

Survey on the distribution of the gene 4 alleles of human rotaviruses by polymerase chain reaction

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SUMMARY

The presence of six gene 4 alleles (or VP4 genotypes) in human rotaviruses has been recognized. Using 16 representative cultivable human rotavirus strains, we confirmed the specificity of VP4 genotyping by polymerase chain reaction (PCR) using the nested oligonucleotides specific to each of the four representative gene 4 alleles. Using the PCR, we surveyed the gene 4 alleles of 199 human rotaviruses in stools collected in Japan and Thailand. Strains with the gene 4 allele, corresponding to P1A serotype, were shown to be the most prevalent, but two strains with P2 gene 4 allele and one strain with P3 gene 4 allele were detected in Thailand and in Japan, respectively.

INTRODUCTION

Group A rotavirus is a major cause of acute non-bacterial gastroenteritis among many avian and mammalian species including humans [1, 2]. Longitudinal studies have shown that the diarrhoeal illness caused by rotavirus infection tends to be severe and often fatal especially in developing countries [1]. Therefore, rotavirus has been given high priority for vaccine development [3].

Rotavirus has two outer capsid proteins VP4 and VP7 which carry independent neutralization antigens [4], and serotype specificity associated with VP4 and VP7 is designated VP4 (P) and VP7 (G) serotypes, respectively. The presence of nine G serotypes (G1–G4, G6, G8–G10, and G12) in human rotaviruses (HRV) has been established [1, 5–8], and four G serotypes (G1–G4) are the major ones. Global distribution of G serotype of HRV has been extensively examined mainly by an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (MAb) [9–11].

Four representative P serotypes (P1A, P1B, P2, and P3) have been identified in HRV [12, 13]. Because antibody to VP4 is important for the prevention of

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rotavirus infection [14], epidemiological surveys of the distribution of P serotypes are essential. However, such surveys have been hindered by the absence of simple, rapid methods. By serological examination of VP4 proteins expressed by baculovirus [12, 13] and comparative nucleotide sequence analysis of the VP4 genes of HRV [15, 16], it was found that P serotype specificity of HRV correlates well with the gene 4 alleles (or VP4 genotypes) identified by nucleotide sequence analysis of their VP4 genes. Four kinds of VP4 genotypes in HRV represented by strains KU, DS-1, 1076, and K8 correspond to P1A, P1B, P2, and P3, respectively [12, 13, 15, 16], and we tentatively designate them here as P1A gene 4 allele, P1B gene 4 allele, P2 gene 4 allele, and P3 gene 4 allele. Recently, Gentsch and colleagues [17] have developed the polymerase chain reaction (PCR) to identify the gene 4 alleles of HRV as a substitute method to assign P serotypes.

Here, we have confirmed the utility of PCR identification of the gene 4 allele of HRV in stools, and used it to survey the distribution of the gene 4 alleles of HRV in Japan and Thailand.

MATERIALS AND METHODS

Viruses

The following cell culture-adapted HRV with different gene 4 alleles were used as reference strains: strains KU, YO, WI-61, and Hosokawa for P1A gene 4 allele, strains DS-1, HN-126, L26, and L27 for P1B gene 4 allele, strains M37, 1076, ST-3, and McN13 for P2 gene 4 allele, and strains K8, AU-1, O265, and M318 for P3 gene 4 allele [16, 18–20]. Viruses were treated with 10 $\mu\text{g}/\text{ml}$ of acetylated trypsin (type V-S from bovine pancreas; Sigma) and were propagated in MA-104 cells in the presence of trypsin (1 $\mu\text{g}/\text{ml}$). The viruses were harvested 1–3 days after infection. Group A rotavirus-positive stool specimens were collected from infants with diarrhoea in Sapporo, Japan (114 specimens), in Osaka, Japan (36 specimens), and in Chiang Mai, Thailand (49 specimens). Approximately 10% stool suspension was prepared in phosphate-buffered saline.

Extraction of double-stranded RNA (dsRNA)

Rotavirus dsRNA was extracted by the method of Gentsch and colleagues [17] with some modifications. Three hundred μl of 10% stool suspension were mixed well with an equal volume of fluorocarbon and centrifuged at 8000 g for 5 min. The 250 μl of the aqueous phase, 320 μl of 6 M guanidine thiocyanate and 10 μl of RNA matrix in RNAID kit (BIO 101, Inc., LaJolla) were added and mixed on a mixer for 10 min. The mixture was centrifuged at 900 g for 1 min, and the pellets were washed three times with 400 μl of wash buffer supplied in the RNAID kit by sequential centrifugation at 2000 g, 3500 g, and 8000 g. The final pellet was dried under vacuum, and suspended in 50 μl of a distilled water, and incubated at 65 °C for 10 min. The suspension was centrifuged at 8000 g for 1 min, and the supernatant stored at –20 °C until use.

PCR for identification of gene 4 allele

PCR was performed as described previously with some modifications [17, 21]. Oligonucleotides specific to each gene 4 allele were synthesized for this study based on the nucleotide sequence alignment of the VP4 genes of numerous HRV strains

Table 1. Primer sequences and their location on the VP4 gene

Primer	Sequence (5'-3')	Strain (P serotype)	Location (Nucleotide no.)
A	(+) TGGCTTCGTTTCATTTATAGACA	KU (P1A)	11-32
B	(-) CTAAATGCTTTTGAATCATCCCA	KU (P1A)	1072-1094
C	(-) ATATTCCTACGAGTTTAGTATC	KU (P1A)	487-508
D	(-) ACTAACATGTGGTTCAACTGCGAT	DS-1 (P1B)	325-348
E	(-) CTGAGCACGTTGATAAGTCAC	1076 (P2)	733-755
F	(-) CGTATATTGATAGTTCATGGG	K8 (P3)	900-921

with different gene 4 allele. Table 1 lists the primer sequences and their location on the VP4 gene.

The first amplification was performed by using a pair of common oligonucleotides corresponding to nucleotide sequence nos. 11-32 (+sense) and 1072-1094 (-sense) of the VP4 gene of strain KU which were found to be conserved well in numerous HRV strains regardless of the difference in their P serotypes or gene 4 alleles. Extracted dsRNA (1 μ l) and 100 pmol each of two common primers (A and B listed in Table 1) were added to 100 μ l of reaction buffer containing 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM MgCl₂, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 5 mM dithiothreitol, 3.5% dimethyl sulfoxide (DMSO), and RNase inhibitor (5 units). The mixture was heated at 97 °C for 5 min and rapidly cooled in ice, and 10 units of avian reverse transcriptase (Seikagaku Kogyo, Tokyo) and 2.5 units of Taq DNA polymerase (Bokusui Brown Inc.) were added. The reaction mixture was covered with one drop of mineral oil, and the tube was placed in a thermal cycler (PC-700; Astec Co., Tokyo). Following incubation for reverse transcription at 42 °C for 30 min, samples were subjected to one cycle of 5 min at 94 °C, 3 min at 40 °C, and 3 min at 72 °C, 25 cycles of 1 min at 94 °C, 2 min at 40 °C, and 3 min at 72 °C and a final cycle of 1 min at 94 °C, 2 min at 40 °C, and 10 min at 72 °C.

In the second amplification, a mixture of primers (25 pmol each of primers C, D, E, and F that are specific to P1A, P1B, P2, and P3 gene 4 alleles, respectively and a primer A common to 5' end of the VP4 gene of HRV listed in Table 1) and the first amplification product (1 μ l) were added to the same reaction buffer (100 μ l) used for the first amplification except for the absence of RNase inhibitor, and the steps of denaturation and reverse transcription were omitted. The samples were subjected to 25 cycles of 94 °C for 1 min, at 42 °C for 2 min, and 72 °C for 1 min. The final extension was allowed to continue for 7 min. PCR products (10 μ l) were analysed by electrophoresis on 1% agarose in Tris-acetate-EDTA buffer containing ethidium bromide (1 μ g/ml).

PCR for G serotype assignment

PCR for G serotype assignment was performed as described previously [21, 22], except that RNAID method described above was used for RNA extraction in some samples.

ELISA for G serotyping

The G serotype of viruses in stool was determined using monoclonal antibodies in an ELISA test described previously [9, 10].

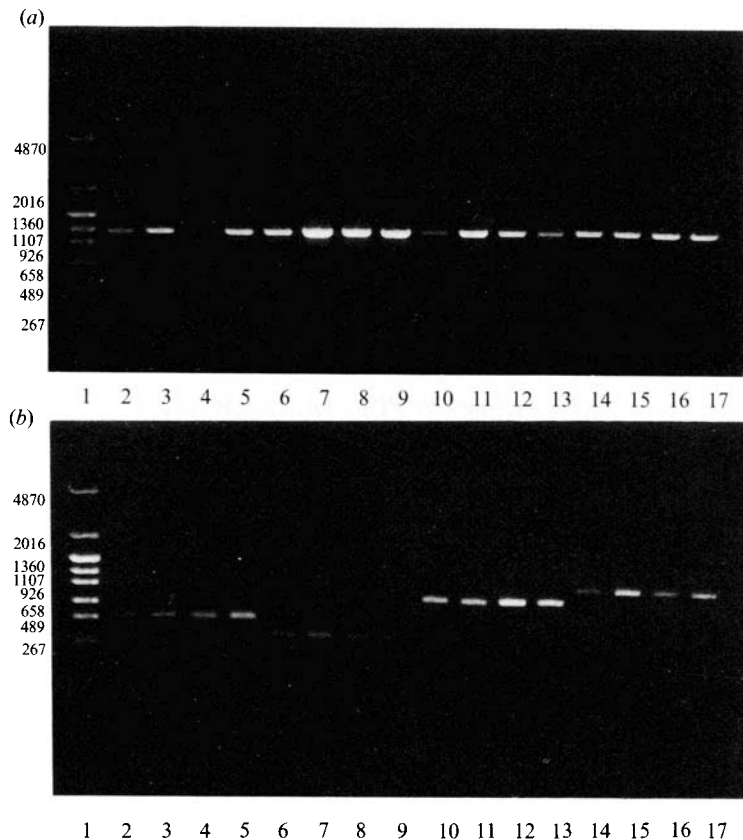


Fig. 1. Electrophoretic analysis of the PCR products after first amplification (a) and after second amplification (b) with cultivable HRV strains. Lanes: 1, molecular weight marker (a mixture of *Hind* III digests of pHY300PLK DNA and pHY300.2PLK DNA; Takara Shuzo Co., Japan); 2, strain KU (G1, P1A gene 4 allele); 3, strain YO (G3, P1A); 4, WI-61 (G9, P1A); 5, strain Hosokawa (G4, P1A); 6, strain DS-1 (G2, P1B); 7, strain HN-126 (G2, P1B); 8, strain L26 (G12, P1B); 9, strain L27 (G12, P1B); 10, strain M37 (G1, P2); 11, strain 1076 (G2, P2); 12, strain ST-3 (G4, P2); 13, strain McN13 (G3, P2); 14, strain K8 (G1, P3); 15, strain AU-1 (G3, P3); 16, strain O265 (G3, P3); and 17, strain M318 (G3, P3).

RESULTS

Specificity of PCR for the identification of the gene 4 allele

The specificity of PCR with nested oligonucleotides specific to each of the four gene 4 allele was examined by using 16 cultivable HRV strains whose antigenic and genomic properties were characterized well. In the first amplification using two common primers, DNA fragments with 1084 base pairs (bp) were produced from the dsRNA of all the HRVs. Figure 1a shows the DNA products of the same size after the first amplification of 16 HRV with different gene 4 allele. In the second amplification, DNA fragments with the expected different sizes (498 bp for P1A gene 4 allele, 338 bp for P1B gene 4 allele, 745 bp for P2 gene 4 allele, and 911 bp for P3 gene 4 allele) were detected (Fig. 1b). Thus, the specificity of the PCR for gene 4 allele identification was confirmed.

Table 2. Gene 4 alleles of rotaviruses in stool specimens as determined by PCR

Source	Gene 4 allele corresponding to						Total (%)
	P1A	P1A + P1B	P1B	P2	P3	ND*	
Sapporo	104	0	8	0	0	2	114 (98.2)
Ehime	21	1	11	0	1	2	36 (94.4)
Chiang Mai	40	1	5	2	0	1	49 (98.0)
Total	165	2	24	2	1	5	199 (97.5)

* ND, not determined.

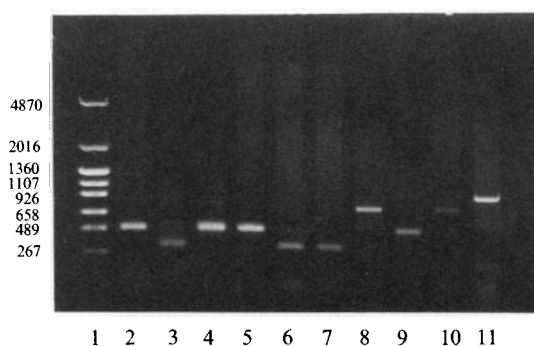


Fig. 2. Electrophoretic analysis of the PCR products after second amplification with stool samples. Lanes: 1. molecular weight marker; 2. stool sample Mc300 (assigned to P1A gene 4 allele); 3. Mc311 (P1B); 4. Mc314 (P1A); 5. Mc322 (P1A); 6. Mc324 (P1B); 7. Mc349 (P1B); 8. Mc359 (P2); 9. Mc361 (P1A); 10. Mc365 (P2); 11. 88-318 (P3).

Identification of the gene 4 allele of HRV in stools by PCR

The PCR was applied to HRV in stool specimens from patients with diarrhoea. In a total of 199 stool specimens positive for group A HRV collected in Japan and Thailand, the gene 4 allele of 194 (97.5%) samples could be classified; 165 were grouped to P1A gene 4 allele, 24 were P1B gene 4 allele, 2 were P2 gene 4 allele, and 1 was P3 gene 4 allele (Table 2). Figure 2 shows representative results. In all the three districts where the stool samples were obtained, samples with P1A gene 4 allele were the most prevalent, followed by samples with P1B gene 4 allele. Two samples produced two DNA bands corresponding to P1A and P1B gene 4 alleles (data not shown). These samples appear to contain two HRV strains because their RNA profiles in polyacrylamide gel electrophoresis of the sample exhibited a mixture of short and long RNA patterns and their G serotype was G1 + G2 (see below).

Correlation between gene 4 allele and G serotype

G serotype of the 199 samples was determined primarily by ELISA using G serotype-specific MAbs. The samples whose G serotype could not be assigned by the ELISA were subjected to PCR and their G serotype was assessed indirectly. G serotype of 194 (97.5%) of the 199 samples could be assigned. Correlation between gene 4 allele and G serotype was examined (Table 3). One hundred and sixty-three samples with G1, G3 or G4 serotype specificity had P1A gene 4 allele, and 23 samples with G2 serotype specificity showed P1B gene 4 allele. In contrast, one sample (from Sapporo) with G1 serotype specificity possessed P1B

Table 3. *Correlation between G serotype and gene 4 allele in the rotaviruses in stool samples as determined by PCR and ELISA*

G serotype	Gene 4 allele corresponding to						Total
	P1A	P1A + P1B	P1B	P2	P3	ND*	
1	152	0	1	2	0	4	159
1 + 2	0	2	0	0	0	0	2
2	0	0	23	0	0	0	23
3	9	0	0	0	1	0	10
4	2	0	0	0	0	0	2
ND	2	0	0	0	0	1	3
Total	165	2	24	2	1	5	199

* ND, not determined.

gene 4 allele. Two samples with P1A + P1B gene 4 allele showed G1 + G2 serotype. G serotype of two samples with P2 gene 4 allele was G1, and one sample with P3 gene 4 allele had G3 serotype specificity.

DISCUSSION

The necessity of the surveys on the distribution of VP4 (P) serotype of HRV worldwide has been increasingly recognized, because VP4 appears to play a role in eliciting protective immune response. However, it is quite difficult to determine the P serotype of HRVs in stool samples by serological assays. As an alternative, genetic analysis to identify the gene 4 allele seems to be effective for predicting P serotype, because the correlation between gene 4 alleles and P serotypes of HRV is well established. The amino acid sequences of the VP8 fragment of VP4 carry strain-specific or P serotype-specific epitopes, while cross-reactive neutralization epitopes are located on the VP5 fragment of VP4 [23–25]. PCR, using the nucleotide sequences in the hyper-variable region on the VP8 which encode P serotype-specific epitopes, is appropriate for predicting P serotype which corresponds to gene 4 allele. Using cultivable HRV strains whose serological and genomic properties have been characterized, the specificity of PCR in assigning P serotype was confirmed in the present study. Although the sensitivity of the PCR was not quantified in this study, the high determination rate of > 95% suggests the high sensitivity of the method. In particular, the use of the RNAID kit was found to be extremely effective compared to several other methods we tested [17; Taniguchi and colleagues, unpublished data], and the RNAID procedure also increased the sensitivity of PCR-typing of G serotype.

In general, there is good correlation between G serotype and P serotype specificities: HRV strains with P1A have G1, G3, G4, or G9 serotype specificity, strains with P1B have G2 or G12 serotype, strains with P2 have G1–G4, and strains with P3 specificity have G1 or G3 serotype. As expected from extensive surveys on G serotype distribution worldwide which show the highest prevalence of G1 serotype, reports on the distribution of gene 4 alleles have described that P1 serotype is the most prevalent, and that the strains with P2 and P3 were isolated in a small number [17, 26]. Similarly, we detected the strains with P1 gene 4 allele in the highest frequency (85.1%). We also detected one strain with unusual

combination of G serotype and gene 4 allele (G1 and P1B gene 4 allele) and three minor strains with P2 or P3 gene 4 allele. Further characterization of these strains, especially of the two symptomatic strains with P2 gene 4 allele which have been usually isolated from asymptomatic infections would be of interest.

In addition to the four gene 4 alleles (or P serotypes), the strains with distinct VP4 sequence (represented by 69M) have been described [27]. Inclusion of the primer corresponding to the fifth P serotype may also be necessary. Furthermore, by comparative analysis of VP4 sequences of various animal rotavirus strains, the presence of at least 12 more gene 4 alleles (or VP4 genotypes) was suggested in animal rotaviruses [28, 31, 32]. Recently, HRV strains with gene 4 alleles specific to animal rotaviruses have also been isolated [8, 29–31]. Conversely, animal strains with HRV gene 4 alleles (such as porcine strain Gottfried) also would be detected. More systematic PCR using primers specific to animal rotavirus gene 4 alleles would be useful to understand fully the ecology of rotavirus infections. Some of the strains whose gene 4 allele could not be identified in this study might have such gene 4 allele.

Although the cost per sample is high when tested by PCR, and relatively few samples can be tested per batch, the high specificity and sensitivity of PCR using specific primers will be quite useful as a proxy method to predict rotavirus P serotype.

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REFERENCES

1. Kapikian AZ, Chanock RM. Rotaviruses. In: Fields BN, Knipe DM, Chanock RM, et al. eds. *Virology*. New York: Raven Press, 1990: 1353–404.
2. Estes M, Cohen J. Rotavirus gene structure and function. *Microbiol Rev* 1989; **53**: 410–49.
3. Kapikian AZ, Flores J, Hoshino Y, et al. Rotavirus: the major etiologic agent of severe infantile diarrhea may be controllable by a 'Jennerian' approach to vaccination. *J Infect Dis* 1986; **153**: 815–22.
4. Hoshino Y, Sereno MM, Midthun K, Flores J, Kapikian AZ, Chanock RM. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. *Proc Natl Acad Sci USA* 1985; **82**: 8701–4.
5. Urasawa S, Urasawa T, Wakasugi F, et al. Presumptive seventh serotype of human rotavirus. *Arch Virol* 1990; **113**: 279–82.
6. Beards G, Xu L, Ballard A, Desselberger U, McCrae MA. A serotype 10 human rotavirus. *J Clin Microbiol* 1992; **30**: 1432–5.
7. Urasawa S, Hasegawa A, Urasawa T, et al. Antigenic and genetic analysis of human rotaviruses prevailing in Chiang Mai, Thailand: evidence for a close relationship between human and animal rotaviruses. *J Infect Dis* 1992; **166**: 227–34.
8. Das M, Dunn SJ, Woode GN, Greenberg HB, Rao D. Both surface proteins (VP4 and VP7) of an asymptomatic neonatal rotavirus strain (I321) have high levels of sequence identity with the homologous proteins of a serotype 10 bovine rotavirus. *Virology* 1993; **194**: 374–9.
9. Taniguchi K, Urasawa T, Morita Y, Greenberg HB, Urasawa S. Direct serotyping of human rotavirus in stools by an enzyme-linked immunosorbent assay using serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies to VP7. *J Infect Dis* 1987; **155**: 1159–66.
10. Urasawa S, Urasawa T, Taniguchi K, et al. Survey of human rotavirus serotypes in different locales in Japan by enzyme-linked immunosorbent assay with monoclonal antibodies. *J Infect Dis* 1989; **160**: 44–51.

11. Woods PA, Gentsch J, Gouvea V, et al. Distribution of serotypes of human rotavirus in different populations. *J Clin Microbiol* 1992; **30**: 781–5.
12. Gorziglia M, Larralde G, Kapikian AZ, Chanock RM. Antigenic relationships among human rotaviruses as determined by outer capsid protein VP4. *Proc Natl Acad Sci USA* 1990; **87**: 7155–9.
13. Li B, Larralde G, Gorziglia M. Human rotavirus K8 strain represents a new VP4 serotype. *J Virol* 1993; **67**: 617–20.
14. Matsui SM, Offit PA, Mackow ER, Benfield DA, Shaw RD, Greenberg HB. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralization domain of VP7 and VP8 fragment of VP4. *J Clin Microbiol* 1989; **27**: 780–2.
15. Gorziglia M, Green K, Nishikawa K, et al. Sequence of the fourth gene of human rotaviruses recovered from asymptomatic or symptomatic infections. *J Virol* 1988; **62**: 2978–84.
16. Taniguchi K, Nishikawa K, Urasawa T, et al. Complete nucleotide sequence of the gene encoding VP4 of a human rotavirus (strain K8) which has unique VP4 neutralization epitopes. *J Virol* 1989; **63**: 4101–6.
17. Gentsch JR, Glass RI, Woods P, et al. Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J Clin Microbiol* 1992; **30**: 1365–73.
18. Taniguchi K, Urasawa T, Kobayashi N, Gorziglia M, Urasawa S. Nucleotide sequence of VP4 and VP7 genes of human rotaviruses with subgroup I specificity and long RNA pattern: implication for new G serotype specificity. *J Virol* 1990; **64**: 5640–4.
19. Urasawa T, Taniguchi K, Kobayashi N, et al. Antigenic and genetic analyses of human rotavirus with dual subgroup specificity. *J Clin Microbiol* 1990; **28**: 2837–41.
20. Isegawa Y, Nakagomi O, Nakagomi T, Ueda S. A VP4 sequence highly conserved in human rotavirus strain AU-1 and feline rotavirus strain FRV-1. *J Gen Virol* 1992; **73**: 1939–46.
21. Taniguchi K, Wakasugi F, Pongsuwanna Y, et al. Identification of human and bovine rotavirus serotypes by polymerase chain reaction. *Epidemiol Infect* 1992; **28**: 276–82.
22. Gouvea V, Glass RI, Woods P, et al. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J Clin Microbiol* 1990; **28**: 276–82.
23. Mackow ER, Shaw RD, Matsui SM, Vo PT, Dang MN, Greenberg HB. Characterization of the rhesus rotavirus VP3 gene: location of amino acids involved in homologous and heterologous rotavirus neutralization and identification of a putative fusion region. *Proc Natl Acad Sci USA* 1988; **85**: 645–9.
24. Taniguchi K, Maloy WL, Nishikawa K, et al. Identification of cross-reactive and serotype-2 specific neutralization epitopes on VP3 of human rotavirus. *J Virol* 1988; **62**: 2421–6.
25. Larralde G, Li BG, Kapikian AZ, Gorziglia M. Serotype-specific epitope(s) present on the VP8 subunit of rotavirus VP4 protein. *J Virol* 1991; **65**: 3213–18.
26. Steele DA, Garcia D, Sears J, Gerna G, Nakagomi O, Flores J. Distribution of VP4 gene alleles in human rotaviruses by using probes to the hyperdivergent region of the VP4 gene. *J Clin Microbiol* 1993; **31**: 1735–40.
27. Qian Y, Green KY. Human rotavirus strain 69M has a unique VP4 as determined by amino acid sequence analysis. *Virology* 1991; **182**: 407–12.
28. Taniguchi K, Urasawa T, Urasawa S. Independent segregation of the VP4 and the VP7 genes in bovine rotaviruses as confirmed by VP4 sequence analysis of G8 and G10 bovine rotavirus strains. *J Gen Virol* 1993; **74**: 1215–21.
29. Gentsch JR, Das BK, Jiang B, Bhan MK, Glass RI. Similarity of the VP4 protein of human rotavirus strain 116E to that of the bovine B223 strain. *Virology* 1993; **194**: 424–30.
30. Li B, Clark HR, Gouvea V. Nucleotide sequence of the VP4-encoding gene of an unusual human rotavirus (HCR3). *Virology* 1993; **196**: 825–30.
31. Taniguchi K, Urasawa T, Urasawa S. Species specificity and interspecies relatedness in VP4 genotypes demonstrated by VP4 sequence analysis of equine, feline and canine rotavirus strains. *Virology*. In press.
32. Sereno M, Gorziglia M. The outer capsid protein VP4 of murine rotavirus Eb represents a tentative new P type. *Virology*. In press.