In this study, phylogenetic analyses of VP6 and VP7 genes have shown a close relatedness between the neonatal NB-150 rotavirus strain and human rotaviruses. In addition, sequence analyses of VP4 and NSP4 genes sustain a close relatedness with the porcine strains.

Evidence for genetic reassortment between human and animal rotaviruses has been reported in several studies (Das *et al.*, 1993). The neonate from whom NB-150 was recovered lived in the outskirts of Belém, Brazil, under poor sanitation and in close contact with domestic animals, including pigs. A study limitation is that no faecal specimens were collected from pigs living in the same environment, so a more firm conclusion could not be drawn as to the interspecies transmission issue. An additional limitation of our study was that the NB-150 strain could not be cell cultured due to local technical constraints. Plaque purification procedures would clearly add further evidence in favour of the hypothesis of mixed infection. Simultaneous surveillance and sequencing of rotavirus genes from animals and humans are essential for a better understanding of the relationship between co-circulating strains with common and uncommon G/P combinations. Moreover, this would significantly contribute to a better assessment of the ample genetic diversity of rotaviruses.

Our study provides evidence to support the hypothesis that there is a dynamic interaction between rotaviruses of human and animal origins. Consequently, reassortment events can lead to a stable introduction and successful spread of animal gene alleles into human rotavirus genomes.

In summary, we have identified a human rotavirus strain that displays an unusual combination with long e-type pattern, G1 and G4 specificities, and belongs to SGII. VP7 and VP6 genes were closely related to rotavirus strains of human origin but phylogenetic analyses demonstrated that VP8* and NSP4 genes were clustering with those of porcine

strains. The findings from our study suggest that animal rotaviruses may cross the species barrier and even cause diarrhoea in humans. This can be regarded as a key step in acquiring a better understanding of rotavirus evolution and ecology. Moreover, interspecies transmission may possibly pose potential difficulties in future strategies toward the development of rotavirus vaccines.

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