Molecular characterization of the VP1, VP2, VP4, VP6, NSP1 and NSP2 genes of bovine group B rotaviruses: Identification of a novel VP4 genotype

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Running title: Molecular characterization of bovine group B rotaviruses

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Abstract

Studies on bovine group B rotaviruses (GBRs) are limited. To date, the VP6 gene of a single bovine GBR strain, and VP7 and NSP5 genes of a few bovine GBR strains only have been sequenced and analyzed. In the present study, by single primer amplification method, we determined the full-length nucleotide sequences of VP1, VP2, VP4, VP6, NSP1 and NSP2 genes of three bovine GBR strains from eastern India. In all these six genes, the bovine GBR strains shared high genetic relatedness among themselves, but exhibited high genetic diversity with cognate genes of human, murine and ovine GBRs. Interestingly, as in group A rotaviruses, the bovine GBR VP1, VP2, VP6 and NSP2 genes appeared to be more conserved than VP4 and NSP1 genes among strains between species. The present study provided important insights into the genetic makeup and diversity of bovine GBRs, and also identified a novel GBR VP4 genotype.

Introduction

Rotaviruses (genus *Rotavirus*, family *Reoviridae*) are major causes of diarrhoea in humans and other animals [8]. Mature rotavirus particle contains eleven segments of double-stranded RNA that encode six structural (VP1-4, VP6-7) and six nonstructural proteins (NSP1-6) [8]. Among the structural proteins, VP1 is an RNA-dependent RNA polymerase (RdRp), VP2 is the major inner core protein, VP3 functions as the virus capping enzyme, VP6 forms the inner capsid, and VP4 and VP7 constitute the outer layer of the viral capsid [8]. The nonstructural proteins are primarily involved in virus replication and morphogenesis [8].

Rotaviruses have been classified into seven groups (A-G) on the basis of antigenicity of the VP6 protein [8]. Among them, group A and C rotaviruses (GARs and GCRs, respectively) cause diarrhoea in infants and children [8], while group B rotaviruses (GBRs) have been primarily associated with diarrhoea in adults [17], though a recent study reported high rates of detection of GBRs among children with diarrhoea [3]. Apart from humans, group B rotaviruses cause diarrhoea in pigs, lambs, rats and cattle [2, 5, 10, 31-32, 34-35]. In cattle, GBRs were detected in sporadic cases and/or outbreaks of diarrhoea in calves and adults from India, Japan and USA [2, 5, 10, 34]. Moreover, antibodies to GBRs have been reported in cattle from Japan and UK [4, 33].

Contrary to GARs, limited sequence data are available for GBR strains. To date, the full genome of a murine and a few human GBR strains have been sequenced [17, 25]. In lambs, NSP1 and NSP5 genes of an ovine GBR strain have been sequenced [31]. In cattle, VP7 gene sequences are available for bovine GBRs from India (Kolkata and RUBV strains), Japan (Nemuro strain) and USA (ATI, Mebus and WD653 strains) [2, 5, 10, 34]. Among other bovine GBR genes, NSP5 genes of Kolkata and RUBV strains and VP6 gene of Nemuro strain have been sequenced [2, 10, 33]. Comparative analyses of these gene sequences revealed high genetic diversity among GBR strains between species.

The rotavirus outer capsid proteins, VP4 and VP7, provide immune protection, and therefore, information on VP4 and VP7 genotypes are important for vaccine development [8, 16]. Moreover, full genome analyses of rotaviruses are necessary to ascertain the true origin of a strain [12, 22]. However, our present knowledge on the bovine GBR genome are limited to only three (VP6, VP7 and NSP5) of the 11 gene segments. Therefore, information on the remaining 8 bovine GBR gene segments are required to completely understand the evolution of bovine GBRs and their genetic relatedness to other GBRs. Moreover, gathering of such sequence information might be helpful to investigate the ecology of human GBRs, since they seem to be rare and it is

not clear if they are true human pathogens or the result of occasional transmission from unidentified animal sources.

In the present study, we report for the first time the full-length nucleotide (nt.) sequences of VP1, VP2, VP4, NSP1 and NSP2 genes of three bovine GBR strains (one Kolkata and 2 RUBV strains). The VP6 genes of these strains were also genetically characterized. Our findings provided new insights into the genetic makeup of bovine GBRs, and identified a novel GBR VP4 genotype.

Materials and methods

Viruses

Bovine GBR strains DB176, RUBV226 and RUBV282, characterized in the present study, were detected from calves with diarrhoea during surveillance studies conducted at a cattle market in the city of Kolkata, eastern India [2, 10]. The sample collection dates for the samples were November of 2001, December of 2004 and February of 2005 for strains DB176, RUBV226 and RUBV282, respectively [2, 10].

RT-PCR, cloning and sequencing

The full-length nt. sequences of the VP1, VP2, VP4, VP6, NSP1 and NSP2 genes of bovine GBR strain DB176 were obtained by the single primer amplification method as described previously [36]. Rotavirus RNA was extracted from the stool sample using

the QIAamp Viral RNA Mini kit (Qiagen Sciences, MD, USA). Thereafter, ligation of the single amino group-linked oligonucleotide primer to 3'- end of both strands of viral double-stranded RNA, column based purification and concentration of the ligated RNA and RT-PCRs were carried out as described previously [36]. The PCR products were cloned into pCR2.1-TOPO vectors (Invitrogen, CA, USA), and six positive clones were sequenced for each of the six genes. Subsequently, 5'- and 3'- end primers and several internal primers were designed from the full-length VP1, VP2, VP4, VP6, NSP1 and NSP2 gene sequences of strain DB176, and used to confirm the obtained data by RT-PCR and sequencing. Moreover, these primers were also employed to amplify and determine the full-length sequences of the six genes of the two other bovine GBR strains, RUBV226 and RUBV282, respectively. Furthermore, the 5'- and 3'- end sequences of the six genes of the three bovine GBR strains were confirmed by a modified single primer amplification method. Briefly, cDNA was generated from viral RNA by the single primer amplification method [36], and then, the 5'- and 3'- portions were amplified and sequenced using the common end primer C [complementary to the ligated primer] [36] and internal primers, designed from the obtained sequences. Throughout the study nt. sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Reaction kit (Applied Biosystems, CA, USA) on an automated

sequencer (ABI PRISM 3100).

Sequence analyses

Sequence comparisons, multiple alignments and plotting of hydropathy profiles were carried out as described previously [2, 10, 25]. Phylogenetic trees were generated by the neighbor-joining method [27] using the MEGA software (version 4.1). The trees were statistically supported by bootstrapping with 1000 replicates, and phylogenetic distances were measured by the Kimura two-parameter model.

Nucleotide sequence accession numbers

The full-length nt. sequences of VP1, VP2, VP4, VP6, NSP1 and NSP2 genes of bovine GBR strains DB176, RUBV226 and RUBV282 were submitted to GenBank database, and were assigned consecutive nt. sequence accession numbers GQ358710- GQ358727.

Results and discussion

Although GBRs have been detected in cattle in different parts of the world, few bovine GBR genes have been sequenced and analyzed. Among them, the VP7 genes of the Kolkata and RUBV strains, detected from a cattle market in eastern India, shared high deduced amino acid (aa) identities (97.8%-100%) among themselves, but exhibited <89% deduced aa identities to those of other bovine GBRs, suggesting the existence of at least two bovine GBR VP7 genotypes [2, 10]. However, the overall VP7 identities

among bovine GBRs were higher than those between bovine and human or murine GBRs [2, 5, 10, 34]. The NSP5 genes of the Kolkata and RUBV strains shared high deduced aa identities (97%- 100%) among themselves, but exhibited low aa identities (48%- 50.6%) to those of human and murine GBR strains, and relatively higher aa identities (81.9%- 83%) to that of ovine strain KB63 [2, 10]. The VP6 gene of the Nemuro strain exhibited high diversity to those of human and murine GBRs [33]. These observations indicated that bovine GBRs might be genetically distinct from other GBRs, but were based on limited data on 3 gene segments.

In the present study, we determined the full-length nt. sequences of the VP1, VP2, VP4, VP6, NSP1 and NSP2 genes of one Kolkata (DB176) and two RUBV (RUBV226 and RUBV282) bovine GBR strains, for which samples were available in sufficient quantities. In all the three bovine GBR strains, each of the four structural (VP1-2, VP4, VP6) and two nonstructural (NSP1-2) genes sequenced exhibited identical sizes, including that of open reading frame (ORF) (Table 1). The 5'- end of all the six bovine GBR genes started with a GG- sequence (Table 1), common to GARs, GBRs and GCRs [8, 16]. On the other hand, the 3'- termini of VP1, VP2, VP4, VP6 and NSP1 genes of the three bovine GBR strains ended with a –AAAACCC sequence, common to those of human GBRs [16, 25]. However, the NSP2 gene of these bovine

GBRs terminated with a –AAGACCC sequence (Table 1).

Molecular characterization of VP4 gene

The bovine GBR VP4 gene was 16bp and 19bp longer than those of murine and most human GBRs, respectively (Table 1). The predicted bovine GBR VP4 protein was 7 aa and 8 aa longer than those of murine and most human GBRs, respectively (Table 1). Comparative analysis of the deduced aa sequences of VP4 gene of GBRs revealed three putative trypsin cleavage sites in the bovine GBR strains that were also conserved in murine strain IDIR, while only the second and third sites were present in the human GBRs (Fig. 1). Interestingly, the predicted third cleavage site of bovine GBRs was best aligned with the preferred second site of GARs (data not shown). Based on the 2nd and 3rd putative trypsin cleavage sites, the VP8* and VP5* portions of VP4 of the bovine GBRs were 215 aa and 536 aa long, respectively. The VP8* to VP5* aa ratio was 1:2.49, within the range (1:2.1-2.6) observed in GARs, GBRs and GCRs. In GARs, higher sequence diversity has been observed in VP8* than in the VP5* portion of VP4 aa sequences [11, 30]. Similarly, the VP8* portion of bovine GBRs appeared to be more diverse than VP5* as well as the whole VP4 protein (Table 2, Supplementary Fig. S1). Moreover, the additional aa present in VP4 of bovine GBRs were mostly located in the VP8* portion (Supplementary Fig. S1).

The VP4 genes of the three bovine GBR strains shared high genetic relatedness among themselves, but exhibited low genetic relatedness to those of human and murine GBRs (Fig. 2, Table 2). With VP4 of novel group strains B219 and J19, related to GBRs [1, 15, 25], the bovine GBR strains exhibited nt. and aa identities of 50.2%- 50.5% and 25.4%- 26.3%, respectively. By multiple alignment of GBR VP4 aa sequences, all the 24 proline and 10 cysteine residues in the bovine GBR strains DB176, RUBV226 and RUBV282 were located at identical positions, and of them, 17 proline and 4 cysteine residues were also conserved in other GBRs (Supplementary Fig. S1). Comparisons of the hydropathy profiles of VP4 aa sequences of bovine, human and murine GBRs revealed common features, such as the presence of hydrophilic regions at the N-terminal and around the putative trypsin cleavage sites, and hydrophobic regions in VP5* portion (Fig. 3). However, the human GBRs lacked a hydrophilic region present in VP8* portion of bovine and murine GBRs (Fig. 3).

Molecular characterization of VP6 gene

The VP6 gene sequences of the three bovine strains exhibited high identities among themselves, and shared moderate nt., but high aa identities to the only other available bovine VP6 gene sequence of Nemuro strain (Table 2). On the other hand, strains DB176, RUBV226 and RUBV282 exhibited low sequence identities to those of human

and murine GBRs (Table 2), and by phylogenetic analysis, clustered together with Nemuro strain to form a bovine lineage, distinct from human and murine GBRs (Fig. 2). Therefore, our findings corroborated previous observations [33] that the genetic relatedness of VP6 among GBRs between species was lower than those observed among GARs and GCRs from different species. However, despite the low genetic relatedness, several of the proline and cysteine residues in the bovine VP6 aa sequences occurred at positions identical to those in human and murine GBRs (Supplementary Fig. S2). The VP6 gene of bovine GBRs exhibited low nt. (53%- 54.1%) and aa (36.9%- 37.4%) identities to those of novel group rotavirus strains, B219 and J19, respectively.

Molecular characterization of VP2 gene

The bovine GBR VP2 gene was 2bp longer than those of most other GBRs, and encoded a predicted protein that was 3 aa longer than those of other GBRs (Table 1). The bovine GBR VP2 gene appeared to be highly conserved among strains DB176, RUBV226 and RUBV282, and exhibited high diversity to those of human and murine GBRs (Fig. 2, Table 3). By multiple alignment of GBR VP2 aa sequences, 46 of the 47 proline and all the 6 cysteine residues were conserved among bovine GBRs, with the additional proline residue being present in the two RUBV strains, and of them, 39 proline and 4 cysteine residues were also conserved in other GBRs (Supplementary Fig.

S3). In GARs, although the VP2 proteins from different strains are highly conserved, they show marked variation in their amino termini [23]. This portion of VP2 is thought to lie inside the core shell and to bind the viral enzyme-RNA complex (VP1/VP3/RNA) [20, 26], and therefore, such variations within this region might reflect differences in protein-protein or protein-RNA interactions and RNA synthesis among strains [23]. Interestingly, the amino termini (1 aa-75 aa) of the VP2 aa sequences of the bovine GBRs also exhibited considerable variation (35.5%- 39.5% aa identities) with those of other GBRs, though the overall VP2 aa identities were much higher (Table 3).

Molecular characterization of VP1 gene

The bovine GBR VP1 gene, which encodes the RdRp, was shorter than those of human and murine GBRs (Table 1). The bovine VP1 gene appeared to be highly conserved among strains DB176, RUBV226 and RUBV282, and exhibited low genetic relatedness to those of human and murine GBRs (Fig. 2, Table 3, Supplementary Fig. S4). However, despite low sequence identities with human and murine GBRs, the specific sequence motifs, conserved in RdRps of double-stranded RNA viruses [6], were retained in the VP1 of bovine GBRs (Fig. 4). Moreover, similar to previous observations in human and murine GBRs [24], the N- and C- terminals of bovine GBR-VP1 appeared to be more diverse than the central part that corresponds to polymerase domain of GAR-VP1

(Supplementary Fig. S4).

Molecular characterization of NSP1 gene

The bovine GBR NSP1 gene exhibited higher nt. identities to that of ovine GBR strain KB63 than those observed to human or murine GBRs (Table 4). However, unlike the ovine strain, the bovine NSP1 gene contained two ORFs as in human and murine GBRs (Table 1). The deduced aa sequences of bovine NSP1 gene ORF 1 and 2 shared low identities with those of human and murine GBRs, while the three peptides of ovine GBR strain KB63 exhibited relatively higher identities to cognate stretches of NSP1 of the three bovine GBR strains, respectively (Table 4). By phylogenetic analysis, the bovine strains and the ovine strain formed a single cluster, distinct from the human and murine GBRs (Fig. 2). Therefore, the NSP1 gene of bovine GBRs appeared to be more related to that of ovine strain KB63 than to those of human and murine GBRs. Although the zinc-finger motif region is absent in NSP1 peptides of GBRs, a previous study [18] had identified regions of conserved cysteine and histidine residues, similar to the cysteine-rich zinc-finger motif region of NSP1 of GARs. The NSP1 peptide 2 sequences of the bovine GBR strains also lacked the zinc-finger motif, but retained these conserved cysteine and histidine residues (Supplementary Fig. S5). Moreover, a conserved cysteine rich motif [C-X-C-X8-C-X9-C2-X9-C-X6-C-X2-C] (aa positions

120- 162) was present in the NSP1 peptide 2 of the bovine GBR strains (Supplementary Fig. S5).

In GARs, the NSP1 protein is considered to be highly divergent among strains within and between species [12-13, 16, 22]. Despite the high divergence, previous studies had shown clustering of GAR NSP1 sequences according to the species of origin, and indicated its significance as a host range restriction factor [7, 19]. Similarly, the NSP1 sequence identities between bovine and other species were the lowest among the bovine GBR genes sequenced so far. On the other hand, phylogenetic clustering of NSP1 sequences of GBR strains from related species (bovine and ovine) was also evident.

Molecular characterization of NSP2 gene

The NSP2 gene of the three bovine GBR strains was 4bp and 6bp shorter than those of murine and human GBRs, respectively (Table 1). The NSP2 sequences of the bovine GBRs shared high genetic relatedness among themselves, but exhibited high genetic diversity to those of human and murine GBRs (Fig. 2, Table 4). With the partial NSP2 sequence of a porcine GBR strain (Strain BRA16-UEL, GenBank accession no. EF577259), nt. and aa identities of 66.4%- 66.9% and 73.3%- 74.1%, respectively, were observed. In GARs, the NSP2 gene has been shown to be highly conserved among

strains within and between species [12, 22]. Interestingly, the NSP2 sequence identities of bovine GBRs to those of other species were highest among the different bovine GBR genes sequenced so far.

Conclusions

Detailed analyses of the full-length VP1, VP2, VP4, VP6, NSP1 and NSP2 gene sequences of strains DB176, RUBV226 and RUBV282 provided new insights into the genetic makeup of bovine GBRs. All the six genes were highly conserved among these strains. However, relatively higher sequence identities were observed between strains RUBV226 and RUBV282 than those between RUBV GBRs and DB176, and might be due to accumulation of point mutations over time, considering the time intervals between detection of the DB and RUBV strains. The bovine GBR VP1, VP2, VP4, VP6, NSP1 and NSP2 genes exhibited high genetic diversity with cognate genes of human, murine and ovine GBRs. Therefore, information on 8 of the 11 bovine GBR gene segments clearly suggested that the bovine GBR genome might belong to a separate evolutionary lineage of GBRs, distinct from human and murine GBRs. These observations further suggested that the GBR genome might be more species specific than that of GARs. Nevertheless, the overall pattern of genetic relatedness observed was similar to GARs, with the bovine GBR VP1, VP2, VP6 and NSP2 genes being more

conserved than VP4 and NSP1 genes among strains between species. In this context, it would have been interesting to study the remaining three bovine GBR genes (VP3, NSP3-4). However, in the present study, further molecular characterization of bovine GBRs could not be performed as sufficient amount of samples were not available.

The present study also identified a novel GBR VP4 genotype. In GARs, nt. and deduced aa sequence identity cutoff values of 80% and 89%, respectively, form the basis of classification of VP4 genes of different strains into at least 31 P genotypes [22, 29]. In GCRs, lack of high aa sequence homology (<80%) among bovine, human and porcine VP4 genes indicated the presence of at least 3 genotypes [14]. Although no formal classification scheme exists for VP4 of GBRs, the sequence identities exhibited by the bovine GBR VP4 genes to those of human and murine GBRs were extremely low (60.1%- 62% nt. and 54.8%- 60.2% aa) compared to the proposed sequence identity cutoff values for assigning VP4 genotypes to GARs, and therefore, the bovine GBR VP4 genes might constitute a novel and the third GBR VP4 genotype. Unlike GARs, the three GBR VP4 genotypes appeared to be species specific (human, murine and bovine). In GARs, the VP4 genotype may not always coincide with VP4 serotype designations [8]. Due to lack of tissue culture adaptation of GBRs, except for a porcine strain [28], no serotyping scheme has been defined for GBRs [21]. However, the high levels of

sequence divergence observed among the three GBR VP4 genotypes indicated that multiple VP4 serotypes might exist in GBRs.

The zoonotic potential of GARs has been well documented [12, 16, 22]. Recently, transmission of GCRs from pigs to humans has been reported [9]. On the other hand, as of now, there is no genetic evidence for animal to human transmission of GBRs. Human and murine GBR strains are genetically diverse [17, 25], and in all the 8 bovine GBR gene segments sequenced, high genetic diversity was observed with human GBRs. However, GBRs have been reported from humans and cattle from the same geographical region (eastern India) [2-3, 10, 17]. In such regions, humans live in close proximity to cattle, and therefore, the possibility of reassortment events involving human and bovine GBRs cannot be ruled out. Further studies are required to understand better the mechanisms of genetic diversity of GBRs and investigate the possibility of their transmission across species.

Acknowledgements

The study was supported in part by the grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant number 20-08463) and Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (Okayama University-National Institute of Cholera and Enteric

Diseases, India).

References

1. Alam MM., Kobayashi N, Ishino M, Ahmed MS, Ahmed MU, Paul SK, Muzumdar BK, Hussain Z, Wang YH, Naik TN (2007) Genetic analysis of an ADRV-N-like novel rotavirus strain B219 detected in a sporadic case of adult diarrhea in Bangladesh. Arch Virol 152:199-208

2. Barman P, Ghosh S, Das S, Varghese V, Chaudhuri S, Sarkar S, Krishnan T, Bhattacharya SK, Chakrabarti A, Naik TN (2004) Sequencing and sequence analysis of VP7 and NSP5 genes reveal emergence of a new genotype of bovine group B rotaviruses in India. J Clin Microbiol 42:2816-2818

3. Barman P, Ghosh S, Samajdar S, Mitra U, Dutta P, Bhattacharya SK, Krishnan T, Kobayashi N, Naik TN (2006) RT-PCR based diagnosis revealed importance of human group B rotavirus infection in childhood diarrhoea. J Clin Virol 36:222-227

4. Brown DW, Beards GM, Chen G.M, Flewett TH (1987) Prevalence of antibody to group B (atypical) rotavirus in humans and animals. J Clin Microbiol 25:316–319

5. Chang KO, Parwani AV, Smith D, Saif LJ (1997) Detection of group B rotaviruses in fecal samples from diarrheic calves and adult cows and characterization of their VP7 genes. J Clin Microbiol 35:2107–2110

6. Cohen J, Charpilienne A, Chilmonczyk S, Estes MK (1989) Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. Virology 171:131-140

7. Dunn SJ, Cross TL, Greenberg HB (1994) Comparison of the rotavirus nonstructural protein NSP1 (NS53) from different species by sequence analysis and northern blot hybridization. Virology 203:178-183

8. Estes MK, Kapikian AZ (2007) Rotaviruses and their replication. In: Fields BN, Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields Virology, 5th edn. Lippincott, Williams & Wilkins, Philadelphia, PA, 1917-1974

9. Gabbay YB, Borges AA, Oliveira DS, Linhares AC, Mascarenhas JD, Barardi CR, Simões CM, Wang Y, Glass RI, Jiang B (2008) Evidence for zoonotic transmission of group C rotaviruses among children in Belém, Brazil. J Med Virol 80:1666-1674

10. Ghosh S, Varghese V, Sinha M, Kobayashi N, Naik TN (2007) Evidence for interstate transmission and increase in prevalence of bovine group B rotavirus strains with a novel VP7 genotype among diarrhoeic calves in Eastern and Northern states of India. Epidemiol Infect 135:1324-1330

11. Gorziglia M, Green K, Nishikawa K, Taniguchi K, Jones R, Kapikian AZ, Chanock RM (1988) Sequence of the fourth gene of human rotaviruses recovered from asymptomatic or symptomatic infections. J Virol 62:2978-2984

12. Heiman EM, McDonald SM, Barro M, Taraporewala ZF, Bar-Magen T, Patton JT (2008) Group A human rotavirus genomics: evidence that gene constellations are influenced by viral protein interactions. J Virol 82:11106-11116

13. Hua J, Mansell EA, Patton JT (1993) Comparative analysis of the rotavirus NS53

gene: conservation of basic and cysteine-rich regions in the protein and possible stem-loop structures in the RNA. Virology 196:372-378

14. Jiang B, Gentsch JR, Tsunemitsu H, Saif LJ, Glass RI (1999) Sequence analysis of the gene encoding VP4 of a bovine group C rotavirus: molecular evidence for a new P genotype. Virus Genes 19:85-88

15. Jiang S, Ji S, Tang Q, Cui X, Yang H, Kan B, Gao S (2008) Molecular characterization of a novel adult diarrhoea rotavirus strain J19 isolated in China and its significance for the evolution and origin of group B rotaviruses. J Gen Virol 89:2622-2629

16. Kobayashi N, Alam M, Kojima K, Mise K, Ishino M, Sumi A (2003) Genomic diversity and evolution of rotaviruses: An overview. In: Kobayashi N (ed) Genomic diversity and molecular epidemiology of rotaviruses. Research Signpost, Trivandrum, India, 75-90

17. Kobayashi N, Naik TN, Ahmed MU (2003) Genomic diversity and molecular

evolution of group B human rotaviruses. In: Kobayashi N (ed) Genomic diversity and molecular epidemiology of rotaviruses. Research Signpost, Trivandrum, India, 91-104

18. Kobayashi,N, Naik TN, Kusuhara Y, Krishnan T, Sen A, Bhattacharya SK, Taniguchi K, Alam MM, Urasawa T, Urasawa S (2001) Sequence analysis of genes encoding structural and nonstructural proteins of a human group B rotavirus detected in Calcutta, India. J Med Virol 64:583-588

19. Kojima K, Taniguchi K, Kobayashi N (1996) Species-specific and interspecies relatedness of NSP1 sequences in human, porcine, bovine, feline, and equine rotavirus strains. Arch Virol 141:1-12

20. Lawton JA, Zeng CQ, Mukherjee SK, Cohen J, Estes MK, Prasad BV (1997) Three-dimensional structural analysis of recombinant rotavirus-like particles with intact and amino-terminal-deleted VP2: implications for the architecture of the VP2 capsid layer. J Virol 71:7353-7360

21. Mackow ER (1995) Group B and C rotaviruses. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL (eds) Infections of the gastrointestinal tract. Raven Press Ltd., New York, 983-1008

22. Matthijnssens J, Ciarlet M, Heiman E, Arijs I, Delbeke T, McDonald SM, Palombo EA, Iturriza-Gómara M, Maes P, Patton JT, Rahman M, Van Ranst M (2008) Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J Virol 82:3204-3219

23. McDonald SM, Patton JT (2008) Molecular characterization of a subgroup specificity associated with the rotavirus inner capsid protein VP2. J Virol 82:2752-2764

24. McDonald SM, Aguayo D, Gonzalez-Nilo FD, Patton JT (2009) Shared and group-specific features of the rotavirus RNA polymerase reveal potential determinants of gene reassortment restriction. J Virol 83:6135-6148

25. Nagashima S, Kobayashi N, Ishino M, Alam MM, Ahmed MU, Paul SK, Ganesh B, Chawla-Sarkar M, Krishnan T, Naik TN, Wang YH (2008) Whole genomic characterization of a human rotavirus strain B219 belonging to a novel group of the genus Rotavirus. J Med Virol 80:2023-2033

26. Prasad BV, Rothnagel R, Zeng CQ, Jakana J, Lawton JA, Chiu W, Estes MK (1996) Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. Nature 382:471-473

27. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

28. Sanekata T, Kuwamoto Y, Akamatsu S, Sakon N, Oseto M, Taniguchi K, Nakata S, Estes MK (1996) Isolation of group B porcine rotavirus in cell culture. J Clin Microbiol 34:759-761

29. Schumann T, Hotzel H, Otto P, Johne R (2009) Evidence of interspecies transmission and reassortment among avian group A rotaviruses. Virology

386:334-343

30. Sereno MM, Gorziglia M (1994) The outer capsid protein VP4 of murine rotavirus strain Eb represents a tentative new P type. Virology 199:500-504

31. Shen S, McKee TA, Wang ZD, Desselberger U, Liu DX (1999) Sequence analysis and in vitro expression of genes 6 and 11 of an ovine group B rotavirus isolate, KB63: evidence for a non-defective, C-terminally truncated NSP1 and a phosphorylated NSP5. J Gen Virol 80:2077-2085

32. Snodgrass DR, Herring AJ, Campbell I, Inglis JM, Hargreaves FD (1984) Comparison of atypical rotaviruses from calves, piglets, lambs and man. J Gen Virol 65:909–914

33. Tsunemitsu H, Kamiyama M, Kawashima K, Katsuda K, Kohmoto M, Saif LJ, Shouji T, Onodera T (2005) Molecular characterization of the major capsid protein VP6 of bovine group B rotavirus and its use in seroepidemiology. J Gen Virol 86:2569-2575

34. Tsunemitsu H, Morita D, Takaku H, Nishimori T, Imai K, Saif LJ (1999) First detection of bovine group B rotavirus in Japan and sequence of its VP7 gene. Arch Virol 144:805–815

35. Vonderfecht SL, Lindsay DA, Eiden JJ (1994) Detection of rat, porcine, and bovine group B rotavirus in fecal specimens by solid-phase enzyme immunoassay. J Clin Microbiol 32:1107-1108

36. Wakuda M, Pongsuwanna Y, Taniguchi K (2005) Complete nucleotide sequences of two RNA segments of human picobirnavirus. J Virol Methods 126:165–169

Figure legends

Fig. 1. Alignment of partial deduced amino acid sequences of VP4 gene of group B rotaviruses. The putative trypsin cleavage sites (★) are indicated. A dot indicates an amino acid identical to DB176, while a dash denotes a gap generated to obtain the best alignment. Amino acid numbers are indicated for the leftmost and rightmost residues of each sequence. Abbreviations: Bov (Bovine), Hu (Human) and Mu (Murine).

Fig. 2. Phylogenetic trees constructed from nucleotide sequences of VP1, VP2, VP4, VP6, NSP1 and NSP2 genes of strains DB176, RUBV226 and RUBV282 with those of other group B rotaviruses and novel group strains B219 and J19. One group A (Wa) and one group C (Bristol) rotavirus strain were also included in the analyses. The positions of strains DB176, RUBV226 and RUBV282 are indicated ( ]). Bootstrap values are shown. Bar, 0.05 substitutions per nucleotide. Abbreviations: Bov (Bovine), Hu (Human), Mu (Murine) and Ov (Ovine).

Fig. 3. Hydrophobicity/hydrophilicity plots for the deduced amino acid sequences of VP4 gene of group B rotavirus strains DB176, IDIR and CAL-1 by the Kyte-Doolittle method. The VP8* and VP5* portions of VP4 have been demarcated. The hydrophilic and hydrophobic regions, mentioned in the text, are shown by dotted and solid lines, respectively. The region containing the putative trypsin cleavage sites has been

highlighted by a double line. Horizontal scale, amino acid numbers.

Fig. 4. Alignment of partial deduced amino acid sequences of VP1 gene of group B rotaviruses. Conserved motifs, observed in RNA-dependent RNA polymerases of different double-stranded RNA viruses, are shaded. A dot indicates an amino acid identical to DB176. Amino acid numbers are indicated for the leftmost and rightmost residues of each sequence. Abbreviations: Bov (Bovine), Hu (Human) and Mu (Murine).

Fig. 1.

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                                ★   ★       ★
DB176/Bov       200 MYSWSDVCQQSRVTLRDTEQNSRIIIYE 227
RUBV226/Bov     200 ............A...N.......... 227
RUBV282/Bov     200 ............A...N.......... 227
Bang373/Hu      196 CFA.DMN.AN----A.S.N.D..L.... 219
CAL-1/Hu        196 CFT.DMN.AN----A.S.N.D..L.... 219
WH-1/Hu         196 CFT.DMN.AN----V.S.K.D..L.... 219
ADRV/Hu         195 CFT.DMN.AN----V.S.K.D..L.... 218
IDIR/Mu         194 S.V.GPCSG--.IKT.VQGND....... 219
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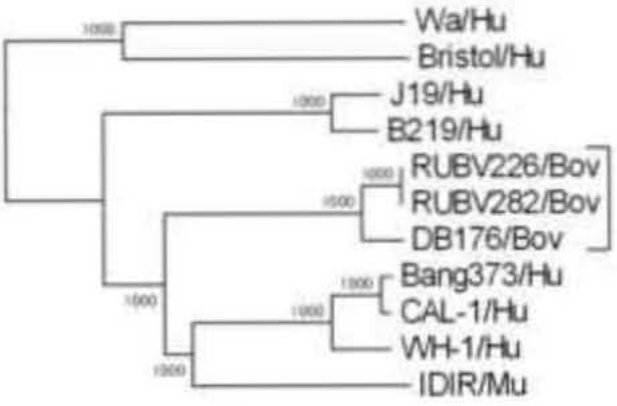
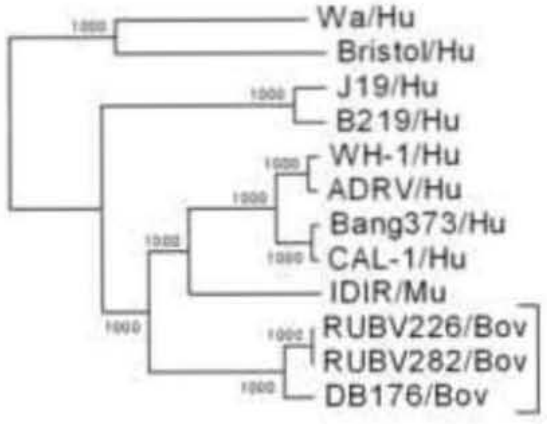
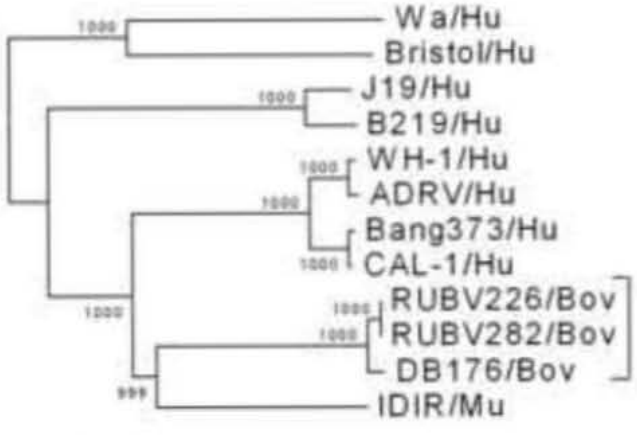
Fig. 2



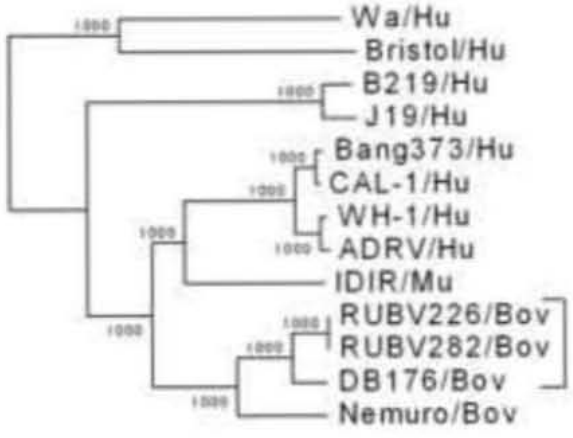
(VP1)



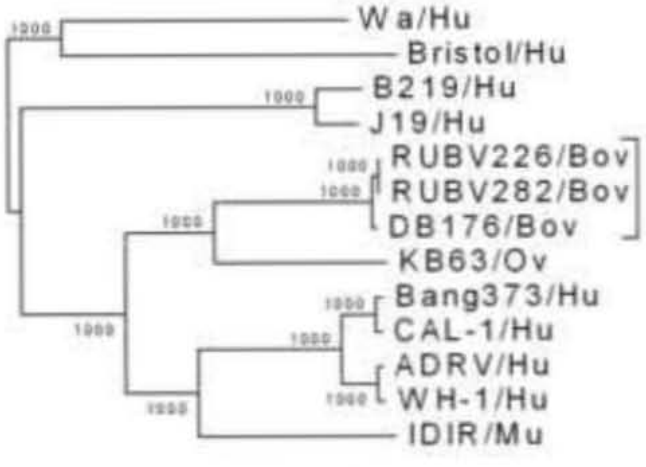
(VP2)



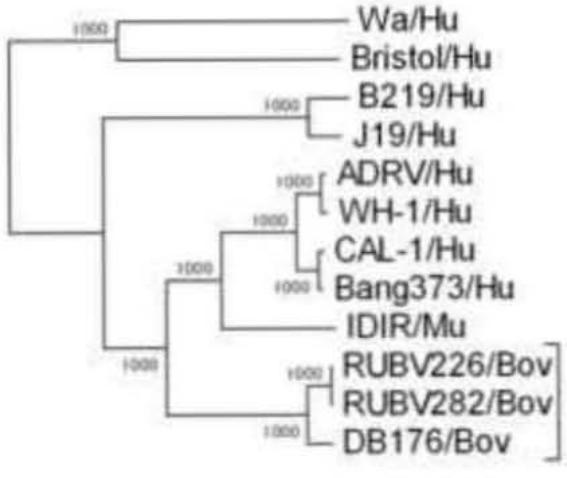
(VP4)



(VP6)



(NSP1)



(NSP2)

Fig. 3

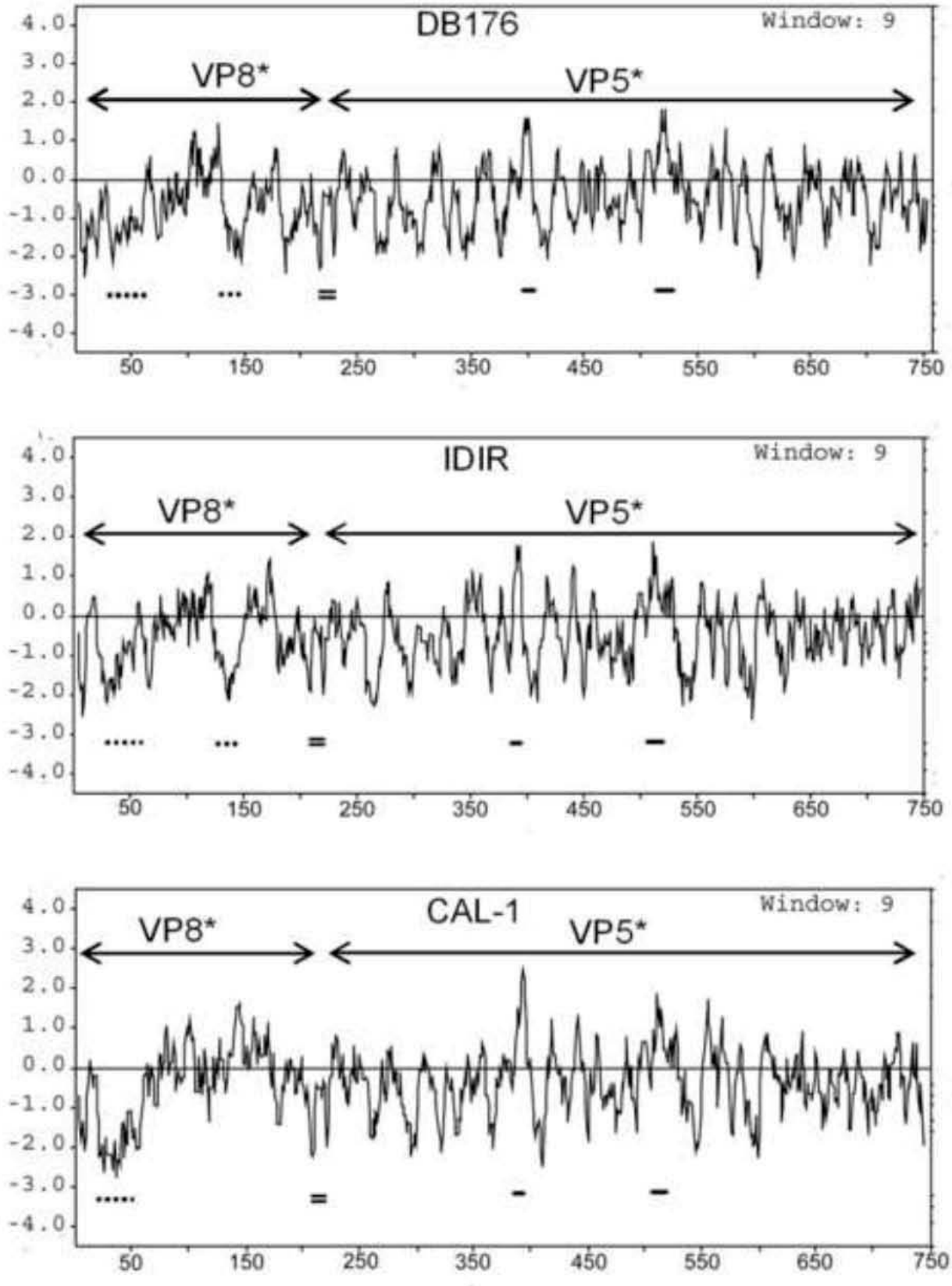

DB176
Window: 9
VP8*
VP5*
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
50
150
250
350
450
550
650
750
IDIR
Window: 9
VP8*
VP5*
CAL-1
Window: 9
VP8*
VP5*

Fig. 4.

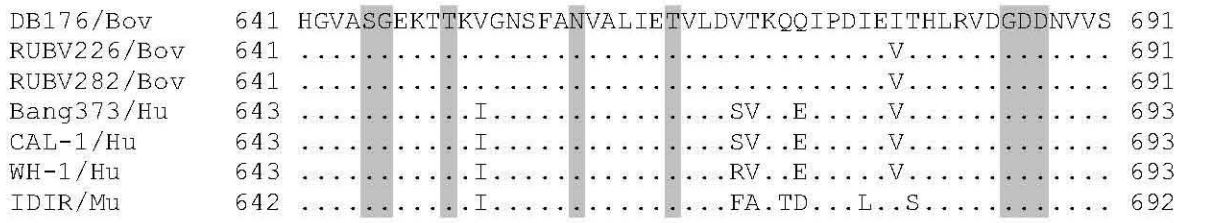

DB176/Bov 641 HGVASGEKTTKVGNSFANVALIETVLDVTKQQIPDIEITHLRVDGDDNVVS 691
RUBV226/Bov 641 .....V..... 691
RUBV282/Bov 641 .....V..... 691
Bang373/Hu 643 .....I.....SV..E.....V..... 693
CAL-1/Hu 643 .....I.....SV..E.....V..... 693
WH-1/Hu 643 .....I.....RV..E.....V..... 693
IDIR/Mu 642 .....I.....FA.TD...L..S..... 692

Table 1. Six genes of bovine group B rotavirus strains analyzed in the present study.

Viral protein gene	Length (nt.)	ORF (nt. nos.)	Length of deduced aa sequence	5'-terminal nt. sequence	3'-terminal nt. sequence
VP1 gene	3504	8- 3484	1158	**GG**CAATAATG-	-TGTA**AAAACCC**
VP2 gene	2849	9- 2822	937	**GG**AAGTATAT-	-ATAA**AAAACCC**
VP4 gene	2325	19- 2295	758	**GG**TATTTAAT-	-AGTA**AAAACCC**
[VP8*]			[215] [a]		
[VP5*]			[536] [a]		
VP6 gene	1269	33- 1208	391	**GG**TATTAATT-	-ACTA**AAAACCC**
NSP1 gene	1276			**GG**TATAATAA-	-AATA**AAAACCC**
[Peptide 1]		[43- 348]	[101]		
[Peptide 2]		[254- 1216]	[320]		
NSP2 gene	1001	57-959	300	**GG**TACAAAA-	-TGAT**AAGACCC**

[a] Based on the 2nd and 3rd putative trypsin cleavage sites, the lengths of VP8* and VP5* portions of VP4 were assumed to be 215 aa and 536 aa, respectively.

Table 2. Nucleotide (amino acid) sequence identities (%) of VP4 and VP6 genes of strains DB176, RUBV226 and RUBV282 to those of other group B rotaviruses.

	VP4 [VP8*, VP5*] [a]		VP6	
Strain	DB176	RUBV226/RUBV282 [b]	DB176	RUBV226/RUBV282 [b]
RUBV226	97.4 (97.1)[95.3, 97.8]		92.9 (99.5)	
Bang373	60.5 (55.3)[30.2, 65.3]	60.8 (55.7)[30.7, 65.6]	66.2 (70.6)	65.6 (71.1)
CAL-1	60.1 (55.5)[29.8, 65.6]	60.5 (55.9)[30.2, 66.0]	66.3 (70.8)	65.9 (71.4)
WH-1	60.6 (55.5)[29.8, 65.6]	60.9 (56.0)[31.2, 65.8]	66.4 (71.1)	66.6 (71.6)
ADRV	60.5 (54.8)[28.8, 65.1]	60.8 (55.3)[30.2, 65.3]	65.9 (69.8)	66.1 (70.3)
IDIR	61.7 (59.6)[37.2, 68.6]	62.0 (60.2)[37.7, 69.3]	67.1 (72.1)	68.5 (72.6)
Nemuro	-[c]	-[c]	82.3 (97.2)	82.3 (96.7)

[a] Identities of the deduced VP8* and VP5* amino acid sequences are shown in third brackets.

[b] Absolute nucleotide sequence identity between strains RUBV226 and RUBV282.

[c] No sequence data was available for VP4 gene of strain Nemuro in the GenBank database.

Table 3. Nucleotide (amino acid) sequence identities (%) of VP1 and VP2 genes of strains DB176, RUBV226 and RUBV282 to those of other group B rotaviruses.

	VP1			VP2		
Strain	DB176	RUBV226	RUBV282	DB176	RUBV226	RUBV282
RUBV226	94.2 (97.4)			93.9 (97.4)		
RUBV282	94.2 (97.4)	99.9 (100)		94.0 (97.4)	99.7 (99.8)	
Bang373	67.5 (69.0)	67.8 (69.3)	67.8 (69.3)	66.2 (69.1)	66.1 (68.6)	66.1 (68.5)
CAL-1	67.5 (69.0)	67.7 (69.3)	67.8 (69.3)	65.6 (68.6)	65.5 (68.2)	65.5 (68.1)
WH-1	67.3 (69.5)	67.0 (69.8)	67.0 (69.8)	66.2 (68.9)	66.5 (68.5)	66.5 (68.4)
ADRV	-[a]	-[a]	-[a]	66.1 (68.7)	66.5 (68.3)	66.5 (68.2)
IDIR	65.5 (68.2)	65.2 (68.1)	65.2 (68.1)	66.5 (69.3)	66.8 (69.3)	66.7 (69.2)

[a] No sequence data was available for VP1 gene of strain ADRV in the GenBank database.

Table 4. Nucleotide (amino acid) sequence identities (%) of NSP1 and NSP2 genes of strains DB176, RUBV226 and RUBV282 to those of other group B rotaviruses.

	NSP1 (Peptide 1, Peptide 2) [a]		NSP2		
Strain	DB176	RUBV226/RUBV282 [b]	DB176	RUBV226	RUBV282
RUBV226	98.8 (99.0, 99.7)		95.5 (98)		
RUBV282	98.8 (99.0, 99.7)	100 (100,100)	95.4 (98)	99.9 (100)	
Bang373	57.6 (35.5, 43.9)	57.0 (34.6, 43.9)	68.6 (71.1)	68.7 (71.1)	68.7 (71.1)
CAL-1	57.4 (35.5, 43.3)	56.8 (34.6, 43.3)	68.5 (70.4)	68.4 (70.4)	68.4 (70.4)
WH-1	56.9 (34.6, 43.6)	56.4 (33.6, 43.6)	68.7 (71.1)	68.6 (71.1)	68.7 (71.1)
ADRV	57.5 (34.6, 43.6)	56.7 (33.6, 43.6)	69.2 (71.1)	69.1 (71.1)	69.3 (71.1)
IDIR	56.2 (38.3, 44.5)	55.2 (38.3, 44.5)	68.7 (74.1)	68.5 (74.1)	68.4 (74.1)
KB63	70.8 [64.4, 65.3, 67.8] [c]	70.6 [65.3, 62.9, 67.8] [c]	- [d]	- [d]	- [d]

[a] Amino acid sequence identities of NSP1 peptides 1 and 2.

[b] Absolute nucleotide sequence identity between strains RUBV226 and RUBV282.

[c] Amino acid sequence identities of NSP1 peptides 1, 2 and 3 of strain KB63 with cognate stretches of strains DB176, RUBV226 and RUBV282.

[d] No sequence data was available for NSP2 gene of strain KB63 in GenBank database.