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Full Genomic Analysis of a Porcine–Bovine Reassortant G4P[6] Rotavirus Strain R479 Isolated From an Infant in China

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During the 2004 surveillance of rotaviruses in Wuhan, China, a G4P[6] rotavirus strain R479 was isolated from a stool specimen collected from a 2-year-old child with diarrhea. The strain R479 had an uncommon subgroup specificity I + II, and analysis of the VP6 gene suggested that it was related to porcine rotaviruses. In the present study, full-length nucleotide sequences of all the RNA segments of R479 were determined and analyzed phylogenetically to identify the origin of individual RNA segments. According to the rotavirus genotyping system based on 11 RNA segments, the genotype of R479 was expressed as G4-P[6]-I5-R1-C1-M1-A1-N1-T7-E1-H1. This genotype includes the porcine-like VP6 genotype (I5) and bovine-like NSP3 genotype (T7). Phylogenetic analysis revealed that R479 genes encoding VP1, VP2, VP3, VP6, VP7, VP8*, NSP1, NSP4, and NSP5 were more closely related to those of porcine rotaviruses than human or other animal rotaviruses. In contrast, it was remarkable that the NSP3 gene of R479 was genetically closely related to only a bovine rotavirus strain UK. The NSP2 gene of R479 was also unique and clustered with only the G5P[8] human strain IAL28 and G3P[24] simian strain TUCH. These results suggested that R479 may be a reassortant virus having the NSP3 gene from a bovine rotavirus in the genetic background of a porcine rotavirus, with an NSP2 gene related to the porcine-human reassortant strain IAL28. To our knowledge, R479 is the first porcine–bovine reassortant rotavirus isolated from a human. ***J. Med. Virol. 82:1094–1102, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: rotavirus; full genome; reassortant; human; porcine

INTRODUCTION

Group A rotavirus is a major cause of acute gastroenteritis in children in both developing and developed countries, being the single most important cause of deaths associated with diarrhea among children [Bresee et al., 2005]. Rotaviruses are also present in various mammals and birds as an intestinal pathogen. A rotavirus has 11 segments of double-stranded RNA (dsRNA) as a genome, which encodes six structural proteins (VPs) and six nonstructural proteins (NSPs). Because of the segmented nature of the genome, reassortment is one of the major processes of genetic evolution of rotaviruses. Neutralization antigens of rotaviruses are present on the two structural proteins VP7 and VP4, which constitute the outermost layer of the rotavirus particle. Based on the VP7 and VP4 gene sequences, group A rotavirus has been classified genetically into a G type and P type, respectively [Estes and Kapikian, 2007]. At least 23 G types and 31 P types have been described so far in rotaviruses from humans and various animal species [Matthijnssens et al., 2008b; Abe et al., 2009; Schumann et al., 2009; Ursu et al.,

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2009]. Among these, some specific G and P types are dominant in individual animal species. In human rotaviruses, genotypes G1, G2, G3, G4, or G9 which are combined with P[4], P[6], or P[8] have been reported on frequently worldwide [Santos and Hoshino, 2005], while G12 is becoming increasingly common [Rahman et al., 2007]. G types of porcine rotaviruses are assigned mostly to G3, G4, G5, G9, or G11, while G6, G8, and G10 are detected commonly in bovine rotaviruses [Martella et al., 2005].

In the previous surveillance of human rotaviruses in Wuhan, China, during the period of 2000–2006, G3P[8] was detected most frequently, followed by G1P[8] [Wang et al., 2007]. In contrast, G4P[6] was identified in only one fecal specimen from which a rotavirus strain R479 was isolated by tissue culture. The strain R479 showed unusual dual subgroup specificity, I + II. Phylogenetic analysis of the VP6 gene indicated that R479 did not belong to the main lineages of subgroup I- or II-human rotaviruses, but clustered with many porcine rotaviruses. Similarly, the VP7 gene of R479 was divergent from most G4 human rotaviruses. These findings suggested that R479 may not have originated from humans, but was related to porcine or other animal rotaviruses. Although the P[6] rotavirus has been associated with symptomatic and asymptomatic infection in humans, P[6] is also regarded as a major P type in porcine rotaviruses [Martella et al., 2006a,b]. Phylogenetic analysis of P[6]-VP8* genes indicated the existence of (1) human lineages, (2) porcine lineages, and (3) lineages containing both human and porcine rotaviruses [Martella et al., 2006a; Nguyen et al., 2007; Li et al., 2008]. Therefore, some P[6] rotaviruses are considered to be distributed commonly to humans and swine, suggesting a relatedness of R479 to porcine rotaviruses.

Among the human rotaviruses, which caused symptomatic diarrhea in humans, viruses of putative porcine origin or porcine-human reassortant rotaviruses have been reported mostly in developing countries [Nguyen et al., 2007; Ahmed et al., 2007; Esona et al., 2009; Bányai et al., 2009; Chitambar et al., 2009]. However, while these reports were based on phylogenetic analysis of several important viral genes, evidences from whole genomic information have been presented for only a few strains [Varghese et al., 2004, 2006; Mukherjee et al., 2009; Bányai et al., 2009]. In the present study, we present a full genomic and full-length sequence characterization of the strain R479 isolated in China, to elucidate the exact origin of all the gene segments. In this study, the newly proposed genotyping system based on 11 RNA segments [Matthijnssens et al., 2008a,b] was employed to describe the whole genomic profile of R479, together with phylogenetic analysis to specify lineage within a genotype for each gene. The VP6 and VP7 gene sequences of R479 determined in the previous study were also reanalyzed, together with more gene sequence data reported recently, to determine phylogenetic relatedness to other rotaviruses. The results indicated that R479 may be the first porcine–bovine reassotant rotavirus that caused symptomatic infection in an infant.

MATERIALS AND METHODS

Virus Strain

Rotavirus A strain R479 was detected in a fecal specimen collected from a 2-year-old male child admitted with acute diarrhea at the Renming Hospital of Wuhan University in Wuhan, China, in December 2004. This strain was isolated by tissue culture in MA-104 cells. R479 showed a long RNA pattern, which was distinct from those of G3 and G1 rotaviruses detected in the same period in Wuhan, as described in the previous report [Wang et al., 2007]. The strain R479 was classified as G4P[6], VP6 genotype II, and NSP4 genotype B by multiplex semi-nested RT-PCR, while the subgroup specificity was determined as I + II by ELISA with monoclonal antibodies [Wang et al., 2007].

RT-PCR and Nucleotide Sequencing

Viral dsRNA was extracted from the stool suspension using guanidine isothiocyanate and an RNaid kit (BIO 101, Inc., La Jolla, CA), and RT-PCR was performed using the conditions as described previously [Ahmed et al., 2004]. Some portions of the VP1–VP4 genes which overlapped with each other and covered whole gene sequence were amplified by RT-PCR using primers designed in this study based on sequences of the Wa strains as a reference (primer sequences not shown). Amplification of NSP1–NSP5 gene sequences were achieved by using primer pairs that have been described previously [Kojima et al., 2000; Varghese et al., 2004; Alam et al., 2008].

PCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Inc., Madison, MI). Sequencing reactions were performed with fluorescent dideoxy chain termination chemistry using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). DNA sequences were determined by using the ABI Prism 3100 genetic analyzer (Applied Biosystems).

Sequence Analysis

A nucleotide BLAST search was performed using the NCBI (National Center for Biotechnology Information, National Institute of Health, Bethesda, MD) website (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) to find similar rotavirus gene sequences. Genotyping numbers of individual RNA segments were assigned based on the cut-off values of sequence identities as proposed previously [Matthijnssens et al., 2008a,b]. GENETYX-Win Version 5.1 (Software Development, Tokyo, Japan) was used to perform pairwise alignment and calculate the sequence identity of viral genes from different strains. Phylogenetic analysis was performed with MEGA software version 4.1 based on the neighbor-joining method. Phylogenetic distances were measured by the Kimura

two-parameter model, and phylogenetic trees were supported statistically by bootstrapping with 1,000 replicates.

Accession Numbers of Rotavirus Genes

The nucleotide sequences of R479 genes encoding VP1–VP4, NSP1–NSP5 analyzed in this study were deposited in the GenBank database under accession numbers GU189551–GU189559, respectively.

RESULTS AND DISCUSSION

Full-length nucleotide sequences of all the RNA segments revealed that strain R479 had a G4-P[6]-I5-R1-C1-M1-A1-N1-T7-E1-H1 genotype, including the porcine-like VP6 genotype (I5) and bovine-like NSP3 genotype (T7) and most of the other genotypes (R1, C1, M1, A1, N1, E1, and H1) related to both human Wa-like rotaviruses or porcine rotaviruses.

VP7, VP4, and VP8* Genes

Phylogenetic analysis of the VP7 genes of G4 rotaviruses indicated that R479 was grouped into lineage G4f, which includes porcine rotavirus strains from Thailand (e.g., CMP77) and some human rotaviruses in Vietnam (e.g., VN592/2003) (Fig. 1A). Most of the common G4 human rotaviruses were clustered in the G4a lineage. The R479-VP7 gene exhibited high sequence identities (>90%) to human G4 strains E931 (China) and VN846/2003 (Vietnam), as well as porcine rotavirus strains (e.g., CMP166) (Table I). It was notable that the R479-VP7 gene sequence was identical to G4 human rotavirus strains reported recently in Vietnam (e.g., VN-4). Although information on these G4 viruses is not available at present, other Vietnamese G4 strains in G4f lineage such as VN592/2003 were considered, through analysis of the VP8* gene, to have originated from swine [Nguyen et al., 2007], that is, a porcine-like human rotavirus. These findings indicated that the VP7 gene of R479 was closely related to those of porcine rotaviruses in Southeast Asia.

In the dendrogram of full-length VP4 gene sequences, R479 was located in an isolated branch distinct from clusters of many human rotaviruses and a porcine rotavirus strain Gottfried, which may have been due to the lack of a sufficient number of full-length VP4 gene sequence (data not shown). However, clearer genetic relatedness of the VP4 gene could be estimated by analysis of the VP8*-encoding portion of the VP4 gene, which had been sequenced for a number of strains. Figure 1B shows a dendrogram of VP8* genes with lineage numbers described by Martella et al. [2006a] previously. The strain R479 was located in the lineage Ic, which included porcine strains (e.g., 134/04-11), human rotavirus strains in Vietnam (e.g., KH210 and VN592/2003) and strain mcs/13-07 in India showing high-level sequence identities to these strains (Table I). However, R479 was distinct from lineage Ia, which contained most of the common human rotaviruses. The strain KH210 (G5P[6]) and the strain mcs/13-07 (G9P[6]) were inferred to be a porcine rotavirus which had transmitted to humans [Ahmed et al., 2007; Mukherjee et al., 2009]. Therefore, VP8* of R479 was considered to be close to porcine rotaviruses genetically.

VP6 Gene

The VP6 gene, which had been characterized previously was analyzed again, by using new genotyping system and phylogenetic analysis with more recent sequence data. Strain R479 was assigned to the I5 genotype containing mostly porcine rotaviruses, while common human rotaviruses were classified into I1 (subgroup II) and I2 (subgroup I) (Fig. 1C). Within the I5 genotype, R479 was located in lineage I5c, which included porcine rotaviruses and human rotavirus strains such as LL3354 (G5P[6]) in China and RMC321(G9P[19]) in India having relation to the porcine rotavirus [Li et al., 2008; Varghese et al., 2004]. Whole genomic characterization indicated that RMC321 may be a reassortant virus having the VP7 gene of human origin and all other genes derived from the porcine rotavirus [Varghese et al., 2004, 2006]. Thus, I5c may represent one of the lineages of porcine rotaviruses. The highest sequence identities of the R479-VP6 gene were found with porcine strains 4F (93.8%), RU172 (93.7%), and human strain RMC321 (93.7%) (Table II).

VP1–VP3, NSP1–NSP5 Genes

The VP1 gene of R479 was assigned into the R1 genotype and clustered with the porcine rotavirus strain HP140 and porcine-like human strain mcs/13-07 in the lineage R1b, while common human rotaviruses were found in other lineages (Fig. 1D). The highest sequence identities were found with mcs/13-07 (94.6%) and HP140 (93.4%) (Table II). The VP2 gene of R479 belonged to the C1 genotype and clustered with the porcine HP140 strain and porcine-like human rotaviruses (mcs/13-07, RMC321) in the lineage C1b (Fig. 1E). Most human rotaviruses were grouped into C1a or C2. The R479-VP3 gene was assigned to the M1 genotype and clustered with strains RMC321 and mcs/13-07 (lineage M1c) exhibiting the highest identities to these strains (94–95%) (Fig. 1F, Table II), whereas most human rotaviruses were grouped into lineage M1a or genotype M2. These findings indicated a close relatedness of R479-VP1–VP3 genes to porcine rotavirus genes.

The R479-NSP1 gene clustered with porcine strains (4F and 4S) and porcine-like human strain RMC321 in the lineage A1c (Fig. 2A).

The NSP2 gene of R479 clustered with only two strains, human rotavirus IAL28 and simian rotavirus TUCH (lineage N1b, Fig. 2B), while most human rotaviruses were grouped into the lineage N1a or genotype N2, and porcine viruses were assigned to N1a. Strain IAL28 isolated in Brazil had a G5P[8] genotype and was considered to be a reassortant having the VP7 gene from the porcine virus and ten other genes

from the human rotavirus of the Wa genogroup [Heiman et al., 2008]. However, it was also revealed that porcine rotaviruses were related to human Wa-like rotaviruses genetically, with common genotypes R1-C1-M1-A1-N1-T1-E1-H1 for VP1-3 and NSP1-5 genes, respectively [Matthijnssens et al., 2008b]. Therefore, the origin of the IAL28-NSP2 gene, whether human or porcine, is not still evident. On the other hand, simian strain TUCH isolated in US [McNeal et al., 2005] had the genotype G3P[24]-I9-R3-C3-M3-A9-N1-T3-E3-H6 which included human AU-1-like genotypes (R3,C3, M3, T3, E3), simian or simian–lapine genotypes A9 and H6, and a unique type I9 (Table II). The genotype N1 is related to the human Wa-like rotavirus; therefore, the TUCH NSP2 gene does not appear to be indigenous to the simian rotavirus, although more genetic data are required for judgment of its origin. Accordingly, in the present study, presumptive origin of the R479-NSP2

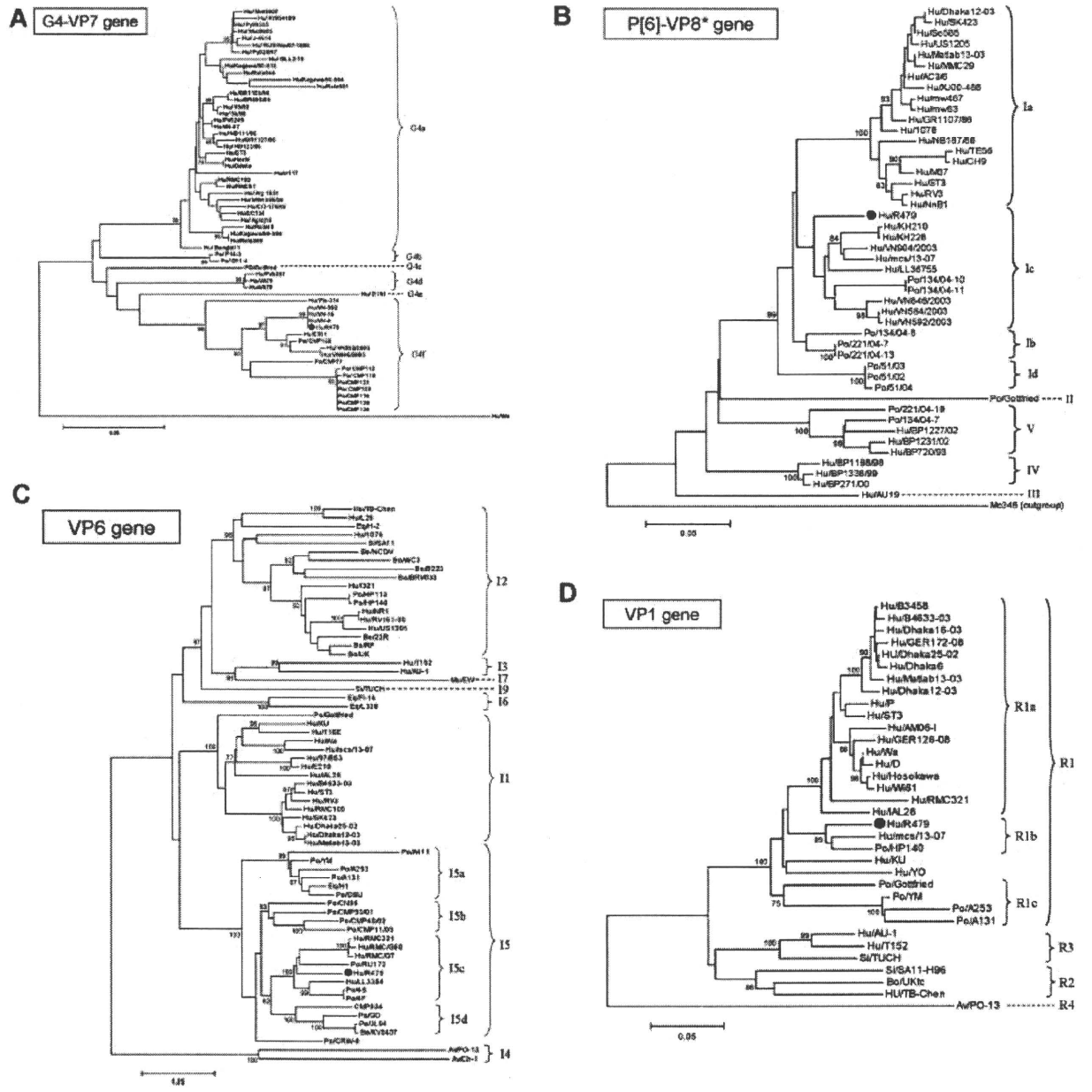

Fig. 1. Phylogenetic dendrograms of the genome segments encoding G4-VP7 (**A**), VP8* region of P[6]-VP4 (**B**), VP6 (**C**), VP1 (**D**), VP2 (**E**), VP3 (**F**), constructed by neighbor-joining method with MEGA.4 program. Solid circle indicates the strain R479. Name of each rotavirus strain is described with animal of its origin (Av, avian; Bo, bovine; Eq, equine; Hu, human; Mu, murine; Po, porcine; Si, simian). The genotypes and/or lineages within a genotype are indicated at the right side. Reference sequences used in the analysis were obtained from the GenBank database. Variation scale (substitution per site) is indicated at the bottom. Percent bootstrap support is indicated by the values at each node, and the values <75 are omitted.

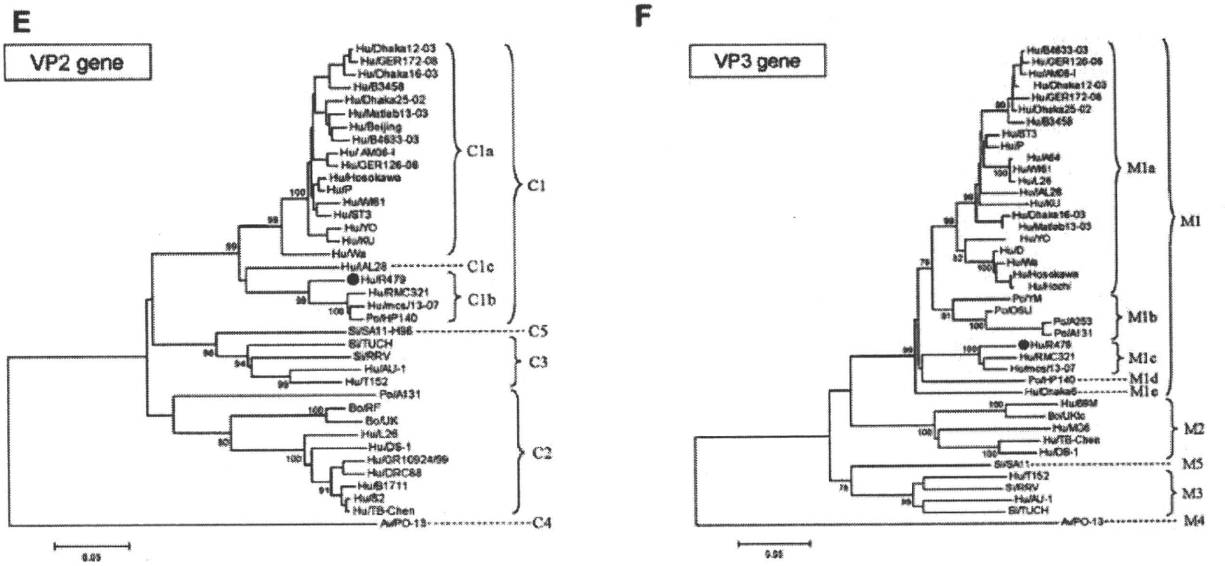

Fig. 1. (*Continued*)

gene was described as either human or swine, because it was not definitive.

It was remarkable that the NSP3 gene of R479 was grouped into T7 and clustered only with bovine rotavirus strain UK (Fig. 2C). The R479-NSP3 gene showed the highest identity (91.6%) to UK, with only lower identities (<86%) to any human and porcine rotaviruses.

The NSP4 gene of R479, grouped into genotype E1, clustered in the lineage E1c with only porcine strains HP113, HP140, and RU172 (Fig. 2D). The R479-NSP5 gene (genotype H1) was located in a lineage (H1d) isolated from human and porcine rotaviruses in the genotype H1 (Fig. 2E). However, extremely high sequence identities (>97%) were found with porcine strains (HP113 and HP140) and porcine-like human rotavirus mcs/13-07.

Gene Constellation of 11 RNA Segments

Genotypes/lineages and sequence identities of individual R479 genes to those of representative rotavirus strains are summarized in Table II. Taken together with the above-mentioned findings of genotypes and lineages

TABLE I. VP7 and VP8* Gene Sequence Identities of R479 to G4 and P[6] Rotavirus Strains

VP7 gene			VP8 gene		
Strain	G4 lineage	Identity to R479 (%)	Strain	P[6] lineage	Identity to R479 (%)
Mvd9907	G4a	87.6	ST3	Ia	91.1
PyO2SR7	G4a	86.0	US1205	Ia	91.7
Kagawa/90-554	G4a	85.3	Dhaka12-03	Ia	91.9
ST3	G4a	87.1	RV3	Ia	91.5
RMC61	G4a	86.6	TE56	Ia	88.4
Bangla71	G4a	86.0	134/04-8	Ib	92.6
P14-3	G4b	86.0	221/04-13	Ib	94.4
O11-4	G4b	85.2	KH210	Ic	93.0
Gottfried	G4c	87.9	VN904/2003	Ic	94.5
PV5257	G4d	87.6	mcs/13-07	Ic	93.5
VA79	G4d	87.6	134/04-11	Ic	93.1
D151	G4e	84.8	VN592/2003	Ic	94.0
Fin-314	G4f	90.6	51/03	Id	90.5
VN-4	G4f	100	51/04	Id	90.3
E931	G4f	96.1	Gottfried	II	82.3
CMP166	G4f	94.9	AU19	III	81.0
VN846/2003	G4f	95.0	BP1198/98	IV	86.5
CMP77	G4f	94.0	BP271/00	IV	86.5
CMP112	G4f	92.9	221/04-19	V	85.7
CMP134	G4f	91.8	BP720/93	V	84.7

TABLE II. Comparison of R479 Viral Protein Genes With Those of Human and Animal Rotaviruses

Strain	Host	G-type	P-type	Genotypes of viral protein genes and nucleotide sequence identities (%) to R479								
				VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Wa	Human	G1	P[8]	I1 83.3	R1a 89.0	C1a 89.4	M1a 87.8	A1a 82.1	N1a 88.7	T1 84.7	E1a 90.5	H1b 94.1
TB-Chen	Human	G2	P[4]	I2 79.1	R2 78.2	C2 79.5	M2 76.6	A2 75.6	N2 83.8	T2 79.4	E2 81.8	H2 82.9
AU-1	Human	G3	P[9]	I3 78.9	R3 80.0	C3 79.9	M3 78.0	A3 65.9	N3 81.5	T3 78.2	E3 81.3	H3 88.2
ST3	Human	G4a	P[6]-Ia	I1 84.2	R1a 88.9	C1a 88.6	M1a 86.8	A1b 82.2	N1a 87.4	T1 85.3	E1a 89.7	H1a 95.3
IAL28	Human	G5	P[8]	I1 83.0	R1a 88.8	C1c 87.7	M1a 86.8	A1b 82.0	N1b 92.1	T1 84.0	E1a 86.2	H1b 94.6
RMC321	Human	G9	P[19]	I5c 93.7	R1a 86.8	C1b 93.7	M1c 94.2	A1c 94.3	N1a 88.1	T1 84.9	E1 92.8	H1c 97.3
mcs/13-07	Human	G9	P[6]-Ic	I1 81.9	R1b 94.6	C1b 93.4	M1c 95.1	A8 78.0	N1a 87.5	T1 84.7	E1a 90.5	H1a 93.9
VN904/2003	Human	G9	P[6]-Ic								E9 86.4	H1a 96.5
CMP034	Porcine	G2	P[27]	I5d 91.0								
A131	Porcine	G3	P[7]	I5a 87.5	R1c 84.3	C2 76.2	M1b 85.3	A1a 78.7	N1a 83.4	T1 80.8	E1 85.2	H1a 89.6
4F	Porcine	G3	P[19}	I5c 93.8				A1c 94.7				
Gottfried	Porcine	G4c	P[6]-II	I1 83.3	R1c 85.9			A8 77.7				
134/04-11	Porcine	G4c	P[6]-Ic									H1a 96.2
SB1A	Porcine	G4	P[7]						N1a 88.8	T1 85.6	E1 88.1	H1a 96.7
HP113	Porcine	G6	P[13]	I2 79.0							E1c 97.1	H1c 97.4
HP140	Porcine	G6	P[13]	I2 78.9	R1b 93.4	C1b 93.4	M1d 88.1				E1c 97.1	H1c 97.3
YM	Porcine	G11	P[7]	I5a 90.0	R1c 86.2		M1b 87.5	A8 78.2			E1 93.5	H1a 96.7
RU172	Porcine	G12	P[7]	I5c 93.7							E1c 97.2	H1a 95.9
UK	Bovine	G6	P[5]	I2 79.6	R2 78.3	C2 78.4	M2 75.7	A3 67.3	N2 85.6	T7 91.6	E2 83.6	H3 88.0
TUCH	Simian	G3	P[24]	I9 80.2	R3 80.3	C3 79.5	M3 77.2	A9 55.2	N1b 91.1	T3 78.2	E3 77.7	H6 88.3
R479	Human	G4f	P[6]-Ic	I5c	R1b	C1b	M1c	A1c	N1b	T7	E1c	H1
Presumptive origin[a]		Po	Po	Po	Po	Po	Po	Po	Po/Hu	Bo	Po	Po

Po, porcine; Hu, human; Bo, bovine.

in each RNA segment, R479 genes encoding VP1–VP4, VP6, NSP1, NSP4, and NSP5 were related to porcine rotaviruses or porcine-like human rotaviruses closely, while the NSP3 gene was the closest to a bovine rotavirus. This conclusion implied that R479 may be a reassortant virus having an NSP3 gene from a bovine strain and all other genes of porcine rotavirus origin. The R479-NSP2 gene may be derived possibly from a human rotavirus, although it is not definitive. To our knowledge, R479 is the first porcine–bovine (or porcine–bovine–human) reassortant rotavirus isolated from an infant with diarrhea. In Wuhan, China, that G4P[6] strain E931, which had a VP7 gene genetically close to R479 (lineage G4f; Fig. 1A), was detected in an infant in 2008 [Wang et al., 2009] suggests the persistence of an R479-like virus in the same area.

It was of note that porcine rotaviruses or porcine-like human rotaviruses, which were close to R479 genetically have been found in eastern India (strains HP140, HP113, RU172, RMC321, and mcs/14-07), Thailand

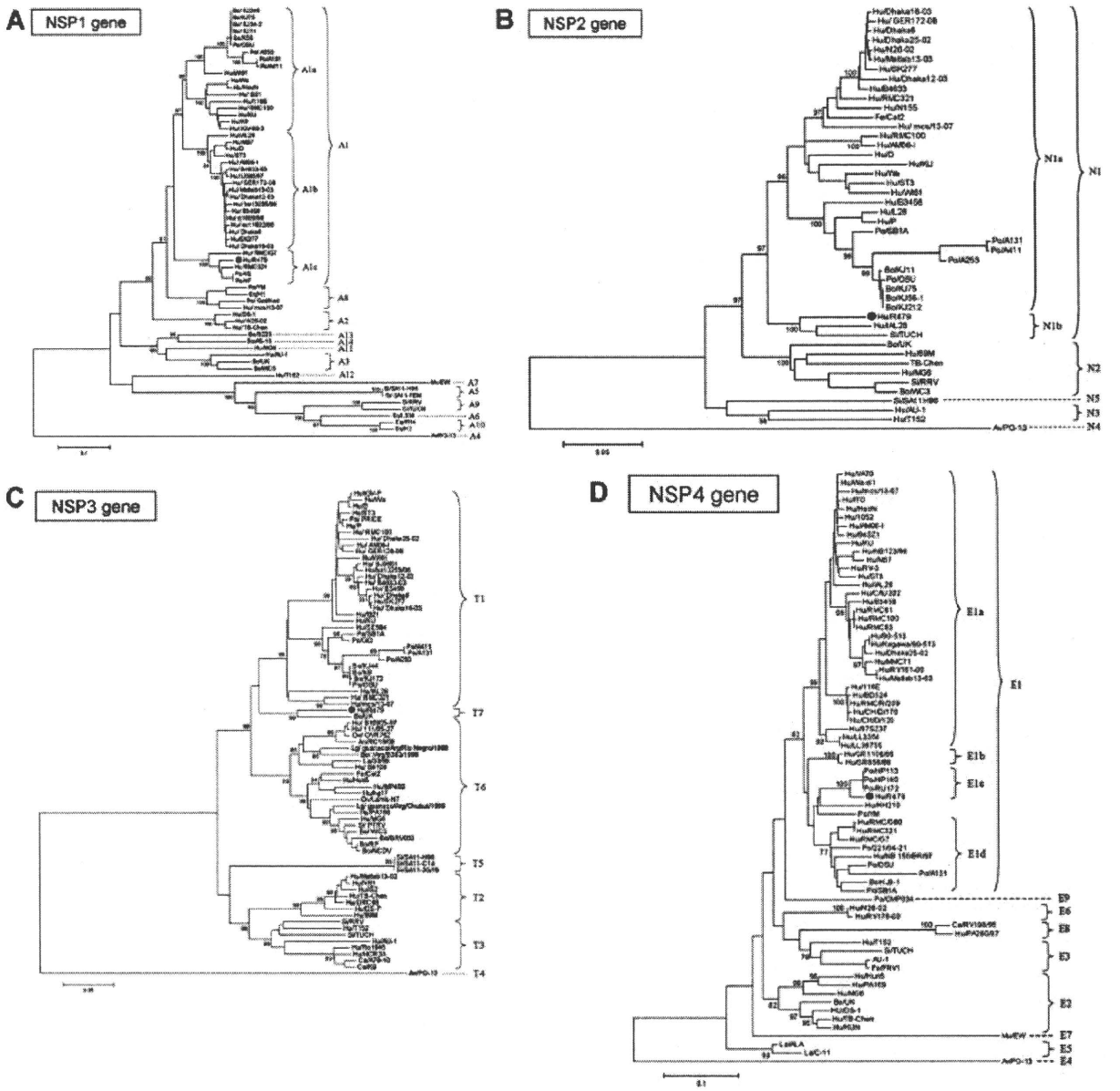

Fig. 2. Phylogenetic dendrograms of the genome segments encoding NSP1 (**A**), NSP2 (**B**), NSP3 (**C**), NSP4 (**D**), NSP5 (**E**), constructed by neighbor-joining method with MEGA.4 program. Solid circle indicates the strain R479. Name of each rotavirus strain is described with animal of its origin (An, antelope; Av, avian; Bo, bovine; Ca, canine; Eq, equine; Fe, feline; Hu, human; La, lapine; Lg, Lama guanicoe; Mu, murine; Ov, ovine; Po, porcine; Si, simian). The genotypes and/or lineages within a genotype are indicated at the right side. Reference sequences used in the analysis were obtained from the GenBank database. Variation scale (substitution per site) is indicated at the bottom. Percent bootstrap support is indicated by the values at each node, and the values <75 are omitted.

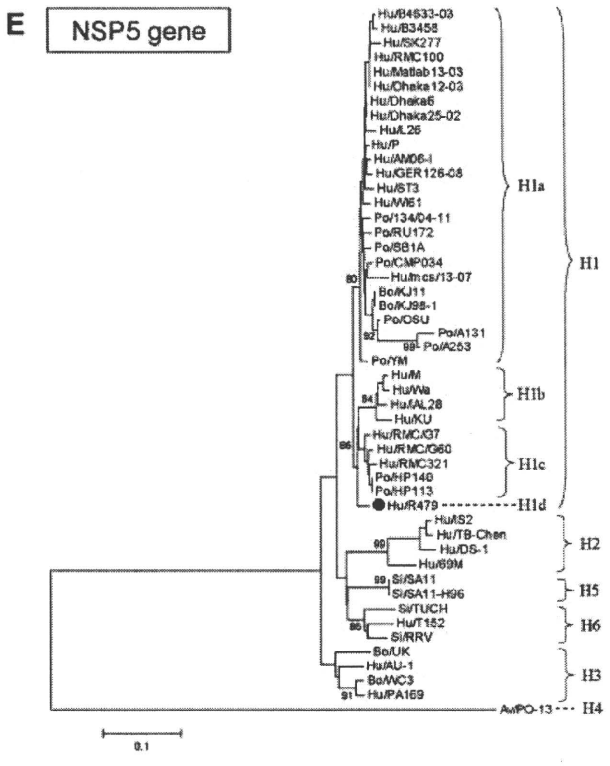

Fig. 2. (*Continued*)

(e.g., strain CMP166), Vietnam (e.g., strain VN904/2003). This may suggest that the strain R479 has originated from porcine rotaviruses, which are distributed to eastern India and Southeast Asia widely, although sufficient sequence information is not available for porcine viruses in this region.

Two reports have described identification of porcine-bovine reassortant rotaviruses in animals. The G5P[1] rotavirus strains KJ44 and KJ75 which were isolated from calves with diarrhea in South Korea have VP3 and VP4 genes of bovine origin in the genetic background of porcine rotaviruses [Park et al., 2006]. The G3P[7] rotavirus strain PP-1 was isolated from calves with diarrhea in UK. The strain PP-1 had porcine-like VP4 and NSP4 and bovine strain UK-like NSP1, and was found to cause symptomatic infection in pigs experimentally [El-Attar et al., 2001]. Therefore, while the genetic constellation of R479 is different from those of KJ44/KJ75 and PP-1, porcine–bovine reassortant R479 is suggested to have infectivity in swine. It is conceivable that R479 may be a naturally occurring reassortant in animals that transmitted to humans subsequently, thereafter undergoing further possible mixed infection with human rotavirus and reassortment of the NSP2 gene. Genetic traits of R479 revealed in this study imply that successive events of co-infection of rotaviruses, reassortment, and interspecies transmission may have occurred in nature. To understand ecological aspects of rotaviruses more accurately, accumulation of more sequence data may be required, especially for animal rotaviruses.

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Method

Identification of P[8]b Subtype in OP354-Like Human Rotavirus Strains by a Modified RT-PCR Method

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SUMMARY: In our previous study, a novel P[8] subtype, i.e., P[8]b was identified for human rotavirus strains MMC38 and MMC71 detected in Bangladesh, of which the P types could not be determined by conventional RT-PCR genotyping methods. In the present study, a modified multiplex RT-PCR method was developed to detect P[8]b as well as common human rotavirus P types. With this method, P[8]b was detected in three strains among the 26 rotavirus specimens which had been judged as mixed P types in the previous study in Bangladesh. The VP4 nucleotide sequences of these strains showed more than 98.9% identities to those of strains MMC38 and MMC71. The newly designed RT-PCR method was considered as useful for identifying P[8]b and avoiding misclassification by the conventional RT-PCR genotyping methods.

INTRODUCTION

Rotavirus, a member of the family *Reoviridae*, has 11 segments of double-stranded RNA encoding six structural proteins and six nonstructural proteins (1). The rotavirus outer capsid consists of two structural proteins VP7 and VP4, which have neutralization antigens and define G and P serotypes, respectively. Based on VP7 and VP4 gene sequences, rotaviruses are classified into G and P genotypes (types), respectively. VP8*, an N-terminal portion of cleavage products of VP4 with trypsin, is highly divergent among rotaviruses and associated with P types (2). The 24 G types and 33 P types of group A rotaviruses have been classified so far (3). In human rotaviruses, the major genotypes are G1, G2, G3, G4, and G9, which are combined with P[4], P[6], and P[8] (4). A large number of epidemiologic studies on human rotavirus conducted to date revealed that predominant G/P types are different depending on countries or regions, and change by year or season (4).

Recently, we have conducted a hospital-based survey of rotaviruses in sporadic diarrheal cases in children and adults in Bangladesh (5). In this study, P genotypes were not identified in the two strains, MMC38 and MMC71, with the RT-PCR developed for P genotyping, which has been commonly used for epidemiologic studies of human rotaviruses (6–8). Therefore, full-length VP4 gene sequences of these strains were determined and analyzed (9). VP4 sequences of MMC38 and MMC71 were genetically distinct from the previously known P genotypes, while relatively close to both P[4] and P[8], the two major P genotypes in human rotaviruses. On the other hand, the VP8* portions of MMC38 and MMC71 showed more than 93.9% nucleotide sequence identity to P[8] variants, i.e., OP354-like P[8] strains (10), being clustered into the same lineage with these strains. Thus, we proposed that the VP4 of these strains should be classified into a subtype of the P[8] genotype (P[8]b) that is distinct from that of common P[8] rotaviruses (P[8]a) (9).

Strain OP354 was reported for the first time in Malawi during 1998–1999 (10). Thereafter, rotavirus strains with OP354-like (P[8]b) VP4 were detected in various countries, e.g., India (11,12), Thailand (13), Vietnam (14), Bangladesh (9), and Finland, suggesting widespread distribution of P[8]b rotaviruses throughout the world. However, to understand the epidemiological nature of P[8]b subtypes in rotaviruses, it was necessary to establish a genotyping method to identify major P types of the human rotavirus, discriminating between P[8]a and P[8]b.

In the present study, we developed a genotyping method to identify P[8]b as well as four other major human P types (P[4], P[6], P[8]a, and P[9]) by a modified multiplex RT-PCR. With this method, we identified additional P[8]b rotaviruses among the Bangladeshi strains which had been classified as mixed P types in an earlier study (5).

MATERIALS AND METHODS

RNA extraction and modified RT-PCR genotyping: Rotavirus RNA was extracted from 10% stool suspension using RNAID kit (BIO101, Inc., La Jolla, Calif., USA) according to the manufacturer's instructions. The 877 bp-cDNA fragment from VP4 gene was amplified RT-PCR as described previously (15) using common primers corresponding to nucleotide sequences of the VP4 gene that are well conserved in human rotavirus

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Table 1. Primer sequences and their positions used for P genotyping by RT-PCR

Name	P genotype	Sequence (5′-3′)	Position	Size of PCR product (bp)	Reference
CON 3		(+) TGGCTTCGCCATTTTATAGACA	11-32	877	(6)
CON 2		(−) ATTTCGGACCATTTATAACC	868-887		(6)
P4-DS-1	P[4]	(−) GCATCCCTACAAGTCTATTACT	488-509	499	This study[1)]
3T-1	P[6]	(−) TGTTGATTAGTTGGATTCAA	259-278	268	(6)
1T-1	P[8]a	(−) TCTACTTGGATAACGTGC	339-356	346	(6)
4T-1	P[9]	(−) TGAGACATGCAATTGGAC	385-402	392	(6)
P8b-MMC38	P[8]b	(−) CTCTTGAGATCTCGGTATTATG	625-646	636	This study[1)]

[1)]: Sequence and position of primers correspond to individual gene sequences deposited in GenBank accession nos. AB118025 (strain DS-1) and EU979379 (strain MMC38).

strains (Table 1). This amplicon was used as a template in a second PCR with a pool of P-type-specific primers to generate fragments with type-specific length (Table 1). The primer mixture selected for the second amplification consisted of primers 1T-1, 3T-1, and 4T-1, described by Gentsch et al. (6), to detect P[8]a, P[6], and P[9] genotypes, respectively, and two newly designed primers P4-DS-1 and P8b-MMC38 specific to the P[4] and P[8]b genotypes, respectively. Sequences of these primers, their position, sizes of each PCR product, and reference strains are indicated in Table 1.

Amplification and sequence analysis of VP4 and VP7 genes: Full-length VP4 and VP7 gene sequences of the strains MMC153, MMC183, and DH389 were determined by RT-PCR and direct sequencing, as described previously (9,15). Sequence analysis and comparison were carried out using the GENETYX-MAC (version 11.2), and multiple sequence alignment and phylogenetic analysis were performed using the MEGA software (version 4.1) with Kimura 2-parameter distances.

Nucleotide sequence accession numbers: The nucleotide sequence data reported in this study were deposited in the GenBank database under the accession numbers GQ869839 (VP4) and GQ869842 (VP7) for strain MMC153, GQ869840 (VP4) and GQ869843 (VP7) for strain MMC183, and GQ869838 (VP4) and GQ869841 (VP7) for strain DH389.

RESULTS

First, the cell culture-adapted reference strains were used to examine the specificity of the modified P-typing method. The results indicated that DNA fragments with the expected different sizes of 268 bp for M37 P[6], 346 bp for KU P[8]a, 392 bp for K8 P[9], 499 bp for DS-1 P[4], and 636 bp for MMC38 and MMC71 P[8]b were amplified (data not shown). When nucleotide sequences of these amplified products were directly determined, these DNA sequences were identical to those of strains representing individual P types (data not shown).

In the previous study, P genotypes of strains MMC38 and MMC71 were not determined as P[4], P[6], P[8], or P[9] by commonly used multiplex RT-PCR P-typing protocols reported (5). However, we observed multiple faint amplicons, i.e., nonspecific products by the RT-PCR generated from the strains MMC38 and MMC71 which might be judged as mixed infection of P[4] and P[6], or P[4] and P[8], by mistake. Therefore, P typing with the newly established RT-PCR method was carried out for 26 stool samples which had been recorded as mixed P types in our previous study in Bangladesh (5), to know whether or not P[8]b is included among them. As a result, P[8]b was identified in three rotavirus samples (strains MMC153, MMC183, and DH389) in which 640 bp-fragment specific to P[8]b was generated (data not shown).

To analyze VP4 genes from the strains MMC153, MMC183, and DH389, full-length VP4 gene sequences of these strains were determined. The VP4 sequences of the three strains were almost identical to each other (99.1–99.2% identity at nucleotide level; 98.8–99.2% identity at amino acid level), and more closely related to those of P[8]b strains MMC38 and MMC71 (98.9–99.6% nucleotide and 98.2–99.2% amino acid sequence identities) than P[8]a strains, e.g., strain Wa (89.2–89.4% nucleotide and 92.1–92.6% amino acid sequence identities). These rotaviruses were assigned to G9 by G typing and their VP7 genes showed an extremely high level of nucleotide and deduced amino acid sequence identity (99.2–100% and 99.1–100%, respectively) to that of the G9P[8]b strain MMC38.

A phylogenetic tree was constructed on the basis of the full-length VP4 nucleotide sequences of the P[8]b strains with those of the P[4] and P[8]a genotypes (Fig. 1A). These newly identified P[8]b strains were clustered with strains MMC38 and MMC71. The VP8* portions of these strains showed more than 99.2% identities to each other, and more closely related to those of the P[8]b strains (94.8–99.1% nucleotide and 95.8–99.5% amino acid sequence identities). In the phylogenetic tree of VP8* sequence, the P[8]b viruses, including three newly identified strains, were located in a single cluster, including OP354 and OP354-like rotaviruses (Fig. 1B).

All the P[8]b rotaviruses which were identified in Bangladesh were detected in stool samples from children. The frequencies of P[8]b among children and the mixed P-types based on conventional P typing were 4.4% (5/113) and 11.5% (3/26), respectively.

DISCUSSION

In the previous study, some nonspecific amplicons were detected from a rotavirus with P[8]b subtype by P genotyping with RT-PCR (5). This finding indicated that the P type of these rotaviruses could not be determined correctly by the RT-PCR, which may cause diagnostic problems in the epidemiologic study of rota-

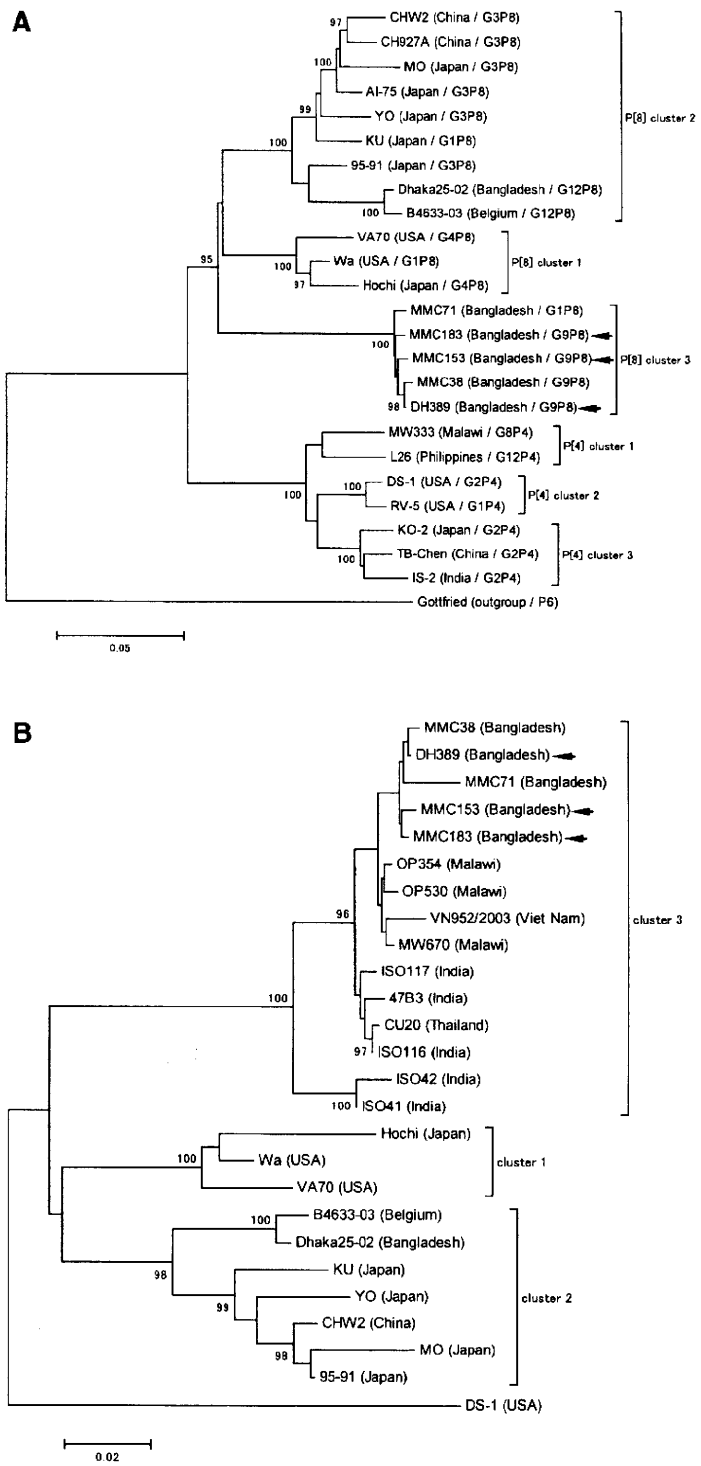

Fig. 1. Phylogenetic trees based on full-length VP4 sequences (2,359-nucleotide sequence [A]) and VP8* sequences (648-nucleotide sequence [B]) for P[8]b strains detected in Bangladesh and other established P[4] and P[8] strains. Arrowheads indicate rotavirus strains analyzed in the present study. P[8]b strains are located in the cluster 3, while P[8]a strains are included in clusters 1 and 2. Bootstrap probability more than 90% is indicated at diverging points of branches. The GenBank accession numbers of the VP4 and VP8* sequences are listed in Ref. 9.

viruses. Therefore, we developed a genotyping method to identify P[8]b as well as four other major human P types (P[4], P[6], P[8]a, and P[9]) by a modified multiplex RT-PCR.

P[8] has been found to be the most frequent P type among rotaviruses worldwide (4). As a component of immunization antigen, two currently available rotavirus vaccines have the P[8]a-VP4 (16), while P[8]b-VP4 is not included. Although it is not evident at present whether or not the rotavirus vaccines are efficient for

both P[8]a and P[8]b rotaviruses equally, in order to obtain any clues relevant to this issue, distribution of P[8]b rotaviruses should also be carefully checked while undertaking epidemiologic surveillance of rotaviruses. For this purpose, the newly established P-typing method in the present study will be useful, and will provide more accurate characteristics of P type distribution in epidemiologic studies of rotaviruses.

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Full Genomic Analysis of a G8P[1] Rotavirus Strain Isolated From an Asymptomatic Infant in Kenya Provides Evidence for an Artiodactyl-to-Human Interspecies Transmission Event

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Group A rotavirus (GAR) G8P[1] strains, found sometimes in cattle, have been reported rarely from humans. Therefore, analysis of the full genomes of human G8P[1] strains are of significance in the context of studies on interspecies transmission of rotaviruses. However, to date, only partial-length nucleotide sequences are available for the 11 genes of a single human G8P[1] strain, while the partial sequences of two other strains have been reported. The present study reports the first complete genome sequence of a human G8P[1] strain, B12, detected from an asymptomatic infant in Kenya in 1987. By nucleotide sequence identities and phylogenetic analyses, the full-length nucleotide sequences of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes of strain B12 were assigned to G8-P[1]-I2-R2-C2-M2-A3-N2-T6-E2-H3 genotypes, respectively. Each of the 11 genes of strain B12 appeared to be more related to cognate genes of artiodactyl (ruminant and/or camelid) and/or artiodactyl-derived human GAR strains than those of most other rotaviruses. Strain B12 exhibited low levels of genetic relatedness to canonical human GAR strains, such as Wa and DS-1, ruling out the possibility of its origin from reassortment events between artiodactyl-like human and true human strains. These observations suggest that strain B12 might have been directly transmitted from artiodactyls to humans. Unhygienic conditions and close proximity of humans to livestock at the sampling site might have facilitated this rare event. This is the first report on a full genomic analysis of a rotavirus strain from Kenya. To our knowledge, strain B12 might be the oldest G8 strain characterized molecularly from the Africa continent. ***J. Med. Virol. 83:367–376, 2011.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: rotavirus; G8P[1]; full genome; zoonosis; artiodactyls; human

INTRODUCTION

Group A rotavirus (GAR; family *Reoviridae*) is a major cause of severe gastroenteritis in the young of humans and animals [Estes and Kapikian, 2007]. Mature virus particles contain eleven segments of double-stranded RNA which encode six structural and six nonstructural proteins [Estes and Kapikian, 2007]. Among them, the outer capsid proteins, VP7 and VP4, are significant antigenically because they contain neutralization epitopes [Estes and Kapikian, 2007]. Therefore, traditionally, GAR strains are dually classified into G and P genotypes based on differences in their VP7- and VP4-encoding genes, respectively [Santos and Hoshino, 2005; Estes and Kapikian, 2007]. In humans, GAR strains exhibiting genotypes G1, G2, G3, G4, or G9 in combination with P[4], P[6], or P[8] have been reported widely, while, recently, G12 is emerging as a globally important

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human genotype [Santos and Hoshino, 2005; Estes and Kapikian, 2007; Greenberg and Estes, 2009]. In addition to these major human genotypes, uncommon G- and P- genotypes, most of which are encountered frequently in animals, have been also reported from humans [Gentsch et al., 2005; Santos and Hoshino, 2005; Martella et al., 2009].

Genotype G8, one of the most common G genotypes found in cattle [Estes and Kapikian, 2007; Martella et al., 2009], was first reported from a child with diarrhea in Indonesia [Hasegawa et al., 1984]. Thereafter, G8 strains have been reported from humans in different countries worldwide [O'Halloran et al., 2000; Gentsch et al., 2005; Santos and Hoshino, 2005; Matthijnssens et al., 2006b; Steyer et al., 2007; Kiulia et al., 2008; Le et al., 2008; Banyai et al., 2009, 2010; Esona et al., 2009; Pietsch et al., 2009; Chandrahasen et al., 2010], with significant rates of detection in some African nations [Santos and Hoshino, 2005; Kiulia et al., 2008; Esona et al., 2010; Nokes et al., 2010]. To date, human G8 strains have been reported in association with a wide variety of P genotypes (P[1], P[2], P[4], P[6], P[8], P[10], P[11], or P[14]) [Santos and Hoshino, 2005; Pietsch et al., 2009; Banyai et al., 2009, 2010; Esona et al., 2009, 2010; Nokes et al., 2010; Nyangao et al., 2010]. These observations suggested that most of the human G8 strains might have originated from complex reassortment events involving human and/or animal rotaviruses or animal-to-human interspecies transmission events [Santos and Hoshino, 2005]. Therefore, determination of the full genome sequences of these viruses is required to pinpoint their exact origin and elucidate their genetic relatedness to other strains. The recently proposed Rotavirus Classification Working Group (RCWG) classification scheme, based on full genome analyses of GAR strains, provided an excellent means of deciphering the true origin of unconventional strains, especially those derived from interspecies reassortment events [Matthijnssens et al., 2008a,b]. Applying this classification scheme, the full-length or nearly full-length nucleotide sequences of the 11 gene segments of a few human G8 (G8P[4], G8P[6], G8P[8], G8P[10], and G8P[14] strains GER1H-09, DRC86, DRC88, 69M, and BP1062/04, respectively) strains were analyzed, yielding vital information on the origin of these strains [Matthijnssens et al., 2006b, 2008a; Heiman et al., 2008; Pietsch et al., 2009; Banyai et al., 2010]. In addition, the evolution of a human G8P[1] strain and few G8P[6] and G8P[8] strains were also studied based on partial-length nucleotide sequences of their 11 gene segments [Banyai et al., 2009; Esona et al., 2009]. However, to date, majority of the studies on human G8 strains are limited to RT-PCR based genotyping and/or sequencing of the VP7 and/or VP4 genes.

G8P[1] rotaviruses are found sometimes in cattle [Reidy et al., 2006; Estes and Kapikian, 2007; Martella et al., 2009; Cashman et al., 2010], and have been reported rarely from humans [Jagannath et al., 2000; Adah et al., 2001; Salu et al., 2003; Banyai et al., 2009]. Therefore, full genomic analyses of human G8P[1] strains are of special significance in context to studies on interspecies transmission of rotaviruses. However, to date, only three G8P[1] strains have been sequenced [Jagannath et al., 2000; Adah et al., 2001, 2003; Banyai et al., 2009]. Among them, partial genome analyses of strain HMG035 (VP4, VP7, and NSP1 genes) from Nigeria [Adah et al., 2001, 2003] and strain MP409 (VP4, VP7, NSP1, NSP3, and NSP4 genes) from India [Jagannath et al., 2000] provided evidence for close genetic relatedness to bovine rotaviruses. Following the RCWG nomenclature, the NSP1 genes of strains HMG035 and MP409 were assigned to A11 genotype, while the NSP3 and NSP4 genes of MP409 were assigned to T6 and E2 genotypes, respectively [Matthijnssens et al., 2008a]. Recently, analysis of the partial-length nucleotide sequences of the 11 gene segments of a human G8P[1] strain, NIC522, from Nicaragua, revealed a G8-P[1]-I2-R2-C2-M2-A13-N2-T2-E2-H3 genotype constellation, pointing towards a possible bovine to human transmission event [Banyai et al., 2009]. However, to our knowledge, to date, the nucleotide sequence accession numbers for the partial-length gene sequences of strain NIC522 are not available in the GenBank database. Therefore, there is a dearth of data available on the human G8P[1] genome. According to the RCWG, information on full-length nucleotide sequences of all the RNA segments of a rotavirus strain might be essential to obtain conclusive data on its origin and pattern of evolution [Matthijnssens et al., 2008b]. In the present study, the full-length nucleotide sequences of the 11 gene segments of a G8P[1] strain, B12, detected from an asymptomatic infant in Kenya in 1987, were analyzed. Full genomic analysis of B12 provided evidence for an artiodactyl (ruminants or camelids)-to-human interspecies transmission event. Moreover, strain B12 might be the oldest G8 strain molecularly characterized from the African continent.

MATERIALS AND METHODS

Virus Strain

GAR strain B12 was detected in a nondiarrheal fecal sample collected from a 2-month-old infant in Bahati division of Nakuru district, Kenya, in January 1987, during the "Research and Control of Infectious Diseases Project" managed by Japan International Cooperation Agency. This strain was successfully isolated by tissue culture in MA-104 cells, and stored at −80°C till further use.

Polyacrylamide Gel Electrophoresis

Fecal sample B12 was screened for the presence of rotaviruses by RNA electrophoresis in polyacrylamide gels using a method described previously [Herring et al., 1982].

Subgrouping

The subgroup specificity of strain B12 was determined by ELISA using subgroup specific monoclonal antibodies as described previously [Taniguchi et al., 1984].

G Serotyping

G serotyping was carried out using serotype-specific monoclonal antibodies against the important human G serotypes (G1–4) as described previously [Taniguchi et al., 1987].

RT-PCR and Nucleotide Sequencing

For RT-PCR, viral RNA was extracted from the tissue culture fluid of strain B12 using the QIAamp Viral RNA Mini kit (Qiagen Sciences, Germantown, MD). RT-PCR-based G- and P- genotyping assays were performed using genotype specific primers described previously [Isegawa et al., 1993; Ghosh et al., 2006; Paul et al., 2008]. The full-length VP6, VP7, NSP1–5 genes and partial-length VP1–4 genes of strain B12 were amplified using primers reported previously [Gentsch et al., 1992; Taniguchi et al., 1992; Ghosh et al., 2010a,b]. Additional primers required for amplification of full-length VP1–4 genes were designed from conserved stretches of cognate genes of published GAR strains (Supplementary Table S1). Nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI PRISM 3100).

Sequence Analysis

Sequence comparisons and phylogenetic analyses were carried out as described previously [Ghosh et al., 2010a,b].

Nucleotide Sequence Accession Numbers

The GenBank accession numbers for the nucleotide sequences of the VP1–4, VP6–7, and NSP1–5 genes of human GAR G8P[1] strain B12 are HM627542–HM627552, respectively.

RESULTS AND DISCUSSION

Human GAR G8 strains have been associated with long, short, and super-short electropherotype patterns [Hasegawa et al., 1984; Gerna et al., 1990; Adah et al., 1997; Rao et al., 2003]. Moreover, G8 strains have been also detected in human GAR samples exhibiting both long and short electropherotypes, highlighting the finding of more unusual combinations due to the close proximity of humans and animals [Santos et al., 1998; O'Halloran et al., 2000]. Interestingly, GAR strain B12 exhibited a long RNA pattern (Supplementary Fig. S1) and subgroup I specificity, a combination found commonly in animal rotaviruses [Kapikian et al., 2001], and could not be serotyped using monoclonal antibodies against the major human G (G1–4) types. By RT-PCR-based genotyping of VP7 and VP4 genes, strain B12 exhibited G8P[1] specificity (data not shown), a combination detected sometimes in bovine rotaviruses [Reidy et al., 2006; Cashman et al., 2010]. The local population at the sampling site lived under extremely unhygienic conditions, with a river and a borehole as the main sources of drinking water, and reared domestic animals (cattle, goats, and sheep) in their homes. Taken together, these observations suggested that strain B12 might be derived from animal rotaviruses, but were not sufficient to ascertain the origin or decipher the pattern of evolution of this unusual strain. Therefore, following the nomenclature of RCWG, the full-length nucleotide sequences of the 11 gene segments of GAR G8P[1] strain B12 were analyzed in the present study. The only other report on the overall genome of a human G8P[1] strain, NIC522, was based on partial-length nucleotide sequences of the 11 gene segments [Banyai et al., 2009]. Hence, to our knowledge, the present study is the first report on the complete genome of a human G8P[1] rotavirus strain.

By nucleotide sequence identities and phylogenetic analyses, the full-length nucleotide sequences of the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes of GAR strain B12 were assigned to the G8-P[1]-I2-R2-C2-M2-A3-N2-T6-E2-H3 genotypes, respectively (Fig. 1 A–K, Table I). Excluding the G and/or P genotypes, the complete genotype constellation of strain B12 was identical to those of bovine (strains NCDV and WC3) and human G6P[14] (strains 111/05-27, B10925-97 and PA169) strains, and differed from those of most other human G8 strains in the NSP1, NSP3, and/or NSP5 genes (Table I). Comparison with the only other overall genotype constellation available for a G8P[1] strain, of that of strain NIC522, revealed differences in the NSP1 and NSP3 genes (Table I). To our knowledge, to date, information on the partial-length nucleotide sequences of the 11 gene segments of strain NIC522 are not available in the GenBank database, and therefore, this strain could not be included in further analysis of B12.

The complete genome of GAR G8P[1] strain B12 was 18,489 bp in size. Among the structural genes, the VP7 gene of B12 shared maximum nucleotide sequence identities of 97.2% and 97.0% with those of G8P[14] strain Arg/Chubut/99 and G8P[1] strain Arg/Rio Negro/98 from guanocos, respectively, followed by those of bovine strain O and ovine strain OVR762, and exhibited relatively low identities to those of other human G8 strains (Table II). With the other Kenyan human G8 strains, 1290, KY6914/02 and KY6950/02, for which VP7 gene sequences (GenBank accession nos. EU488721, FJ386445, and FJ386446, respectively) are available in the GenBank database, identities of 84.7%, 84.4%, and 84.7% were observed, respectively. By phylogenetic analysis, strain B12 clustered with bovine G8 strains, away from the clusters comprising other human G8 strains (Fig. 1F). Although strain B12 was assigned to the P[1] genotype, the VP4 gene of B12 exhibited extremely low nucleotide sequence identities (<82%) to those of other P[1] strains (Table II), and by phylogenetic analysis, clustered separately within the P[1] genotype (Fig. 1D).

Among the other structural genes, the VP6 gene of strain B12 shared high nucleotide sequence identities of 95.8–96.8% and 95.6–96.3% with those of bovine

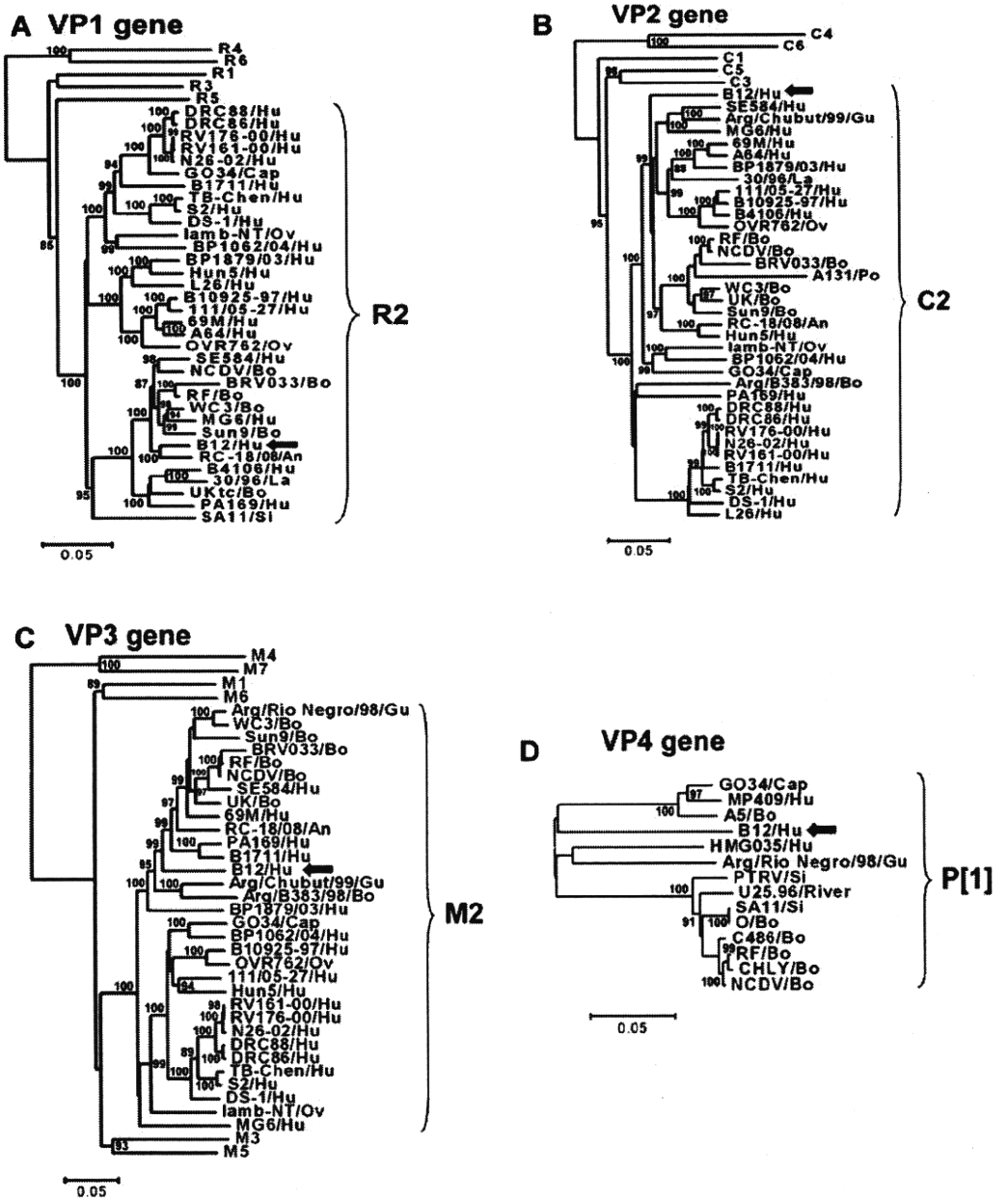

Fig. 1. **A–K**: Phylogenetic trees constructed from nucleotide sequences of VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4, and NSP5 genes of rotavirus strain B12 with those of group A rotavirus strains representing the 6 R, 6 C, 7 M, P[1], 13 I, G8, 16 A, 6 N, 8 T, 12 E, and 8 H genotypes, respectively. Phylogenetic trees were constructed by the neighbor-joining method [Saitou and Nei, 1987] using MEGA (v4.1) software. The trees were statistically supported by bootstrapping with 1,000 replicates, and phylogenetic distances measured by the Kimura two-parameter model. In all trees, the position of strain B12 is indicated by an arrow. Bootstrap values ≥85% are shown. Bar, 0.05 substitutions per nucleotide. Abbreviations: An, antelope; Bo, bovine; Cap, caprine; Eq, equine; Gu, guanaco; Hu, human; La, lapine; Ov, ovine; Po, porcine; Si, simian.

(strains NCDV, UK, and WC3) and human G6P[14] (strains 111/05-27 and PA169) strains (Table II), respectively, and by phylogenetic analysis, clustered near the cluster of human G6 strains (G6P[9] strain SE584 and G6P[14] strain PA169) and bovine strains NCDV and RF (Fig. 1E). Strain B12 was closely related to the VP1 gene of strain RC-18/08 from an antelope (Fig. 1A, Table II). On the other hand, the VP2 and VP3 genes of strain B12 exhibited maximum (but low) nucleotide sequence identities of 90.0% and 89.2% to those of ruminant

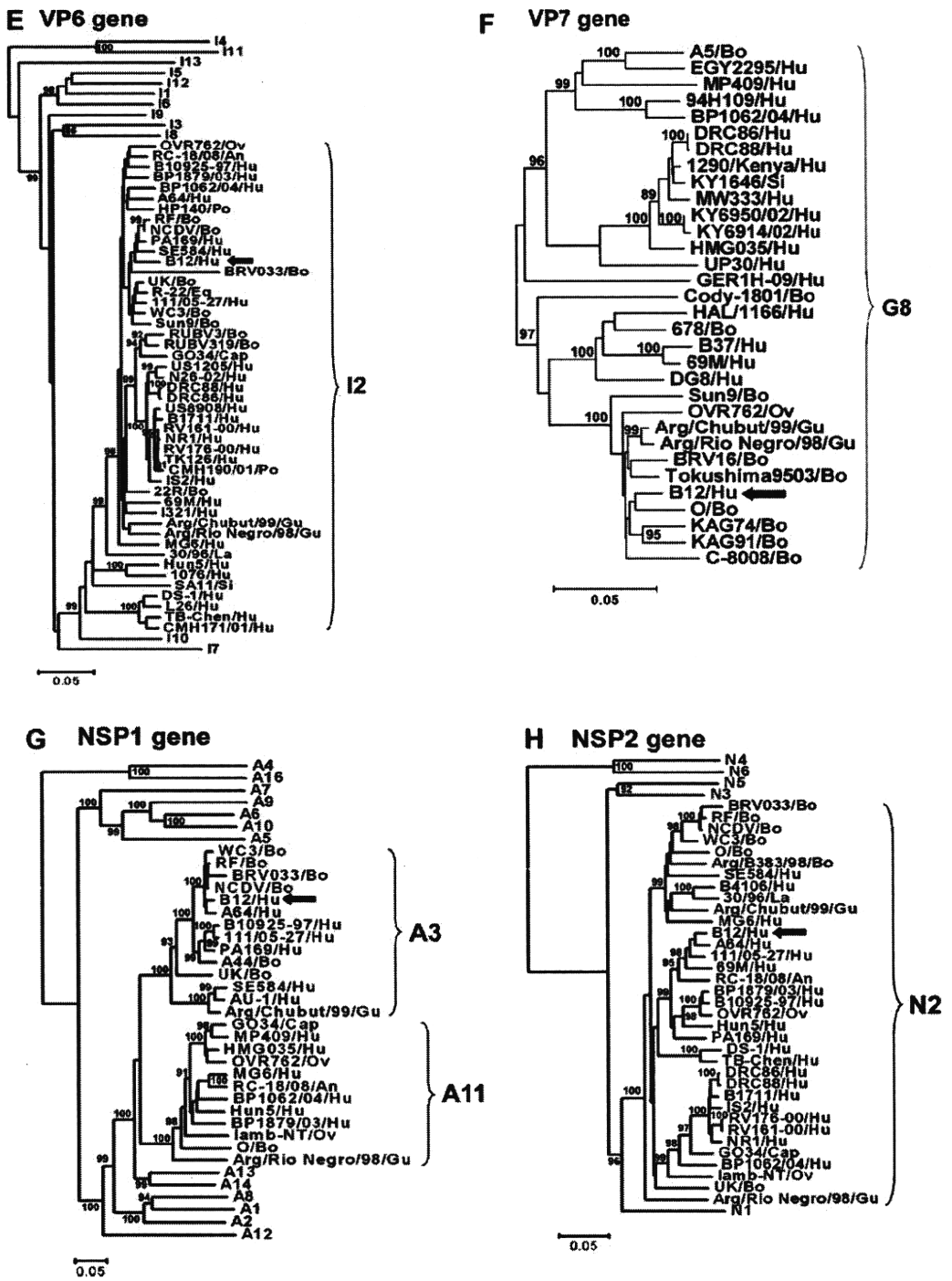

Fig. 1. (*Continued*)

strains NCDV and RC-18/08, respectively (Table II). By phylogenetic analysis, the B12 VP2 gene appeared to be distantly related (but nearest) to the cluster primarily consisting of ruminant, camelid and artiodactyl-like human P[14] strains (Fig. 1B), whilst its VP3 gene clustered near the cluster formed by ruminant, camelid, human G6 (G6P[6] strain B1711, G6P[9] strain SE584, and G6P[14] strain PA169) and G8 (G8P[10] strain 69M) strains (Fig. 1C).

Among the nonstructural genes, the NSP1 gene of strain B12 exhibited high genetic relatedness to those of bovine strains and artiodactyl-like human G10P[14]

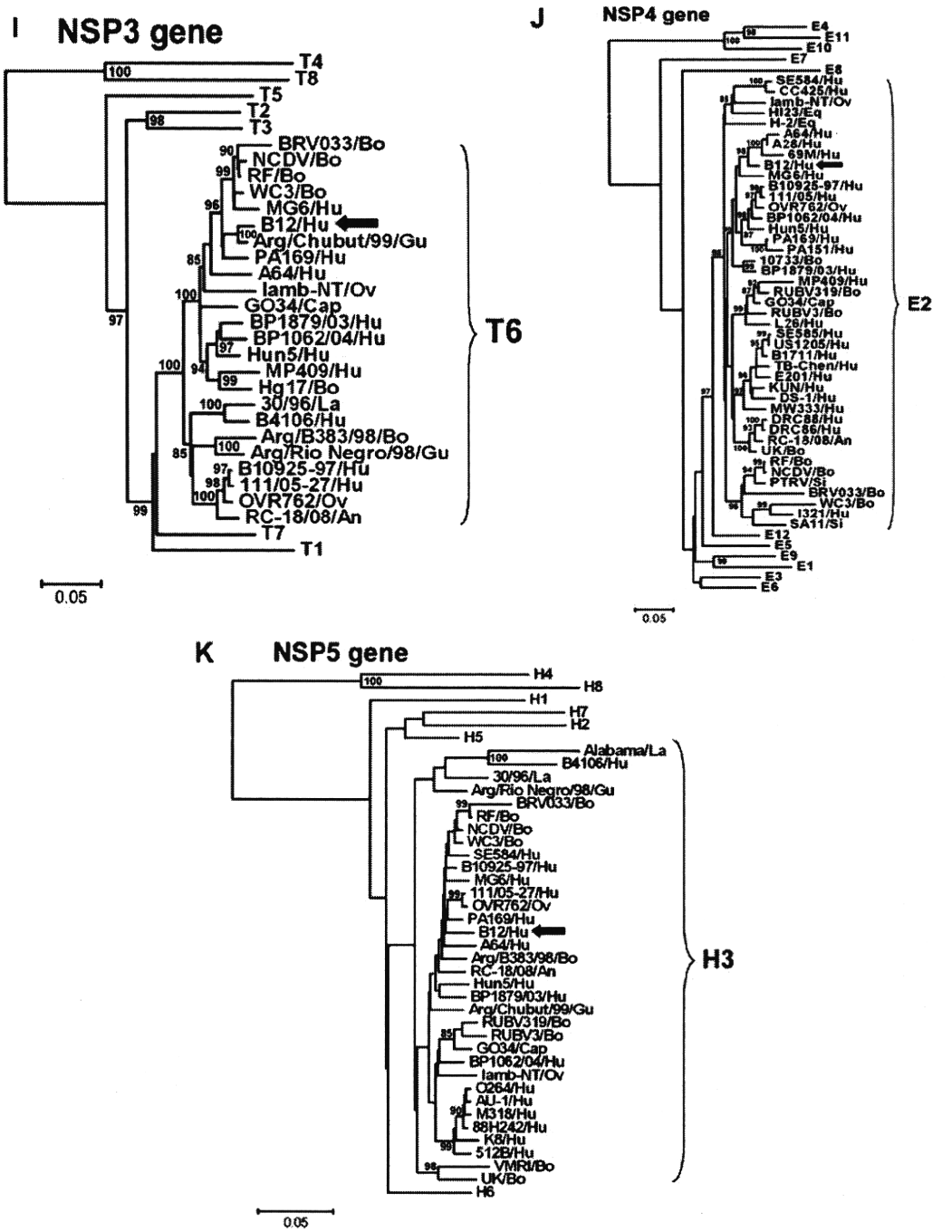

Fig. 1. (*Continued*)

strain A64 (Fig. 1G, Table II). The NSP2 gene of B12 exhibited maximum genetic relatedness to that of human G10P[14] strain A64, and was also closely related to those of human G6P[14] strain 111/05-27 and G8P[10] strain 69M, and strain RC-18/08 from an antelope (Fig. 1H, Table II). Strain B12 was closely related to the NSP3 gene of camelid strain Arg/Chubut/99 (Fig. 1I, Table II). The NSP4 gene of strain B12 shared a maximum nucleotide sequence identity of 94.9% with that of G10P[14] strain A64, followed by those of human G6P[14] strain MG6 and G8P[10] strain 69M (Table II). By phylogenetic analysis, the NSP4 gene of B12 formed a cluster with strains A64 and 69M, near strain MG6, and taken together, these strains were distantly related (but nearest) to the cluster of artiodactyl-like human P[14] strains and ovine G8P[14] strain