that 16 genogroup II strains would be bound by our genogroup II-specific primer, suggesting that the genogroup of the 20 strains could possibly be determined by the RT-PCR method. However, the two genogroup I strains would form mismatches with our genogroup I-specific primer at the 3′ terminus, which might impede the PCR. A similar situation was observed for the VP6 gene of the 116E strain, which varied from the genogroup II-specific primer at the last nucleotide of the 3′ terminus. In the present study, reference strains, including at least four genogroup I (two P[4]G2, one P[2]G3, and RotaTeq vaccine strains) and nine genogroup II strains (two P[8]G1, one P[4]G2, two P[8]G3, one P[8]G4, and three P[8]G9), and 754 rotavirus samples with more than 52 electropherotypes recovered from a 25-year period, representing a wide range of genetic variation, could be easily genogrouped by RT-PCR. For the rare strains, if a negative result is obtained from the RT-PCR assay, we would suggest that the VP6 genogroup be determined by sequence analysis.

The association of subgroup and other genes has been discussed previously. For the associations among the VP6, VP7, and VP4 genes, it has been noticed that G1, G3, and G4 frequently are associated with P[8] and subgroup II, and G2 frequently is associated with P[4] and subgroup I (17). The association of subgroup and RNA electropherotype has also been reported previously for human rotaviruses of group A (20). However, Svensson et al. reported that the subgroup specificity could not be predicted by the migration of gene segments 10 and 11 (29). Iturriza-Gomara et al. have demonstrated the independent segregation of the VP4, VP6, and VP7 genes (18), and these authors also found a 100% linkage of the VP6 subgroup and NSP4 genotype, association of NSP4 genotype A with subgroup I and of NSP4 genotype B with subgroup II, in common and reassortant human rotaviruses (16). In the present study, excluding the five samples characterized as containing rotaviruses with genogroup I+II, all of the rotaviruses with common G- and P-type combinations, and most of those with uncommon G- and P-type combinations, had such gene associations between VP6 genogroups and P genotypes: P[8] associated with VP6 genogroup II, and P[4] associated with VP6 genogroup I. Only two P[4] strains, one P[4]G3 strain and one P[4]G9 strain, had a VP6 gene of genogroup II. Excluding the five rotavirus samples with VP6 genogroup I+II, the associations between VP6 genogroups and NSP4 genotypes, NSP4A being associated with genogroup I and NSP4B associated with genogroup II, were observed in the 23 rotavirus strains with uncommon G and P combinations.

It has been suggested that there are no true human subgroup I+II or subgroup non-I non-II strains to be found and that all such strains are from animals (9, 14, 15). It frequently happens that human rotaviruses reassort with animal strains, and it increases the possibility that subgroup I+II or subgroup non-I non-II could be found in human strains. In the present study, five rotavirus samples were determined to be genogroup I+II. However, after cloning the PCR amplicons of the VP6 gene, we found that all of the clones analyzed belonged to either genogroup I or genogroup II. These rotavirus samples appeared more likely to be the result of coinfection with two different rotavirus strains, one with genogroup I and the other with genogroup II. We also used EIA to confirm the results and found that these samples reacted with both subgroup I-specific and subgroup II-specific MAbs. Furthermore, we did not find any sequences possessing both primer sites specific for the genogroup I and genogroup II. Therefore, there was no true genogroup I+II in the rotavirus samples tested in the present study.

The RT-PCR assay established in the present study to determine the VP6 genogroup was successfully applied to reference rotavirus strains, including RotaTeq vaccine strains, and 754 Taiwanese rotavirus strains recovered between 1981 and 2005. The assay appears to be a reliable and convenient method for determining the VP6 genogroups of human rotaviruses with a wide range of genetic variation.

ACKNOWLEDGMENTS

This study was supported by grants from the National Science Council (grants NSC-92-2314-B-002-355, NSC-94-3112-B-002-022, and NSC-95-3112-B-002-004) and the Department of Health (grant DOH92-DC-1203) of Taiwan, Republic of China.

REFERENCES

1. **Au, K. S., W. K. Chan, J. W. Burns, and M. K. Estes.** 1989. Receptor activity of rotavirus nonstructural glycoprotein NS28. J. Virol. **63**:4553–4562.
2. **Ball, J. M., P. Tian, C. Q. Zeng, A. P. Morris, and M. K. Estes.** 1996. Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. Science **272**:101–104.
3. **Ciarlet, M., F. Liprandi, M. E. Conner, and M. K. Estes.** 2000. Species specificity and interspecies relatedness of NSP4 genetic groups by comparative NSP4 sequence analyses of animal rotaviruses. Arch. Virol. **145**:371–383.
4. **Cunliffe, N. A., P. A. Woods, J. P. Leite, B. K. Das, M. Ramachandran, M. K. Bhan, C. A. Hart, R. I. Glass, and J. R. Gentsch.** 1997. Sequence analysis of NSP4 gene of human rotavirus allows classification into two main genetic groups. J. Med. Virol. **53**:41–50.
5. **Estes, M. K.** 2001. Rotaviruses and their replication. p. 1747–1785. *In* D. M. Knipe, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott/Williams & Wilkins, Philadelphia, PA.
6. **Estes, M. K.** 1996. Rotaviruses and their replication. p. 1625–1655. *In* B. N. Fields, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields virology, 3rd ed. Lippincott-Raven Press, Philadelphia, PA.
7. **Estes, M. K., and J. Cohen.** 1989. Rotavirus gene structure and function. Microbiol. Rev. **53**:410–449.
8. **Gentsch, J. R., R. I. Glass, P. Woods, V. Gouvea, M. Gorziglia, J. Flores, B. K. Das, and M. K. Bhan.** 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. J. Clin. Microbiol. **30**:1365–1373.
9. **Gorziglia, M., Y. Hoshino, K. Nishikawa, W. L. Maloy, R. W. Jones, A. Z. Kapikian, and R. M. Chanock.** 1988. Comparative sequence analysis of the genomic segment 6 of four rotaviruses each with a different subgroup specificity. J. Gen. Virol. **69**(Pt. 7):1659–1669.
10. **Gouvea, V., R. I. Glass, P. Woods, K. Taniguchi, H. F. Clark, B. Forrester, and Z. Y. Fang.** 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J. Clin. Microbiol. **28**:276–282.
11. **Greenberg, H., V. McAuliffe, J. Valdesuso, R. Wyatt, J. Flores, A. Kalica, Y. Hoshino, and N. Singh.** 1983. Serological analysis of the subgroup protein of rotavirus, using monoclonal antibodies. Infect. Immun. **39**:91–99.
12. **Gulati, B. R., R. Deepa, B. K. Singh, and C. D. Rao.** 2007. Diversity in Indian equine rotaviruses: identification of genotype G10,P6[1] and G1 strains and a new VP7 genotype (G16) strain in specimens from diarrheic foals in India. J. Clin. Microbiol. **45**:972–978.
13. **Horie, Y., O. Masamune, and O. Nakagomi.** 1997. Three major alleles of rotavirus NSP4 proteins identified by sequence analysis. J. Gen. Virol. **78**(Pt. 9):2341–2346.
14. **Hoshino, Y., M. Gorziglia, J. Valdesuso, J. Askaa, R. I. Glass, and A. Z. Kapikian.** 1987. An equine rotavirus (FI-14 strain) which bears both subgroup I and subgroup II specificities on its VP6. Virology **157**:488–496.
15. **Hoshino, Y., R. G. Wyatt, H. B. Greenberg, A. R. Kalica, J. Flores, and A. Z. Kapikian.** 1983. Isolation, propagation, and characterization of a second equine rotavirus serotype. Infect. Immun. **41**:1031–1037.
16. **Iturriza-Gomara, M., E. Anderton, G. Kang, C. Gallimore, W. Phillips, U. Desselberger, and J. Gray.** 2003. Evidence for genetic linkage between the gene segments encoding NSP4 and VP6 proteins in common and reassortant human rotavirus strains. J. Clin. Microbiol. **41**:3566–3573.
17. **Iturriza-Gomara, M., B. Isherwood, U. Desselberger, and J. Gray.** 2001. Reassortment in vivo: driving force for diversity of human rotavirus strains

isolated in the United Kingdom between 1995 and 1999. J. Virol. 75:3696–3705.
18. **Iturriza-Gomara, M., C. Wong, S. Blome, U. Desselberger, and J. Gray.** 2002. Molecular characterization of VP6 genes of human rotavirus isolates: correlation of genogroups with subgroups and evidence of independent segregation. J. Virol. 76:6596–6601.
19. **Iturriza-Gomara, M., C. Wong, S. Blome, U. Desselberger, and J. Gray.** 2002. Rotavirus subgroup characterisation by restriction endonuclease digestion of a cDNA fragment of the VP6 gene. J. Virol. Methods 105:99–103.
20. **Kalica, A. R., H. B. Greenberg, R. T. Espejo, J. Flores, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1981. Distinctive ribonucleic acid patterns of human rotavirus subgroups 1 and 2. Infect. Immun. 33:958–961.
21. **Kerin, T. K., E. M. Kane, R. I. Glass, and J. R. Gentsch.** 2007. Characterization of VP6 genes from rotavirus strains collected in the United States from 1996–2002. Virus Genes 35:489–495.
22. **Khamrin, P., N. Maneekarn, S. Peerakome, W. Chan-it, F. Yagyu, S. Okitsu, and H. Ushijima.** 2007. Novel porcine rotavirus of genotype P[27] shares new phylogenetic lineage with G2 porcine rotavirus strain. Virology 361:243–252.
23. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5:150–163.
24. **Lee, C. N., Y. L. Wang, C. L. Kao, C. L. Zao, C. Y. Lee, and H. N. Chen.** 2000. NSP4 gene analysis of rotaviruses recovered from infected children with or without diarrhea. J. Clin. Microbiol. 38:4471–4477.
25. **Lin, Y. P., S. Y. Chang, C. L. Kao, L. M. Huang, M. Y. Chung, J. Y. Yang, H. Y. Chen, K. Taniguchi, K. S. Tsai, and C. N. Lee.** 2006. Molecular epidemiology of G9 rotaviruses in Taiwan between 2000 and 2002. J. Clin. Microbiol. 44:3686–3694.
26. **Mathieu, M., I. Petitpas, J. Navaza, J. Lepault, E. Kohli, P. Pothier, B. V. Prasad, J. Cohen, and F. A. Rey.** 2001. Atomic structure of the major capsid protein of rotavirus: implications for the architecture of the virion. EMBO J. 20:1485–1497.
27. **Matsui, S. M., E. R. Mackow, and H. B. Greenberg.** 1989. Molecular determinant of rotavirus neutralization and protection. Adv. Virus Res. 36:181–214.
28. **Mori, Y., M. A. Borgan, N. Ito, M. Sugiyama, and N. Minamoto.** 2002. Diarrhea-inducing activity of avian rotavirus NSP4 glycoproteins, which differ greatly from mammalian rotavirus NSP4 glycoproteins in deduced amino acid sequence in suckling mice. J. Virol. 76:5829–5834.
29. **Svensson, L., L. Grahnquist, C. A. Pettersson, M. Grandien, G. Stintzing, and H. B. Greenberg.** 1988. Detection of human rotaviruses which do not react with subgroup I- and II-specific monoclonal antibodies. J. Clin. Microbiol. 26:1238–1240.
30. **Tang, B., J. M. Gilbert, S. M. Matsui, and H. B. Greenberg.** 1997. Comparison of the rotavirus gene 6 from different species by sequence analysis and localization of subgroup-specific epitopes using site-directed mutagenesis. Virology 237:89–96.
31. **Taniguchi, K., T. Urasawa, S. Urasawa, and T. Yasuhara.** 1984. Production of subgroup-specific monoclonal antibodies against human rotaviruses and their application to an enzyme-linked immunosorbent assay for subgroup determination. J. Med. Virol. 14:115–125.
32. **Tian, P., M. K. Estes, Y. Hu, J. M. Ball, C. Q. Zeng, and W. P. Schilling.** 1995. The rotavirus nonstructural glycoprotein NSP4 mobilizes Ca^{2+} from the endoplasmic reticulum. J. Virol. 69:5763–5772.
33. **Wyatt, R. G., H. B. Greenberg, W. D. James, A. L. Pittman, A. R. Kalica, J. Flores, R. M. Chanock, and A. Z. Kapikian.** 1982. Definition of human rotavirus serotypes by plaque reduction assay. Infect. Immun. 37:110–115.

Journal of Medical Virology 80:2023–2033 (2008)

Whole Genomic Characterization of a Human Rotavirus Strain B219 Belonging to a Novel Group of the Genus Rotavirus

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Novel rotavirus strains B219 and ADRV-N derived from adult diarrheal cases in Bangladesh and China, respectively, are considered to belong to a novel rotavirus group (species) distinct from groups A, B, and C, by genetic analysis of five viral genes encoding VP6, VP7, NSP1, NSP2, and NSP3. In this study, the nucleotide sequences of the remaining six B219 gene segments encoding VP1, VP2, VP3, VP4, NSP4, and NSP5 were determined. The nucleotide sequences of the group B human rotavirus VP1 and VP3 genes were also determined in order to compare the whole genome of B219 with those of group A, B, and C rotavirus genomes. The nucleotide and deduced amino acid sequences of all B219 gene segments showed considerable identity to the ADRV-N (strain J19) sequences (87.7–94.3% and 88.7–98.7%, respectively). In contrast, sequence identity to groups A–C rotavirus genes was less than 61%. However, functionally important domains and structural characteristics in VP1-VP4, NSP4, and NSP5, which are conserved in group A, B, or C rotaviruses, were also found in the deduced amino acid sequences of the B219 proteins. Hence, the basic structures of all B219 viral proteins are considered to be similar to those of the known rotavirus groups. ***J. Med. Virol. 80:2023–2033, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: rotavirus; novel group; B219; RNA segment

INTRODUCTION

Rotavirus, a member of the family Reoviridae, is the most common cause of severe diarrheal illness in human and animals. Rotavirus has eleven segments of double-stranded RNA, and the viral particle is composed of three concentric layers: the outer capsid, the inner capsid, and the core [Estes and Kapikian, 2007]. The outer capsid consists of two structural proteins VP4 and VP7, which have neutralization antigens, while the inner capsid comprises VP6, the most abundant protein in the particle. The core is composed of the VP2 shell, which contains an enzymatic complex of VP1 and VP3, and genomic RNA segments.

Rotaviruses have been classified into seven serologically and genetically distinct groups A–G, which were discriminated originally by the antigenicity of the inner capsid protein VP6 [Estes and Kapikian, 2007]. The electrophoretic migration pattern of the eleven RNA segments in polyacrylamide gel (RNA pattern) is specific to each rotavirus group. Rotavirus groups A, B, and C are associated with acute gastroenteritis in both humans and animals, while groups D, E, F, and G have been detected only in animals [Mackow, 1995; Estes and Kapikian, 2007]. Group A rotavirus is the most common virus causing diarrheal diseases in children younger than 5 years of age. Group C rotavirus causes gastroenteritis in children, although it is less prevalent than

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan (Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (Okayama University—National Institute of Cholera and Enteric Diseases, India) Grant-in-Aid for Scientific Research; Grant number: 18406018 (partial support); Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant sponsor: Ministry of Health, Labour, and Welfare of Japan (International Health Cooperation Research (18-5)).

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Accepted 25 June 2008
DOI 10.1002/jmv.21286
Published online in Wiley InterScience (www.interscience.wiley.com)

group A rotavirus. In contrast, group B rotavirus causes severe cholera-like diarrhea primarily in adults, and has been detected only in China, India, and Bangladesh [Hung et al., 1983; Krishnan et al., 1999; Sanekata et al., 2003; Yang et al., 2004a].

Recently, human rotaviruses designated ADRV-N and B219, which are not classified into groups A, B, or C, were reported in China and Bangladesh, respectively [Yang et al., 2004b; Alam et al., 2007]. ADRV-N was found as a causative agent of an outbreak of diarrhea in adults [Yang et al., 1998], while B219 was detected in a sporadic case of diarrhea in adult [Alam et al., 2007]. Both exhibited similar RNA patterns, which were distinct from those of group A-G rotaviruses. Sequence analysis of the three viral protein genes (VP6, NSP1, and NSP3) of ADRV-N and five genes (VP6, VP7, NSP1, NSP2, and NSP3) of B219 indicated that these two viruses were related closely with the 87–95% nucleotide sequence identities, while distinguishable genetically from groups A–C rotaviruses showing less than 60% identity [Yang et al., 1998, 2004b; Alam et al., 2007]. These findings indicate that ADRV-N and B219 may belong to a novel species of human rotaviruses other than groups A–C rotaviruses.

In the present study, the complete nucleotide sequences of the B219 gene segments 1, 2, 3, 4, 10, and 11, which encode VP1, VP2, VP4, VP3, NSP4, and NSP5, respectively, were determined. Accordingly, whole genomic data have become available for the B219 virus, together with sequence data of ADRV-N deposited in GenBank database recently. The VP1 and VP3 gene sequences of human group B rotaviruses were determined in order to complete the comparative analysis of all gene sequences from group A-C human rotaviruses, because sequence data of these genes were not available at the beginning of this study. With the sequence data obtained in this study, the characteristics of the whole B219 genome and deduced protein products from the viral genes were analyzed, and compared with those of groups A–C rotaviruses. The results indicated that viral proteins of strain B219 shared similar structural characteristics to those of groups A–C rotaviruses, albeit with a high level of sequence diversity to the known rotavirus groups.

MATERIALS AND METHODS

Rotavirus Strains

The novel, non-group A, B, or C human rotavirus B219, and three group B human rotaviruses (CAL-1, Bang373, and WH-1) were analyzed. The strain B219 was found in a stool specimen from a 65-year-old female patient with severe diarrhea in Bangladesh in 2002 [Alam et al., 2007], and group B rotavirus strains WH-1, CAL-1, and Bang373 were detected in stool specimens from adult patients with diarrhea in China (Wuhan) [Yang et al., 2004a], India (Kolkata) [Krishnan et al., 1999], and Bangladesh (Mymensingh) [Ahmed et al., 2004], respectively. Stool specimens with these virus strains were processed as 10% suspensions with PBS and kept at −80°C.

RNA Extraction and RT-PCR

Rotavirus RNA was extracted from fecal specimens using the RNAID kit (BIO101, Inc., La Jolla, CA) according to the manufacturer's instructions. Viral genes from the strain B219 were amplified by reverse transcription and the polymerase chain reaction (RT-PCR) as described previously [Ahmed et al., 2004]. For the amplification of the VP1, VP2, VP3, VP4, NSP4, and NSP5 genes of B219, oligonucleotide primers were designed based on the sequence data of J19 (GenBank accession numbers DQ113897–DQ113900, DQ113906, and DQ113907), which is a tissue culture adapted isolate from ADRV-N [Ji et al., 2002]. Sequence of primers and their positions are indicated in Table I.

Sequence Analysis of B219 Genes

The amplified cDNA products by RT-PCR from VP1, VP2, VP3, and VP4 genes of B219 were purified with the Wizard SV gel and PCR Clean-Up system (Promega, Madison, WI), and cloned into the pCR 2.1-TOPO vectors in the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Three clones for each gene were selected and used for sequencing. In contrast, the nucleotide sequences of NSP4 and NSP5 genes of B219 were determined directly from PCR products. The nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Reaction kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI PRISM 3100). Sequence analysis and comparisons were performed using the GeneWorks software package (IntelliGenetics, Inc., Mountain View, CA).

Sequence Analysis of Group B Human Rotaviruses

Cloning of the VP1 and VP3 genes of the group B human rotavirus strain WH-1 was carried out by the single primer amplification method [Wakuda et al., 2005]. After RNA extraction, a single amino group-linked oligonucleotide primer A comprising 42 nucleotides (5′-PO_4-CCCTCGAGTACTAACTAGTTAACTGA-TCACCTCTAGACCTTT-NH_2-3′) was ligated to the 3′-end of both strands of dsRNA by using T4 RNA ligase. Next, a reverse transcription was carried out with primer B, which is complementary to the primer A sequence. Subsequently, the cDNA was amplified by a two-step PCR using primer C (5′-GGTCTAGAGGTGA-TCAGTTAACTAGTTAGTACTC-3′; first PCR) and primer D (5′-TCAGTTAACTAGTTAGTACTCGAGGG-3′; second PCR). The PCR products were cloned into the pCR 2.1-TOPO vectors and sequenced as described above. Based on the sequence data of VP1 and VP3 genes of WH-1, cDNAs of these genes were amplified by RT-PCR from CAL-1 and Bang373, and the resultant products were used for direct sequencing.

Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported in this study were deposited in the GenBank database under the

TABLE I. Primer Sequences and Their Positions Used for RT-PCR Amplification of B219 Genes

Viral protein gene	Primer name	Primer sequence (5′–3′)	Position[a]	Size of PCR product (bp)
VP1	J19 S1-1P	(+) GGCACTATGGAACCTGAGAA	1–20	1,338
	J19 S1-1338 M	(–) AGATTCAGCTGTAACGTCAG	1,338–1,319	
	J19 S1-1134 P	(+) TTCAGCCACATTCCACGTTG	1,134–1,153	1,328
	J19 S1-2461 M	(–) TAACTGGTGACAACAGGGAC	2,461–2,442	
	J19 S1-2271 P	(+) CACTGCAGAACGTCCATATG	2,271–2,290	1,268
	J19 S1-3538 M	(–) GGGTATATTAATGGATGTACTCTC	3,538–3,515	
VP2	J19 S2-1 P	(+) GGCACTTAAGCGCTGCAAGA	1–20	870
	J19 S2-870 M	(–) TGCATTGCTGCTCTTCTAGC	870–851	
	J19 S2-821 P	(+) GTAATGGATCGCACCGATAGAG	821–842	1,200
	J19 S2-2020 M	(–) CGTTATGCCCATAACTAAGGACAG	2,020–1,997	
	J19 S2-1654 P	(+) TGTGCGGAGAAATGCTCACG	1,654–1,673	1,316
	J19 S2-2969 M	(–) GGGTATATTGAATAAGCTAG	2,969–2,950	
VP3	J19 S3-1 P	(+) GGCACTTAATGGCTAAGTTA	1–20	1,287
	J19 S3-1287 M	(–) CCAATTGTGTTAATTTCGGATGG	1,287–1,265	
	J19 S3-981 P	(+) GCGTATGATCCAAAATATCG	981–1,000	1,224
	J19 S3-2204 M	(–) GGGTATATTACAGCAACTCA	2,204–1,985	
VP4	J19 S4-1 P	(+) GGCACTTAAGATGTCTCTCAGAAG	1–20	991
	J19 S4-991 M	(–) CTTCCCGTTTCTCTGGTACTC	991–971	
	J19 S4-691 P	(+) CCAAGATTTAATACCTACTATGAC	691–714	1,020
	J19 S4-1710 M	(–) ATAACTCCCGAAGTAAGATC	1,710–1,691	
	J19 S4-1557 P	(+) CACTGTTGCCATCTGATCCAG	1,557–1,577	956
	J19 S4-2512 M	(–) GGGTATATTTAAAGATCTCTTTC	2,512–2,490	
NSP4	J19 S10-1 P	(+) GGCATTTTGTTCATCACAAATCAC	1–24	739
	J19 S10-739 M	(–) GGGTATATTGCATTGAGCATG	739–719	
NSP5	J19 S11-1 P	(+) GGAACTTAAAACATCAATCG	1–20	649
	J19 S11-649 M	(–) GGGTATACTATTGACCTCAG	649–630	

[a]Sequence and positions of primers correspond to individual gene sequences of strain J19 (ADRV-N) deposited in GenBank accession numbers DQ113897 (VP1), DQ113898 (VP2), DQ113900 (VP3), DQ113899 (VP4), DQ113906 (NSP4), and DQ113907 (NSP5).

accession numbers EF453355 (VP1), EF453356 (VP2), EF453357 (VP3), EF453358 (VP4), EF453359 (NSP4), and EF453360 (NSP5) for B219, EU490413 (VP1) and EU490416 (VP3) for WH-1, EU490414 (VP1) and EU490417 (VP3) for CAL-1, and EU490415 (VP1) and EU490418 (VP3) for Bang373.

RESULTS

VP1 and VP3 Genes Sequences of Group B Human Rotaviruses

The nucleotide sequences of VP1 and VP3 genes of group B rotavirus strains WH-1, CAL-1, and Bang373 were determined in order to obtain the whole genome data of group B human rotaviruses. Both gene segments of strain WH-1 determined by the single primer amplification method had 5′-end terminal GG-sequence common to the genus Rotavirus and 3′-end sequences -UAAAACCC (segment 1) and -AAGAACCC (segment 3), which are identical or similar to those of murine group B rotavirus IDIR [Eiden et al., 1992]. The VP1 genes were 3,510 (WH-1) or 3,511 (CAL-1 and Bang373) nucleotides in length, and contained an ORF encoding a product of 1,160 amino acids. The VP3 genes of the three strains comprised 2,341 nucleotides and contained an ORF encoding a product of 763 amino acids. The sizes of VP1 and VP3 from the human group B rotaviruses were almost identical to those of IDIR (1,159 and 763 amino acids, respectively).

Identities of VP1 and VP3 genes sequences among group B rotaviruses, and those between group B and group A or C rotaviruses are shown in Table II. Between CAL-1 and Bang373, extremely high identities (98.5–98.6%) were observed, while slightly lower identities of these strains were found in WH-1 (90.9–91.7%).

Determination of B219 Gene Sequences

Complete nucleotide sequences of B219 RNA segments 1 (VP1), 2 (VP2), 3 (VP4), 4 (VP3), 10 (NSP4), and 11 (NSP5) were determined. The RNA segments 1, 2, 4, 10, and 11 contained ORFs encoding products of 1,167, 973, 719, 213, and 176 amino acids, respectively, which are identical amino acid numbers to those of the strain J19 (ADRV-N) (Table III). The RNA segment 3 (VP4 gene, 2,521 nucleotides) of B219 was nine nucleotides longer than that of J19 in coding region, thereby the VP4 protein product of B219 (826 amino acids) was considered to be longer than J19 by three amino acids. In a previous study [Alam et al., 2007], sequences of five B219 genes encoding NSP1, VP6, NSP3, NSP2, and VP7 were determined, and B219 NSP1, VP6, and NSP3 genes were found to be identical in length to those of J19. The VP7 and NSP2 genes sequences of J19, which were deposited to the GenBank database, also had identical amino acid lengths to those of B219. It may be concluded that RNA segments of B219 and J19 are mostly identical in size.

Sequence Comparison Among B219, J19, and Groups A–C Rotaviruses

B219 gene segments for VP2 and VP4 were longer than those of group A-C human rotaviruses (strains KU,

TABLE II. Nucleotide (Amino Acid) Sequence Identity (%) of VP1 and VP3 Gene of Group B Human Rotaviruses (WH-1, CAL-1, and Bang373) to Groups A–C Rotaviruses

Rotavirus strains (origin)	VP1			VP3		
	WH-1	CAL-1	Bang373	WH-1	CAL-1	Bang373
Group A						
KU (human)[a]	49.8 (25.2)	49.3 (25.2)	49.6 (25.2)	47.7 (18.8)	47.2 (18.1)	46.8 (18.0)
UK (bovine)[a]	48.9 (26.0)	48.4 (26.1)	48.3 (26.0)	45.8 (20.4)	47.1 (17.9)	46.5 (18.1)
Group B						
CAL-1 (human)	91.7 (95.9)			91.2 (94.0)		
Bang373 (human)	91.1 (95.6)	98.6 (98.9)		90.9 (94.1)	98.5 (99.0)	
IDIR (murine)[a]	70.1 (79.5)	70.0 (79.4)	70.2 (79.4)	68.3 (71.7)	68.8 (72.9)	68.7 (72.7)
Group C						
Bristol (human)[a]	48.9 (24.3)	48.4 (24.6)	48.6 (24.5)	48.0 (12.5)	47.2 (11.9)	47.3 (11.7)
Cowden (porcine)[a]	49.8 (24.8)	49.7 (25.3)	49.0 (25.2)	47.4 (15.3)	46.7 (13.6)	47.0 (14.3)

[a]Reference sequences (Bank accession numbers) used in this analysis: KU, AB022765 (VP1), AB022767 (VP3); UK, X55444 (VP1), AY300927 (VP3); IDIR, M97203 (VP1), X16949 (VP3); Bristol, NC_007547 (VP1), NC_007574 (VP3); Cowden, M74216 (VP1), M74219 (VP3).

CAL-1 and Bristol, respectively) by more than 100 nucleotides, while the NSP3 gene was shorter than that of groups A–C viruses (Table III). The VP1 gene segment of B219, as well as VP6, VP7, NSP2, NSP4, and NSP5 genes, were most similar in size to those of CAL-1. The size of B219 VP3 gene was between those of the group B and C rotaviruses. Remarkably, the total nucleotide number of all the B219 RNA segments (17.97 kb) was similar to group B (17.93 kb) and group C (17.91 kb) rotaviruses, while slightly shorter than group A rotavirus (18.50 kb).

Between B219 and J19, nucleotide and deduced amino acid sequence identities of RNA segments were 87.7–94.3% and 88.7–98.7%, respectively (Table III). Extremely high identities were observed in VP1, VP2, VP6, and NSP2 amino acid sequences (97.0–98.7%), while the lowest identity was observed in NSP4 (88.7%). In contrast, nucleotide sequence identities of six B219 genes (VP1, VP2, VP3, VP4, NSP4, and NSP5) to representative groups A, B, and C rotavirus strains were considerably low (less than 61.0%) (Table IV). An identity of more than 60% was observed in VP1 between B219 and group B rotaviruses. Deduced amino acid sequences of B219 showed less than 57% identity to those of all rotavirus groups. Similarly, it was reported in a previous study that B219 genes encoding VP6, VP7, NSP1-NSP3 showed less than 57.1% identity to those from groups A–C [Alam et al., 2007]. In conclusion, it was confirmed genetically that B219 and J19 belong to a different group from groups A–C rotaviruses, based on significant genetic diversity in all the gene segments.

Characterization of B219 VP1 and VP4 Sequences

Rotavirus VP1 has RNA-dependent RNA polymerase activity [Cohen et al., 1989; Valenzuela et al., 1991].

TABLE III. Sizes of Whole Gene (Amino Acid) Sequences of B219 and J19, and Comparison with Groups A, B, and C Human Rotaviruses

Viral protein gene	Nos. of nucleotide (amino acid)[a]					Nucleotide (amino acid) sequence identity (%) between B219 and J19
	KU [group A]	CAL-1 [group B]	Bristol [group C]	B219 [Novel group]	J19 [Novel group]	
VP1 gene	3,302 (1,088)	3,511 (1,160)	3,309 (1,090)	3,538 (1,167)	3,538 (1,167)	93.1 (97.2)
VP2 gene	2,723 (892)	2,847 (934)	2,736 (884)	2,969 (973)	2,969 (973)	93.4 (98.0)
VP3 gene	2,591 (835)	2,341 (763)	2,166 (693)	2,204 (719)	2,204 (719)	91.6 (93.7)
VP4 gene	2,359 (775)	2,306 (750)	2,283 (744)	2,521 (826)	2,512 (823)	91.3 (95.0)
VP8*	(240)	(207)	(231)	(249)	(245)	89.0 (89.6)
VP5*	(529)	(536)	(496)	(563)	(563)	91.9 (97.1)
NSP1 gene	1,564 (486)	1,276 (107, 321)	1,270 (394)	1,307 (395)	1,307 (395)	92.4 (94.9)
VP6 gene	1,356 (397)	1,269 (391)	1,353 (395)	1,287 (396)	1,287 (396)	93.9 (98.7)
NSP3 gene	1,075 (310)	1,179 (347)	1,350 (402)	932 (262)	932 (262)	88.4 (93.5)
NSP2 gene	1,058 (317)	1,007 (301)	1,037 (312)	1,006 (297)	1,004 (297)	92.8 (97.0)
VP7 gene	1,062 (326)	814 (249)	1,063 (332)	818 (258)	820 (258)	94.3 (96.5)
NSP4 gene	750 (175)	751 (219)	613 (150)	739 (213)	739 (213)	87.7 (88.7)
NSP5 gene	664 (197)	631 (170)	730 (212)	649 (176)	649 (176)	93.7 (93.2)

[a]GenBank accession numbers of genes from KU, CAL-1, and Bristol which have been published previously and used in this analysis are as follows. KU: AB0022765 (VP1), AB022766 (VP2), AB022767 (VP3), AB222784 (VP4), AB022769 (NSP1), AB022768 (VP6), AB022771 (NSP3), AB022770 (NSP2), D16343 (VP7), AB022772 (NSP4), AB022773 (NSP5); CAL-1: EU490414 (VP1), AB037932 (VP2), EU490417 (VP3), AF184084 (VP4), AF230975 (NSP1), AB037931 (VP6), AF230974 (NSP3), AY238383 (NSP2), AF184083 (VP7), AY238387 (NSP4), AY238386 (NSP5); Bristol: NC_007547 (VP1), NC_007546 (VP2), NC_007574 (VP3), NC_007572 (VP4), NC_007544 (NSP1), NC_007570 (VP6), NC_007543 (NSP3), NC_007545 (NSP2), NC_007571 (VP7), NC_007573 (NSP4), NC_007569 (NSP5).Sequence data of J19 were obtained from GenBank database, accession nos. DQ113897–DQ113907.

TABLE IV. Sequence Identities of B219 Gene Segments to Representative Rotavirus Strains of Groups A, B, and C

Rotavirus strains (origin)	Nucleotide (amino acid) sequence identity (%) of B219 genes					
	VP1	VP2	VP3	VP4	NSP4	NSP5
Group A						
KU (human)	50.4 (23.2)	46.1 (15.6)	42.8 (17.9)	9.1 (10.9)	24.6 (11.0)	9.1 (12.3)
UK (bovine)[a]	50.1 (22.6)	16.6 (13.1)	43.9 (18.0)	46.3 (12.8)	24.3 (15.5)	22.4 (11.1)
Group B						
WH-1 (human)[a]	60.8 (56.3)	57.3 (46.7)	52.2 (48.9)	49.5 (30.2)	48.2 (19.5)	52.5 (27.3)
CAL-1 (human)	60.4 (55.9)	55.6 (44.6)	51.9 (48.1)	49.1 (24.5)	21.2 (15.7)	32.9 (26.7)
Bang373 (human)[a]	60.5 (56.1)	56.5 (48.2)	52.1 (48.1)	49.6 (30.6)	47.4 (16.7)	53.8 (26.7)
ADRV (human)[a]	—	56.1 (45.4)	—	46.5 (21.5)	21.0 (14.6)	31.2 (16.4)
IDIR (murine)[a]	61.0 (55.6)	20.9 (45.7)	40.9 (25.3)	46.1 (23.1)	11.2 (14.5)	32.5 (24.6)
Group C						
Bristol (human)	51.0 (21.6)	9.8 (12.7)	20.7 (17.7)	32.6 (15.4)	42.9 (13.0)	16.5 (16.5)
Cowden (porcine)[a]	49.8 (21.0)	38.6 (15.2)	50.3 (17.9)	18.0 (16.9)	41.9 (16.8)	35.6 (10.9)

[a]GenBank accession numbers of sequences used in this analysis are as follows: UK, X5544 (VP1), X52589 (VP2), AY300927 (VP3), M22306 (VP4), K03384 (NSP4), K03385 (NSP5); WH-1, AY539859 (VP2), AY539857 (VP4), AY539864 (NSP4), AY539863 (NSP5); Bang373, AY238390 (VP2), AY238388 (VP4), AY238384 (NSP4), AY238394 (NSP5); ADRV, M91433 (VP2), M91434 (VP4), AY548957 (NSP4), M34380 (NSP5); IDIR, M97203 (VP1), U00673 (VP2), U03556 (VP3), X16949 (VP4), U03557 (NSP4), D00912 (NSP5); Cowden, M74216 0 (VP1), M74217 (VP2), M74219 (VP3), M74218 (VP4), AF093202 (NSP4), X65938 (NSP5). References of KU, CAL-1, and Bristol sequences, see footnote of Table III.

RNA-dependent RNA polymerase of positive-sense and negative-sense single-stranded RNA viruses and double-stranded RNA viruses possess specific sequence motifs, which are suggested to be essential for enzymatic activity [Kamer and Argos, 1984; Poch et al., 1989; Bruenn, 1991; Jablonski and Morrow, 1995]. This functional domain has been found to exist in VP1 of group A rotaviruses [Mitchell and Both, 1990; Ito et al., 2001]. In the present study, it was found that the three group B human rotaviruses, B219, and J19 possess this domain in the central region of VP1, as well as group B IDIR strain and group C rotaviruses (Fig. 1). This finding suggests that B219 VP1 may function as an RNA-dependent RNA polymerase, as well as other rotavirus groups.

Between the VP4 sequences from B219 and J19, most of the cysteines and prolines (4 and 25 residues, respectively) were conserved (Fig. 2). In group A rotavirus, the outer capsid protein VP4 is cleaved into VP8 and VP5 with the presence of protease, which facilitates infectivity of the rotavirus [Estes and Kapikian, 2007]. In the B219 VP4, putative trypsin cleavage

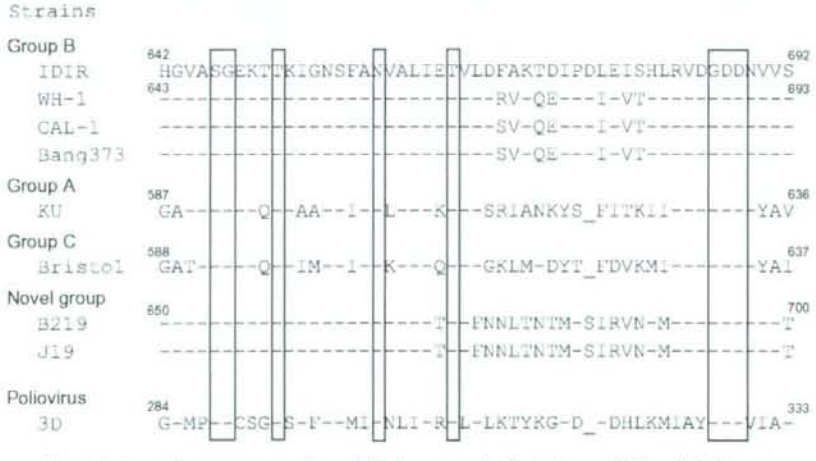

Fig. 1. Amino acid sequence comparison of VP1 from groups A–C rotaviruses, B219, and J19. Sequence of the RNA polymerase of poliovirus is arranged at the bottom as a reference. Identical residues to IDIR sequence are indicated by dashes, and the gap is shown by an underscore. At both ends of sequences, amino acid numbers are indicated. Identical amino acids in the RNA polymerases of various RNA viruses noted by Cohen et al. [1989] are boxed. The GDD sequence indicated by the rightmost box is functionally essential for RNA polymerase [Jablonski and Morrow, 1995].

```
B219    MSLRSLLITT EAVGETTQTS DHQTSFSTRT YNEINDRPSL RVEKDGEKAY     50
J19     .......... .......... .......... .......... .I........     50

B219    CFKNLDPVRY DTRMGEYPFD YGGQSTENNQ LQFDLFTKDL MADTDIGLSD    100
J19     .......... .......... .......... .......... K.....D...    100

B219    DVRDDLKRQI KEYYQQGYRA IFLIRPQNQE QQYIASYSST NLNFTSQLSV    150
J19     ..K....... M......... ...V...... .H........ .......P.I    150

B219    GVNLSVLNKI QENKLHIYST QPHIPSVGCE MITKIFRTDV DNENSLINYS    200
J19     .....I.... .....Y.... .......... ..A......I ..........    200

B219    VPVTVTISVT KATFEDTFVW NQNNDYPNMN YKDLIPAVTK NSIYHDVKRI    250
J19     A......... ....G....C .H----.... .Q....TM.. ..........    246

B219    TKIHEYINSK KKK-NGVGKI GGIQIAESKD GFWKILTKNY QIKLKFGIEG    299
J19     .......... ...K.STS.. .......... .......... .......V..    296

B219    YGVMGGTFGN WLIDSGFKTV ETNYEYQRNG KTINATTVAS VKPSRKCGTR    349
J19     .......... .......... .......... .......... ..........    346

B219    SPVFGQLQFS GEMMVLSHND ILTVFYTERE WALSNAIYAK NFATDFKRQF    399
J19     .......... .......... .......... .......... ..........    396

B219    EVTAQSDELL VRTNVVPHTI KNTPGKALME YSHGGFGQID TSDYTGMALT    449
J19     .I........ .......... .......... .......... ..........    446

B219    FRFRCVSEDL PEGYYDKDKA LTFANVGLTS FQDRQETNGT YWVYNTSTVG    499
J19     .....I.... ......R... .A........ ......A... ..........    496

B219    FGSCYPKKEF EYDINVTYTT LLPSDPEFTT GGTNYAQSVT AVLEESFINL    549
J19     .......... .......... .......... .......... ..........    546

B219    QNQVNEMLTR MNISDLTSGV MSVFSVATSF PQILDGISDL LKAASSAFKK    599
J19     .......... .......... .......... .......... ..........    596

B219    VKGKVGNVAK RLRGKRYVRL FDEDISIEET PRFLDSIRSS RRPSILSNMF    649
J19     ......S... .......... ...NV..... .......... ..........    646

B219    NDDETFTALH TLASRTNSVA SDVTYIQPII TTRIANSTPP VIAPASSVTY    699
J19     .......... .......... .....L.... .......... ..........    696

B219    AKLKDISKII NAEIDPKSIM EFNQVSNTIS ILDSTKKLAQ YAVDPDVIDG    749
J19     .......... .......... ....I..... .......... ......I...    746

B219    ILNKMVGGHA RSLFSLKVRK HLLDAVEKDA FVKYNYHDLM GKLLNDRELL    799
J19     .......... .......... .......... .......... ..........    796

B219    DITNNLSSQK QFELAKEFRD LLINALA                             826
J19     .......... .......... .......                             823
```

Fig. 2. Comparison of deduced amino acid sequences of VP4 between B219 and J19. Dots indicate amino acids identical to those of B219, while a dash and box denote a gap generated to obtain the best alignment. Conserved cysteine and proline residue are indicated by solid squares and circles, respectively, while the position of non-conserved cysteine and proline is indicated by a hollow square or circle. Putative trypsin cleavage sites are indicated by arrows. Amino acid numbers are indicated for the rightmost residues at individual lines of sequence.

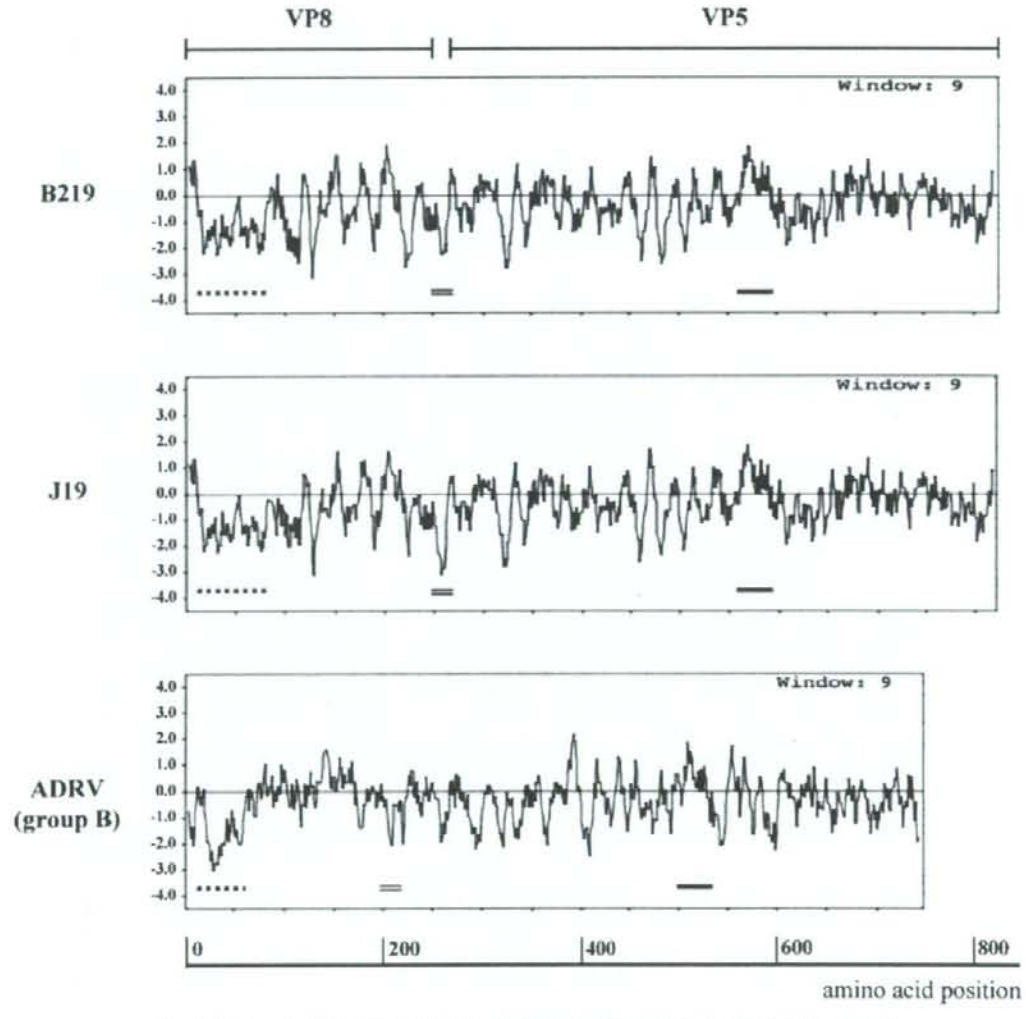

Fig. 3. Comparison of hydrophobicity/hydrophilicity plots for the VP4 amino acid sequences of B219, J19, and ADRV by the Kyte-Doolittle method. The N-terminal hydrophilic regions in VP8 are shown by dotted lines, while the hydrophobic regions near the middle portion of VP5 are indicated by solid lines. Positions of the putative trypsin cleavage sites are indicated by double lines.

sites were located on the C-terminal side of amino acids 249 and 263, through which 249- and 563-amino acid products (VP8 and VP5, respectively) are assumed to be generated (Fig. 2). Figure 3 shows the hydropathicity profiles of the VP4 proteins for B219 and J19, as well as ADRV for comparison purposes. The profile of B219 VP4 was similar to that of J19, and both profiles were also similar to ADRV VP4 with respect to the presence of hydrophilic regions near the N-terminal side of VP8 and around the trypsin cleavage site, and hydrophobic regions in the middle portion of VP5.

In B219, the ratio of amino acid length of VP8 to VP5 is 1:2.3, which is similar to the values in groups A, B, and C rotaviruses (1:2.1–2.6). As mentioned above, the B219 VP4 gene comprises 2,521 nucleotides, which are nine nucleotides longer than that of J19 (2,512 nucleotides) (Table III). B219 had an additional twelve nucleotides encoding four amino acids in the VP8 region, and lacked three nucleotides at the trypsin cleavage site, compared with the J19 VP4 gene (Fig. 2). When the coding regions of putative VP8 (747 nucleotides) and VP5 (1,689 nucleotides) of B219 were compared with those of J19,

the VP5-coding region showed slightly higher amino acid sequence identities (97.1%) than the VP8-coding region (89.6%) (Table III). Similarly, more sequence conservation in VP5 has also been reported in groups A and B rotaviruses [Gorziglia et al., 1988; Sereno and Gorziglia, 1994; Kobayashi et al., 2001; Ahmed et al., 2004]. These findings suggest the structural similarity of VP4 between B219 (J19) and rotaviruses of other groups.

Characterization of B219 NSP4 Sequence

The predicted NSP4 sequence of B219 consists of 213 amino acids, which are 38 and 63 amino acids longer than those of KU (group A) and Bristol (group C), respectively, and six amino acids shorter than the NSP4 of CAL-1 (group B). It has been reported that NSP4 of group A rotavirus has three evident hydrophobic domains comprised of 15–20 amino acids (h1–h3) in the N-terminal region [Chan et al., 1988], as shown in the hydropathicity plots of Figure 4. In the NSP4 of group B and C rotaviruses, two regions in the N-terminal side are evidently hydrophobic, while additional short hydrophobic sequence is found in group B NSP4. In contrast, hydrophilic domains were located on the C-terminal side of this protein [Taylor et al., 1996]. Similar to the NSP4 structure of groups A–C rotaviruses, NSP4 sequences of B219 and J19 were found to have a minor and two major hydrophobic domains in the N-terminal region, and hydrophilic domains in the C-terminal region (Fig. 4).

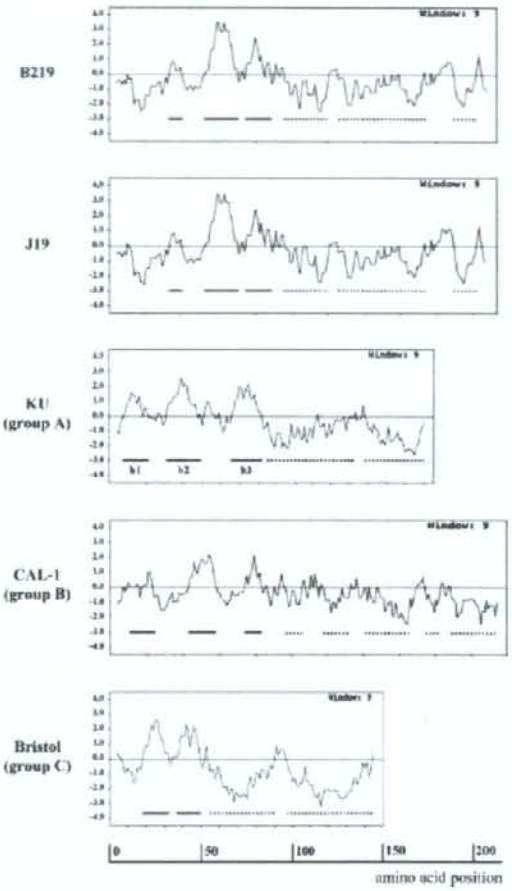

Fig. 4. Hydrophobicity/hydrophilicity plots for the NSP4 amino acid sequences of B219, J19, and group A (KU), B (CAL-1), and C (Bristol) human rotaviruses by the Kyte-Doolittle method. Hydrophilic and hydrophobic regions are shown by dotted lines and solid lines, respectively. Three hydrophobic domains (h1–h3) in the N-terminal region of group A rotavirus are indicated.

DISCUSSION

In the present study, complete VP1 and VP3 sequences of human group B rotaviruses were determined. Among the three strains, extremely high sequence identities of VP1 and VP3 were observed between CAL-1 and Bang373, while these strains showed slightly lower identities to WH-1 (Table II). A similar level of sequence identities of all other genes among these group B rotaviruses was discussed previously [Yang et al., 2004a], suggesting that genomic evolution of these group B human rotaviruses known so far may have occurred evenly in all the gene segments, from a common ancestral virus. VP1 and VP3 genes of human group B rotavirus showed approximately 70% identities to those of IDIR strain, and a similar level of identities (60–79%) was observed for other gene segments [Kobayashi et al., 2001; Ahmed et al., 2004; Yang et al., 2004a]. VP1 and VP3 sequence identities of group B human rotavirus to representative groups A and C rotaviruses were considerably low (45.8–49.8%), as observed for other viral gene segments among different rotavirus groups (less than 58%) [Alam et al., 2007].

The VP2 is the major component of the viral core proteins and has RNA-binding activity for both single- and double-stranded RNA [Boyle and Holmes, 1986]. Labbé et al. [1994] reported that the RNA-binding domain was located in the N-terminus 132 amino acids of VP2 of the bovine rotavirus RF strain. It was also predicted that an alpha-helix occurs within the hydrophilic N-terminal region (amino acids 55–89 in simian rotavirus SA11, 61–94 in human rotavirus Wa), which is suggested to be involved in the dimerization of the VP2 and RNA binding [Ernst and Duhl, 1989; Mitchell and Both, 1990]. Apart from this structure, two leucine-rich regions (amino acids 537–558 and 666–687), including putative leucine zipper motifs have been identified in mammalian rotaviruses [Kumar et al., 1989]. In the VP2 of B219, the presence of alpha-helix was predicted in the N-terminal region, amino acids 71–100 which corresponds to a hydrophilic region (data not shown).

However, the leucine rich regions including leucine zipper motifs were not detected.

The VP3 is a viral guanylyltransferase associated with the 5'-end capping of the viral mRNA [Pizarro et al., 1991; Liu et al., 1992], and two sequence motifs (KXTAMDXEXP and KXXGNNH) were reported as putative enzymatic active sites in VP3 of groups A and C rotaviruses [Cook and McCrae, 2004]. However, these motifs were not detected in the VP3 sequences of B219, J19, and three human group B rotaviruses. However, Ito et al. [2001] reported the presence of a single conserved sequence VALYSLSN in the middle portion of VP3 in groups A and C rotaviruses, and group B IDIR strain. Interestingly, this sequence, with minor variation, was detected also in B219 and J19, and group B human rotaviruses (Fig. 5). Hence, this sequence is suggested to have an essential functional role of this protein, although its relatedness as guanylyltransferase activity has not yet been elucidated.

Between B219 and J19, outer capsid proteins VP4 and VP7 amino acid sequences showed 95.0% and 96.5% identities, respectively. Among group A rotavirus, viruses belonging to a same antigenic type of VP4 (P-type) and VP7 (G-type) show generally more than 90% sequence identity of these outer capsid proteins [Green et al., 1987; Gorziglia et al., 1990]. This suggested that B219 and J19 may have identical antigenic specificity of VP4 and VP7.

The NSP4 of group A rotavirus has been shown to mobilize intracellular Ca_2^+ [Tian et al., 1994; Dong et al., 1997; Morris et al., 1999] and inositol 1,4,5-triphosphate (IP3) [Dong et al., 1997], and cause age-dependent diarrhea in suckling mice [Ball et al., 1996; Morris et al., 1999]. These reports indicate that the NSP4 of group A rotavirus may represent a viral enterotoxin. Remarkably, it has been demonstrated that a short peptide corresponding to NSP4 amino acids 114–135 in the C-terminal portion, caused diarrhea in mice [Ball et al., 1996; Zhang et al., 2000] and the sequence corresponding to this peptide is conserved highly among group A rotaviruses. A previous study reported that the synthetic peptides corresponding to amino acids 99–128 and 191–219 of group B rotavirus (CAL-1) NSP4 caused diarrhea in mice [Ishino et al., 2006], and enterotoxigenic activity was described also for the two-thirds C-terminal portion of group C rotavirus NSP4 [Sasaki et al., 2001]. In the NSP4 of B219 and J19, an enterotoxin (or enterotoxin-like) sequence which is identical or similar to those reported for groups A and B rotaviruses was not detected. This finding may be reasonable because the enterotoxin sequence in group A and enterotoxin-like sequences in group B are highly divergent. While between B219 and J19, NSP4 showed lowest sequence identity (88.7%) among the eleven viral proteins, and the C-terminal half was more conserved than N-terminal half (data not shown). Hence, it is probable that novel sequences in C-terminal region of B219 NSP4 may be responsible for enterotoxin activity of this novel rotavirus.

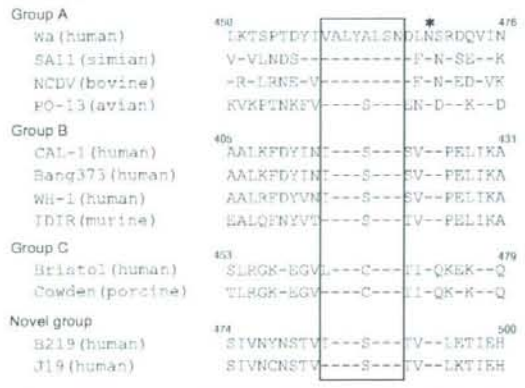

Fig. 5. Comparison of partial VP3 amino acid sequences from groups A–C rotaviruses, B219, and J19. Amino acid numbers are indicated at residues of both ends. Identical residues to Wa (group A) sequence are indicated by dashes. A conserved amino acid region among different groups is boxed. An asterisk denotes a conserved amino acid (N) near the conserved sequence.

The rotavirus nonstructural protein NSP5 has a critical role in the formation of viroplasm and rotavirus replication within infected cells [Campagna et al., 2005; Jiang et al., 2006]. It has been reported that C-terminal domain of NSP5 mediates some functions including dimmer formation and the increased insolubility of NSP5 [Eichwald et al., 2004; Campagna and Burrone, 2006], and C-terminal one-thirds amino acids of NSP5 were found to be sufficient to direct the formation of viroplasms [Mohan et al., 2003; Sen et al., 2006]. In the analysis of group A rotavirus NSP5, the presence of alpha-helix is predicted in the C-terminal 21 amino acid sequence, and two tandem DXDXD motifs are located just upstream of the alpha-helix [Sen et al., 2007]. These structures are considered as functional determinants that regulate viroplasm formation. In the NSP5 of the group B rotavirus CAL-1, predicted alpha-helix domains are found in the C-terminal half regions (amino acids 116–136, 147–161), while group C rotavirus Bristol has two alpha-helix regions in the C-terminal side (amino acids 121–141, 190–204). In the B219 NSP5, the presence of alpha-helix was predicted in a wide region (amino acids 98–139) in the C-terminal half of this protein (data not shown). However, the DXDXD motif was not found in the NSP5 of the strains CAL-1, Bristol, and B219.

In the present study, overall sequence similarity of the B219 and J19 gene segments, and considerable genetic diversity of these viruses to groups A–C rotaviruses were demonstrated. The basic structures of B219 viral proteins were found to be homologous to those of other rotavirus groups. These findings are considered as sufficient evidence to recognize that B219 and J19 are members of the genus rotavirus but belong to a novel

group, which is discriminated from the known rotavirus groups.

REFERENCES

Ahmed MU, Kobayashi N, Wakuda M, Sanekata T, Taniguchi K, Kader A, Naik TN, Ishino M, Alam MM, Kojima K, Mise K, Sumi A. 2004. Genetic analysis of group B human rotaviruses detected in Bangladesh in 2000 and 2001. J Med Virol 72:149–155.

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.

Ball JM, Tian P, Zeng CQ, Morris AP, Estes MK. 1996. Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. Science 272:101–104.

Boyle JF, Holmes KV. 1986. RNA-binding proteins of bovine rotavirus. J Virol 58:561–568.

Bruenn JA. 1991. Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerase. Nucl Acids Res 19:217–226.

Campagna M, Burrone OR. 2006. Fusion of tags induces spurious phosphorylation of rotavirus NSP5. J Virol 80:8283–8284.

Campagna M, Eichwald C, Vascotto F, Burrone OR. 2005. RNA interference of rotavirus segment 11 mRNA reveals the essential role of NSP5 in the virus replicative cycle. J Gen Virol 86:1481–1487.

Chan WK, Au KS, Estes MK. 1988. Topography of the simian rotavirus nonstructural glycoprotein (NS28) in the endoplasmic recticulm membrane. Virology 164:435–442.

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.

Cook JP, McCrae MA. 2004. Sequence analysis of the guanylyltransferase (VP3) of group A rotaviruses. J Gen Virol 85:929–932.

Dong Y, Zeng CQ, Ball JM, Estes MK, Morris AP. 1997. The rotavirus enterotoxin NSP4 mobilizes intracellular calcium in human intestinal cells by stimulating phoapholipase C-mediated inositol 1,4,5-trisphosphate production. Proc Natl Acad Sci USA 94:3960–3965.

Eichwald C, Jacob G, Muszynski B, Allende JE, Burrone OR. 2004. Uncoupling substrate and activation functions of rotavirus NSP5: Phosphorylation of Ser-67 by casein kinase 1 is essential for hyperphosphorylation. Proc Natl Acad Sci USA 101:16304–16309.

Eiden JJ, Vonderfecht S, Petric M. 1992. Terminal sequence conservation among the genomic segments of a group B rotavirus (IDIR strain). Virology 191:495–497.

Ernst H, Duhl JA. 1989. Nucleotide sequence of genomic segment 2 of the human rotavirus Wa. Nucl Acids Res 17:4382.

Estes MK, Kapikian AZ. 2007. Rotaviruses. In: Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE, editors. Fields virology. 5th edition. Philadelphia, PA: Lippincott Williams & Wilkins Co. pp 1917–1974.

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.

Gorziglia M, Larralde G, Kapikian AZ, Chanock RM. 1990. Antigenic relationships among human rotaviruses as determined by outer capsid protein VP4. Proc Natl Acad Sci USA 87:7155–7159.

Green KY, Midthun K, Gorziglia M, Hoshino Y, Kapikian AZ, Chanock RM, Flores J. 1987. Comparison of the amino acid sequences of the major neutralization protein of four human rotavirus serotypes. Virology 161:153–159.

Hung T, Chen G, Wang C, Chou Z, Chao T, Ye W, Yao H, Meng K. 1983. Rotavirus-like agent in adult non-bacterial diarrhoea in China. Lancet 2:1078–1079.

Ishino M, Mise K, Takemura H, Ahmed MU, Alam MM, Naik TN, Kobayashi N. 2006. Comparison of NSP4 protein between group A and B human rotaviruses: Detection of novel diarrhea-causing sequences in group B NSP4. Arch Virol 151:173–182.

Ito H, Sugiyama M, Masubuchi K, Mori Y, Minamoto N. 2001. Complete nucleotide sequence of a group A avian rotavirus genome and a comparison with its counterparts of mammalian rotaviruses. Virus Res 75:123–138.

Jablonski SA, Morrow CD. 1995. Mutation of the aspartic acid residues of the GDD sequence motif of poliovirus RNA-dependent RNA polymerase results in enzymes with altered metal ion requirements for activity. J Virol 69:1532–1539.

Ji S, Bi Y, Yang YH, Yang F, Song J, Tao X, Cui X. 2002. Cultivation and serial propagation of a new rotavirus causing adult diarrhea in primary human embryo kidney cells. Zhonghua Yi Xue Za Zhi (Natl Med J China) 82:14–18 (article in Chinese; English abstract).

Jiang X, Jayaram H, Kumar M, Ludtke SJ, Estes MK, Prasad BV. 2006. Cryoelectron microscopy structures of rotavirus NSP2-NSP5 and NSP2-RNA complexes: Implications for genome replication. J Virol 80:10829–10835.

Kamer G, Argos P. 1984. Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. Nucl Acids Res 12:7269–7282.

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.

Krishnan T, Sen A, Choudhury JS, Das S, Naik TN, Bhattacharya SK. 1999. Emergence of adult diarrhoea rotavirus in Calcutta, India. Lancet 353:380–381.

Kumar A, Charpilienne A, Cohen J. 1989. Nucleotide sequence of the gene encoding for the RNA binding protein (VP2) of RF bovine rotavirus. Nucl Acids Res 17:2126.

Labbé M, Baudoux P, Charpilienne A, Poncet D, Cohen J. 1994. Identification of the nucleic acid binding domain of the rotavirus VP2 protein. J Gen Virol 75:3423–3430.

Liu M, Mattion NM, Estes MK. 1992. Rotavirus VP3 expressed in insect cells possesses guanylyltransferase activity. Virology 188:77–84.

Mackow ER. 1995. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL, editors. Infectious of the gastrointestinal tract. New York: Raven Press. pp 983-1008.

Mitchell DB, Both GW. 1990. Completion of the genomic sequence of the simian rotavirus SA11: Nucleotide sequences of segments 1, 2 and 3. Virology 177:324–331.

Mohan KV, Muller J, Som I, Atreya CD. 2003. The N- and C-terminal regions of rotavirus NSP5 are the critical determinants for the formation of viroplasm-like structures independent of NSP2. J Virol 77:12184–12192.

Morris AP, Scott JK, Ball JM, Zeng CQ, O'Neal WK, Estes MK. 1999. NSP4 elicits age-dependent diarrhea and Ca^{2+} mediated I^- influx into intestinal crypts of CF mice. Am J Physiol 277:G431–G444.

Pizarro JL, Sandino AM, Pizarro JM, Fernández J, Spencer E. 1991. Characterization of rotavirus guanylyltransferase activity associated with polypeptide VP3. J Gen Virol 72:325–332.

Poch O, Sauvaget I, Delarue M, Tordo N. 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J 8:3867–3874.

Sanekata T, Ahmed MU, Kader A, Taniguchi K, Kobayashi N. 2003. Human group B rotavirus infections cause severe diarrhea in children and adults in Bangladesh. J Clin Microbiol 41:2187–2190.

Sasaki S, Horie Y, Nakagomi T, Oseto M, Nakagomi O. 2001. Group C rotavirus NSP4 induces diarrhea in neonatal mice. Arch Virol 146:801–806.

Sen A, Agresti D, Mackow ER. 2006. Hyperphosphorylation of the rotavirus NSP5 protein is independent of serine 67, [corrected] NSP2, or [corrected] the intrinsic insolubility of NSP5 is regulated by cellular phosphatases. J Virol 80:1807–1816.

Sen A, Sen N, Mackow ER. 2007. The formation of viroplasm-like structures by the rotavirus NSP5 protein is calcium regulated and directed by a C-terminal helical domain. J Virol 81:11758–11767.

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.

Taylor JA, O'Brien JA, Yeager M. 1996. The cytoplasmic tail of NSP4, the endoplasmic reticulum-localized non-structural glycoprotein of rotavirus, contains distinct virus binding and coiled coil domains. EMBO J 15:4469–4476.

Tian P, Hu Y, Schilling WP, Lindsay DA, Eiden J, Estes MK. 1994. The nonstructural glycoprotein of rotavirus affects intracellular calcium levels. J Virol 68:251–257.

Valenzuela S, Pizarro J, Sandino AM, Vásquez M, Fernández J, Hernández O, Patton J, Spencer E. 1991. Photoaffinity labeling of rotavirus VP1 with 8-azido-ATP: Identification of the viral RNA polymerase. J Virol 65:3964–3967.

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

Yang H, Chen S, Ji S. 1998. A novel rotavirus causing large scale of adult diarrhea in Shi Jiazhuang. Zhonghua Liu Xing Bing Xue Za Zhi (Chin Epidemiol) 19:336–338 (article in Chinese; English abstract).

Yang J-H, Kobayashi N, Wang YH, Zhou X, Li Y, Zhou DJ, Hu ZH, Ishino M, Alam MM, Naik TN, Ahmed MU. 2004a. Phylogenetic analysis of a human group B rotavirus WH-1 detected in China in 2002. J Med Virol 74:662–667.

Yang H, Makeyev EV, Kang Z, Ji S, Bamford DH, Dijk AA. 2004b. Cloning and sequence analysis of dsRNA segments 5, 6 and 7 of a novel non-group A, B, C. adult rotavirus that caused an outbreak of gastroenteritis in China. Virus Res 106:15–26.

Zhang M, Zeng CQ, Morris AP, Estes MK. 2000. A functional NSP4 enterotoxin peptide secreted from rotavirus-infected cells. J Virol 74:11663–11670.

Arch Virol (2008) 153:555–559
DOI 10.1007/s00705-007-0004-7

BRIEF REPORT

Identical rearrangement of NSP3 genes found in three independently isolated virus clones derived from mixed infection and multiple passages of Rotaviruses

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Received: 14 August 2007 / Accepted: 7 November 2007 / Published online: 19 December 2007

Abstract Three rotavirus variants with a rearranged RNA segment derived from the NSP3 gene were isolated in three independent experiments of coinfection and multiple passages of simian rotavirus strain SA11 and single-VP7-gene- or NSP1-gene-substitution reassortants having genetic background of SA11. Sequence analysis indicated that the three rearranged NSP3 genes had almost identical sequences and genomic structures organized by partial duplication of the open reading frame in a head-to-tail orientation following the termination codon. The junction site of the original NSP3 gene (first copy) and the duplicated portion (second copy) was identical among the three rearranged genes, while a direct repeat, i.e., a homologous sequence between the first copy and second template for duplication, typically located at the junction site, was not detected. However, short similar sequences were present at the end of the first copy and beginning of the second copy. These findings suggest that rearrangement of the NSP3 gene may occur at a certain preferential site which is related to sequence similarity between 3′-untranslated region and a region near the 5′-end of ORF.

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Rotavirus is widely distributed in mammals and birds, and causes acute diarrheal disease via intestinal infections. *Rotavirus* is a genus of the family *Reoviridae*, and it is differentiated into at least seven groups (A–G). The rotavirus genome is a double-stranded (ds) RNA which is divided into 11 segments. Each RNA segment encodes one of the six structural proteins (VP1-4, VP6, VP7) or six nonstructural proteins (NSP1-NSP6) [5]. Because of the difference in size of each segment, the 11 RNA segments are separated and observed as an RNA pattern in polyacrylamide gel electrophoresis (PAGE) that is specific for an individual rotavirus group. Due to the segmented nature of the genome, reassortment is one of the major mechanisms of genomic evolution of rotavirus, as is point mutation [15].

Rearrangement is another mechanism causing genomic diversity of rotavirus. In the rearrangement, a radical change in the size of the RNA segment occurs by concatemerization or deletion within a single RNA segment [4, 14, 15]. The rearranged genome is detected as a segment at an unusual position in the RNA pattern in the absence of an RNA segment at a normal position. The most frequently observed type of rearrangement is a head-to-tail sequence duplication, which has been reported for RNA segments encoding NSP1-NSP5 and VP6 [14]. Since the sequence duplication mostly initiates at the 3′ untranslated region (3′UTR), coding frames for these viral proteins are not affected. However, in a few instances, the sequence duplications begin within the 3′-end of the ORF, thereby causing minor alteration of the protein product [6]. Less frequently, the rearrangement results from deletion of a sequence within an ORF [17].

Rearranged RNA segments have been detected in rotaviruses isolated from chronically infected children with immunodeficiency and apparently immunocompetent

humans and animals [4]. Rotavirus variants with a rearranged genome have also been isolated by serial passage of rotavirus at high multiplicity of infection (m.o.i.) in vitro. Evidence from the genomic organization of the rearranged genome has suggested a mechanism by which partial duplication is caused: RNA-dependent RNA polymerase may disengage from the RNA template and then reattach at an upstream location on the same template, during either plus strand synthesis (transcription) or minus strand synthesis (replication) [4]. Evidence that supports the occurrence of rearrangement in replication [6] as well as transcription [8] has been provided by sequence analysis of the rearranged genomes. However, rearranged genomes have been genetically analyzed for limited number of rotavirus variants, and the definite mechanism of rearrangement is still unknown.

We previously analyzed preferential selection of rotavirus RNA segments (VP7 gene or NSP1 gene) in the genetic background of simian rotavirus SA11, through mixed infection at high m.o.i. and multiple passages among SA11-L2 and/or single-gene reassortant(s) [10, 12]. In these studies, unexpectedly, three variant clones with a rearranged gene (Ga613, Ao123, and Ar171) were detected by observation of the RNA patterns in PAGE for virus clones isolated from mixed-infection culture fluids. Although the three rearranged variants were derived from independent series of mixed infections and multiple passages, all of the rearranged genes migrated at the same position in PAGE, between SA11 RNA segments 4 and 5, in the absence of SA11 RNA segment 7 or 8 at the normal position (Fig. 1a). In our present study, we analyzed the rearranged genes from the three viruses genetically to clarify whether or not the rearrangements in the three clones have same genetic organization and to investigate putative mechanism causing these gene rearrangements, comparing them with those proposed previously.

Ga613, a plaque-purified clone from an isolate designated as Cl-13 in our previous study, was isolated from mixed infection in MA104 cells with simian rotavirus strain SA11 (SA11-L2 clone [16]) and single-VP7 gene-substitution reassortant SNR1, which has a VP7 gene derived from canine rotavirus K9 in the genetic background of SA11 [12]. Ga613 was the only variant with rearrangement among the 42 virus clones isolated from the sixth passage culture fluid, and no other variants were detected in the clones from the first passage (67 clones) and third passage (44 clones) examined in the study.

Ao123 and Ar171 were isolated from culture fluids of SA11-L2 coinfected with single-NSP1 gene-substitution reassortants SOF and SRF, respectively [10]. SOF and SRF possess an NSP1 gene (RNA segment 5) derived from porcine rotavirus strain OSU and simian rotavirus strain

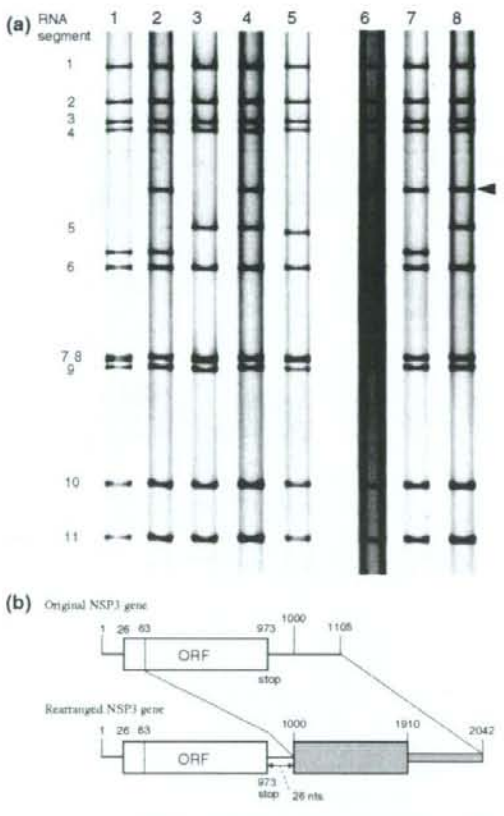

Fig. 1 Rearranged NSP3 genes detected by PAGE (**a**) and their genetic organization (**b**). **a** RNA patterns of the original strain SA11-L2 and single-NSP1 gene reassortants SRF and SOF used for coinfection, and virus clones with a rearranged genome. *Lane* 1, SOF; *lane* 2 and 7, Ao123; *lane* 3, SA11-L2; *lane* 4 and 8, Ar171; *lane* 5, SRF; *lane* 6, Ga613. An *arrowhead* indicates rearranged genes. RNA segments 1-11 assigned for SA11-L2 are indicated on the *left*. **b** Schematic diagram of the rearranged NSP3 gene detected in Ga613, Ao123, and Ar171 compared to the original NSP3 gene of SA11-L2 strain. *Shaded boxes* indicate the duplicated part of the original NSP3 gene

RRV, respectively. Ao123 and Ar171 were single variants with a rearranged gene, found among 50 and 36 clones, respectively, isolated from culture fluids of the tenth passage, and no other variants were detected in either of the mixed-infection cultures. Ao123 has a single foreign gene, i.e., RNA segment 5 derived from SOF (OSU) in the genetic background of SA11.

The three virus clones, Ga613, Ao123, and Ar171 were purified three times by plaque isolation. Nucleotide sequences of the rearranged genes were determined by RT-PCR and direct sequencing, as described previously [8].

For sequencing of the rearranged genome, firstly, a region containing the putative junction site of the original sequence and the duplicated portion was amplified by RT-PCR using a pair of primers, NS3-1 (5′-CACCAGAA TCGTCCATCACA-3′) and NS3-2 (5′-CATCTCTCAACA GCAAGCAC-3′) that are complementary to nucleotide (nt.) nos. 200–181 (minus sense) and 661–680 (plus sense) of the SA11-4F NSP3 gene sequence (GenBank accession no. M87502), respectively. While nucleotide synthesis by these primers progresses in opposite directions, outward from each other, in the normal NSP3 gene, amplification of a PCR product was expected if the rearranged genome had a head-to-tail duplication. As a result, PCR products of approximately 480 bp were obtained from the three clones. Although RT-PCR was attempted similarly to amplify a putative junction site in the NSP2 gene when outward-directed primers were used, no product was amplified, and further sequence analysis demonstrated that these clones had a normal NSP2 gene (data not shown). After determination of the sequence around the junction site, sequences of the original NSP3 gene containing the ORF (first copy) and the duplicated portion (second copy) were amplified by using primers NS3-5E1 (GGCATTTAA TGCTTTTCAGTGG, nt. 1–22) and NS3-3 (CAATCGAA GAAGTAA GGGATCCA, nt. 761–739), and primers NS3-3E1 (GGCCACATAACGCCCCTATA, nt. 1105–1086) and NS3-4 (GGGAATAGAATAT GATTATCAGG, nt. 124–146), respectively. The NSP3 gene sequence of the SA11-L2 clone was also determined to compare it to that of two SA11 clones (SA11-4F, SA11-Cl14) whose sequence data are available. The nucleotide sequences of the NSP3 gene determined in this study were deposited in GenBank under the following accession nos: SA11-L2, EF460843; Ao123, EF460844; Ga613, EF460845.

The NSP3 gene of the original strain SA11-L2 sequenced in the present study was 1105 nucleotides long with a 5′UTR and 3′UTR of 25 and 132 nucleotides, respectively. The rearranged genome of the three variants had 2042 nucleotides and contained the 5′UTR and the NSP3 gene ORF, and a partial duplication (second copy) corresponding to nucleotides 63–1105 of the original SA11-L2 strain (Fig. 1b). The second copy started 26 nucleotides downstream of the termination codon. This genomic organization of the rearranged NSP3 genes and their sequences from the three clones were identical except for a single nucleotide difference in the ORF of Ga613.

The NSP3 gene sequence of SA11-L2 determined in this study differed from those of SA11-4F (GenBank accession no. M87502) and SA11-Cl14 (AY06584) by four nucleotides and one nucleotide, respectively, among which a single site corresponding to nt 305 of the SA11-4F sequence was associated with a different amino acid among the SA11 clones (Table 1). The four nucleotides in SA11-L2 that were different from those of SA11-4F were commonly retained in the first and second copies of rearranged genes of the three variants, proving that the duplication originated from the SA11-L2 clone. In addition, a single nucleotide substitution at nt 749 was detected in the ORF (first copy) of Ga613. Because the nucleotide at the corresponding site in the second copy (nt.1686) was different from that of nt. 749 in Ga613, but the same as that in SA11-L2, this mutation was suggested to have occurred after the rearranged genome was completed during the multiple passages of viruses. By this mutation, the amino acid

Table 1 Nucleotide differences in the NSP3 genes of SA11-L2, Ga613, Ao123, and Ar171 compared with SA11-4F and SA11-Cl14

Location	Nucleotide (amino acid) position	Nucleotide (codon[a] and amino acid) at the indicated position in rotavirus strain					
		SA11-4F[b]	SA11-Cl14[b]	SA11-L2	Ga613	Ao123	Ar171
First copy	305 (94)	C (CCA, Pro)	G (GCA, Ala)	G (GCA, Ala)	G (GCA, Ala)	G (GCA, Ala)	G (GCA, Ala)
	316 (97)	G (TTG, Leu)	G (TTG, Leu)	A (TTA, Leu)	A (TTA, Leu)	A (TTA, Leu)	A (TTA, Leu)
	604 (193)	C (AAC, Asn)	T (AAT, Asn)	T (AAT, Asn)	T (AAT, Asn)	T (AAT, Asn)	T (AAT, Asn)
	749 (242)	A (ACT, Thr)	A (ACT, Thr)	A (ACT, Thr)	C (CCT, Pro)	A (ACT, Thr)	A (ACT, Thr)
	838 (271)	C (GCC, Ala)	G (GCG, Ala)	G (GCG, Ala)	G (GCG, Ala)	G (GCG, Ala)	G (GCG, Ala)
Second copy	1242 (305)[c]				G	G	G
	1253 (316)				A	A	A
	1541 (604)				T	T	T
	1686 (749)				A	A	A
	1775 (838)				G	G	G

[a] Nucleotide at the indicated position is underlined

[b] GenBank accession nos. of NSP3 genes: SA11-4F, M87502; SA11-Cl14, AY065843

[c] Nucleotide position corresponding to that in the first copy is indicated in parentheses

change is considered to occur at position 242, which is located in the eIF4G-binding region of NSP3 [3].

In the nucleotide sequence around the junction region of the templates for the first copy and second copy, no apparent direct repeat was detected, although three identical nucleotides were found just upstream from the junction (Fig. 2a). However, similar six-base-long sequences (CAAT-A) were found just before the junction in the first copy and at the beginning of the second copy. To check for the presence of such similar sequences in other rearranged NSP3 genes, previously published sequence data of the three virus clones (IGV-F, IGV-S, and H57) [9, 11] were examined (Fig. 2b–d). A typical direct repeat sequence at the junction site was not detected in any the clones, although a few nucleotides were identical in IGV-F and IGV-S, and direct-repeat-like sequences were found in H57. However, similar 5- to 6-base-long sequences were observed in these three rearranged genes in the first template sequence and just after the junction site in the second template.

Among the complete sequences of the rearranged genes reported, a direct repeat has been detected in the NSP1 gene, NSP4 gene, and NSP5 gene with rearrangement [1, 8, 9]. A typical direct repeat is a short identical or similar sequence close to the junction site in the first copy and upstream region of the second copy. It has been hypothesized that rearrangement may be caused by dissociation of RNA-dependent RNA polymerase from the RNA template and reassociation at a distant site on the same template [4]. In this process, the direct repeat is considered to provide a relative advantage for the reinitiation of transcription, although the direct repeat might not be indispensable for generating a rearrangement. Even when a direct repeat is not present, a short strech of similarity (even a two-nucleotide-long sequence) has been suggested to be significant for determining a reassociation site [9]. However, even such a short repeat is not found in some rearranged genes [2, 7, 11].

In contrast, it may be notable that similar sequences are found between the first and second templates, as observed in the present study and in some rearranged genes reported previously [7, 13], although their locations are different from that of a typical direct repeat. In case of the rearranged NSP1 gene of the SA11-5S clone, an eight nucleotide sequence just before the junction site in the first template is found at a position six nucleotides upstream from the junction in the second template [13]. Similarly, direct-repeat-like sequences are found in the H57 NSP3 gene [11], as shown in the present study. These observations suggest that similar sequences between the first strand and the second strand may be generally related to template switching of RNA-dependent RNA polymerase. Furthermore, it may be speculated that RNA-dependent RNA polymerase may often shift slightly upstream or downstream from the similar sequence on the second template when it reattaches to the RNA template, for an unknown reason.

Fig. 2 Sequences around the junction site of the first (*upper*) template and second (*lower*) template that are copied into the rearranged NSP3 genes: **a** Ga613, Ao123, Ar171; **b** IGV-F (accession no. AF190172); **c** IGV-S (accession no. AF190171); **d** strain Wa, cloneH57 (accession no. S41238); **e** strain M, clone M1, (accession no. AF338247). The sequence of the rearranged genome is indicated in *boldface* with nucleotide nos. of templates at the end of the first copy and the beginning of the second copy. *Dotted lines* denote identical nucleotides (**a**–**c**) or those forming direct-repeat-like sequence **d**. *Underlines* in **a**–**d** indicate similar sequences between the first and second templates. *Underlines* in **e** show portions of the first and second templates which may form presumptive base paring in the secondary structure according to the directions indicated by *dotted arrows* (6)

Gault and coworkers proposed a new mechanism for rearrangement in which secondary structures might facilitate the transfer of RNA-dependent RNA polymerase from the 5′-end to the 3′-end of the plus strand RNA during the replication step, based on analysis of rearranged genomes derived from genes 7 and 11 [6] (Fig. 2e). This mechanism is based on the prediction that the 5′ and 3′ ends of the mRNA of these genes may form base pairing in a long panhandle structure, and this may explain the occurrence of rearranged genomes in which evident direct repeat or similar sequence are not found. In the NSP3 gene of SA11-L2, base paring of the sequences around nts 63 and 999 was not found in the predicted secondary structure using the *mfold* program version 3.1 [18] and CLC RNA Workbench version 1.0 (CLC bio, Cambridge, MA) (data not shown). Thus, it is not likely that the occurrence of rearrangement found in Ga613 (Ao123, Ar171) was attributable to the secondary structure of the mRNA.

In our present study, an identical rearrangement in the NSP3 gene was detected in the three virus clones isolated from an independent series of multiple passages of rotaviruses. This finding suggests that the common genetic organization, i.e., junction site in the 3′UTR of the first copy and initiating point of duplication in the NSP3 gene ORF, might have been preferentially selected for the process of rearrangement. It is probable that except for the typical direct repeat and secondary structure of mRNA, there may be a critical factor serving to make the template switch of RNA-dependent RNA polymerase efficient. To elucidate this, sequence analysis of more rearranged genomes will be required.

Acknowledgment This study was supported in part by a Grant-in-Aid for Scientific Research (No.15406028) and the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (Okayama University–National Institute of Cholera and Enteric Diseases, India) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and a Grant for International Health Cooperation Research (18-5) from the Ministry of Health, Labour, and Welfare of Japan.

References

1. Ballard A, McCrae MA, Desselberger U (1992) Nucleotide sequences of normal and rearranged RNA segments 10 of human rotaviruses. J Gen Virol 73:633–638
2. Blackhall J, Fuentes A, Magnusson G (1996) Genetic stability of a porcine rotavirus RNA segment during repeated plaque isolation. Virology 225:181–190
3. Deo RC, Groft CM, Rajashankar KR, Burley SK (2002) Recognition of the rotavirus mRNA 3′ consensus by an asymmetric NSP3 homodimer. Cell 108:71–81
4. Desselberger U (1996) Genome rearrangements of rotaviruses.Arch Virol 12(Suppl):37–51
5. Estes MK, Kapikian AZ (2007) Rotaviruses, Chap 53. In: Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE (eds) Fields Virology, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp 1917–1974
6. Gault E, Schnepf N, Poncet D, Servant A, Teran S, Garbarg-Chenon A (2001) A human rotavirus with rearranged genes 7 and 11 encodes a modified NSP3 protein and suggests an additional mechanism for gene rearrangement. J Virol 75:7305–7314
7. Gonzalez SA, Mattion NM, Bellinzoni R, Burrone OR (1989) Structure of rearranged genome segment 11 in two different rotavirus strains generated by a similar mechanism. J Gen Virol 70:1329–1336
8. Kojima K, Taniguchi K, Urasawa T, Urasawa S (1996) Sequence analysis of normal and rearranged NSP5 genes from human rotavirus strains isolated in nature: implications for the occurrence of the rearrangement at the step of plus strand synthesis. Virology 224:446–452
9. Kojima K, Taniguchi K, Kawagishi-Kobayashi M, Matsuno S, Urasawa S (2000) Rearrangement generated in double genes, NSP1 and NSP3, of viable progenies from a human rotavirus strain. Virus Res 67:163–171
10. Mahbub Alam M, Kobayashi N, Ishino M, Naik TN, Taniguchi K (2006) Analysis of genetic factors related to preferential selection of the NSP1 gene segment observed in mixed infection and multiple passage of rotaviruses. Arch Virol 151:2149–2159
11. Mendez E, Arias CF, Lopez S (1992) Genomic rearrangements in human rotavirus strain Wa: analysis of rearranged RNA segment 7. Arch Virol 125:331–338
12. Okada J, Kobayashi N, Taniguchi K, Urasawa S (1998) Preferential selection of heterologous G3-VP7 gene in the genetic background of simian rotavirus SA11 detected by using a homotypic single-VP7 gene-substitution reassortant. Antiviral Res 38:15–24
13. Patton JT, Taraporewala Z, Chen D, Chizhikov V, Jones M, Elhelu A, Collins M, Kearney K, Wagner M, Hoshino Y, Gouvea V (2001) Effect of intragenic rearrangement and changes in the 3′ consensus sequence on NSP1 expression and rotavirus replication. J Virol 75:2076–2086
14. Patton JT, Vasquez-Del Carpio R, Tortorici MA, Taraporewala ZF (2007) Coupling of rotavirus genome replication and capsid assembly. Adv Virus Res 69:167–201
15. Taniguchi K, Urasawa S (1995) Diversity of rotavirus genomes. Sem Virol 6:123–131
16. Taniguchi K, Nishikawa K, Kobayashi N, Urasawa T, Wu H, Gorziglia M, Urasawa S (1994) Differences in plaque size and VP4 sequence found in SA11 virus clones having simian authentic VP4. Virology 198:325–30
17. Taniguchi K, Kojima K, Urasawa S (1996) Nondefective rotavirus mutants with an NSP1 gene which has a deletion of 500 nucleotides, including a cysteine-rich zinc finger motif-encoding region (nucleotides 156 to 248), or which has a nonsense codon at nucleotides 153–155. J Virol 70:4125–4130
18. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415

Arch Virol
DOI 10.1007/s00705-008-0212-9

ORIGINAL ARTICLE

Phylogenetic analysis of rotaviruses with genotypes G1, G2, G9 and G12 in Bangladesh: evidence for a close relationship between rotaviruses from children and adults

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Received: 13 July 2008 / Accepted: 8 September 2008

Abstract To clarify the phylogenetic relatedness of rotaviruses causing gastroenteritis in children and adults, an epidemiologic investigation was conducted in Mymensingh, Bangladesh, during the period between July 2004 and June 2006. A total of 2,540 stool specimens from diarrheal patients from three hospitals were analyzed. Overall, rotavirus-positive rates in children and adults were 26.4 and 10.1%, respectively. Among the 155 rotavirus specimens examined genetically from both children and adults, the most frequent G genotype was G2 (detection rate: 54.0 and 47.6%, respectively), followed by G1 (21.2 and 26.2%, respectively), and G9 (15.9 and 9.5%, respectively). G12 was also detected in five specimens (3.2% in total; four children and one adult). Sequence identities of VP7 genes of G2 rotaviruses from children and adults were higher than 97.8%, while these Bangladeshi G2 viruses showed generally lower identities to G2 rotaviruses reported elsewhere in the world, except for some strains reported in African countries. Similarly, extremely high sequence identities between children and adults were observed for VP7 genes of G1, G9 and G12 rotaviruses, and also for the VP4 genes of P[4], P[6], and P[8] viruses. Rotaviruses from children and adults detected in this study were included in a single cluster in phylogenetic dendrograms of VP7 or VP4 genes of individual G/P types. Rotaviruses with two emerging types, G9 and G12, had VP7 genes that were phylogenetically close to those of individual G-types recently reported in Bangladesh and India and were included in the globally spreading lineages of these G-types. These findings suggested that genetically identical rotaviruses, including those with the emerging types G9 and G12, were circulating among children and adults in city and rural areas of Bangladesh.

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Introduction

Group A rotaviruses are one of the major etiological causes of severe gastroenteritis in infants and young children worldwide. It is estimated that rotavirus accounts for more than a third of all diarrhea-related hospital admissions, causing about 527,000 deaths per year in children less than 5 years of age, mostly in developing countries [24, 39]. To reduce the burden of severe diarrheal illness associated with rotavirus infections, an effort to develop a safe and effective rotavirus vaccine has been made. At present, two rotavirus vaccines have been developed and employed for voluntary or routine administrations for children in many countries [8].

The rotaviruses belong to the family *Reoviridae* and were classified into at least seven established groups (A–G) or a newly proposed group [3, 10]. The genome of rotavirus consists of 11 segments of double-stranded RNA

(dsRNA) encoding six structural viral proteins (VP) and six nonstructural proteins (NSP). Rotavirus is a non-enveloped virus, and the virion is comprised of three concentric protein layers, i.e., the outer capsid, inner capsid, and inner core. Two outer capsid proteins, VP7 and VP4, independently elicit a neutralizing response and define different serotype specificities, G serotype and P serotype, respectively. Based on the VP7 and VP4 gene sequences, antigenic types of group A rotavirus have been discriminated as G genotype (G-type) and P genotype (P-type), respectively [10]. Accordingly, the antigenicity of group A rotavirus strains has been described by the dual classification system with G-type and P-type. At least 15 G-types and 28 P-types have been described so far in rotaviruses from humans and various animal species. In human rotaviruses, the major genotypes are G1, G2, G3, G4, and G9, which are combined with P[4], P[6], and P[8] [30]. The inner capsid protein VP6 contains antigenicities associated with group (A–G) specificity and subgroup specificities I or II for group A rotaviruses. Based on the sequence diversity of NSP4, at least five genotypes (A–E) have been discriminated [10].

Rotaviruses are known to show great antigenic and genomic diversity. In Bangladesh, different G and P types have been becoming predominant and changing by year and season [6, 26, 36]. It has been observed that mixed infections of rotaviruses with different types have occurred frequently, possibly causing increased genomic reassortment. This may result in diversity of rotavirus genotypes. According to recent report in Bangladesh, during the 2005–2006 season, G2P[4], which was previously less prevalent, became most predominant, and the uncommon type G12 became more prevalent than in previous seasons [26]. Because of such situations, continuous investigation of rotavirus genotypes in Bangladesh is considered to be important for knowing the trend of prevalent rotavirus strains, which may be relevant for estimating rotavirus vaccine efficacy.

Despite their low frequency, group A rotaviruses cause gastroenteritis in adults, which has been described as epidemic outbreaks, travel-related gastroenteritis, and endemic cases [4, 15]. In some reports, G2 rotavirus has been described as the common cause of outbreaks in adults [12, 16, 35]. However, epidemiologic and genetic information of rotaviruses causing sporadic gastroenteritis cases in adults is limited [17, 20, 25]. Phylogenetic relatedness of rotaviruses between children and adults has not yet been well studied, except for a recent report in China, in which a close relationship of predominant G3 rotaviruses was demonstrated between children and adults [38]. In Bangladesh, genetic characteristics of rotaviruses from adults have never been investigated; therefore, the significance of rotavirus diarrhea in adults and its possible influence on rotavirus infection in children are still unknown. The present study was carried out to analyze genetic characteristics and phylogenetic relatedness of rotaviruses causing sporadic diarrhea in both children and adults in Mymensingh, Bangladesh.

Materials and methods

Specimens

The present study was conducted as a hospital-based survey of rotaviruses in sporadic diarrheal cases. Fecal specimens were collected from inpatients and outpatients in three medical facilities, Mymensingh Medical College (MMC) Hospital, SK Hospital, and Dharmapasha Thana Health Complex (DH), during a 2-year period between July 2004 and June 2006. The MMC hospital and SK hospital are located in Mymensingh city, about 200 km to the north of Dhaka. DH is a local health facility located about 100 km to the east of Mymensingh. In the SK hospital and DH, fecal specimens were collected from both children and adults, while in the MMC hospital, specimens were collected only from children up to 10 years old. In the present study, patients less than 16 years old were categorized as children. A total of 2,540 fecal specimens from 1,627 children (less than 16 years) and 913 adults (16 years or older) were collected in the study period.

Detection of rotavirus

The presence of rotavirus in stool specimens was determined by detection of dsRNA segments of rotavirus in polyacrylamide gel electrophoresis (PAGE). Viral dsRNA was extracted from 400 µl of 10% stool suspension with sodium dodecyl sulfate (SDS) and phenol and precipitated with ethanol as described previously [19]. RNA segments of rotavirus were separated by PAGE and stained with silver nitrate as described previously [14]. The electrophoretic migration patterns (electropherotypes) of the RNA segments were also analyzed for further genetic discrimination of rotaviruses.

Genotyping of rotavirus

Rotavirus G-type and P-type were determined by reverse transcription-polymerase chain reaction (RT-PCR) as described previously [11, 34]. The viral dsRNA was extracted from stool suspension using guanidine isothiocyanate and an RNaid kit (BIO 101, Inc., La Jolla). When G-type was not assigned to G1-G4, G8 or G9, G type was analyzed by sequencing and phylogenetic analysis of the whole VP7 gene as described below.