Table 1. Representative reverse genetics systems for segmented double-stranded RNA viruses.

Family	Genus	Species	Number of genome segments	Need for the strong selection systems	Ref.
Cystoviridae	*Cystovirus*	Φ6	3	No	[13]
				No	[14]
		Φ8	3	No	[15]
Birnaviridae	*Avibirnavirus*	IBDV	2	No	[10]
				No	[11]
	Aquabirnavirus	IPNV	2	No	[12]
Reoviridae	*Orthoreovirus*	Reovirus	10	Yes	[16]
	Rotavirus	Rotavirus	11	Yes	[20]

IBDV: Infectious bursal disease virus; IPNV: Infectious pancreatic necrosis virus.

Future perspective

The introduction of mutations that change the biological characteristics of rotaviruses would help to define the precise functions of all of the viral proteins, including those of nonstructural proteins. The UTRs of the genome could also be studied by means of mutagenesis, leading to an improved understanding of the regulatory signals present in viral RNAs. Furthermore, this reverse genetics system provides the opportunity to identify attenuation markers within the rotavirus genome segments and, thus, will facilitate the development of attenuated recombinant viruses that could be used as vaccine or vaccine vector candidates. Clearly, there are limitations to helper virus-dependent rotavirus reverse genetics system owing to the requirement of a strong selection system, such as a neutralizing antibodies selection system. For gene segments that do not encode viral surface proteins, other gene-specific selection procedures, such as host-range restriction and temperature sensitivity, will be required. Furthermore, the low efficiency of the present system, which might prevent the rescue of transfectant rotavirus harboring mutations associated with significant decreased fitness, should be improved by the modification of the experimental conditions. Despite its current limitations, the new and powerful approaches have immediate promise in several areas, particularly with respect to the study of the functions of viral proteins and the signals involved in the transcription, replication and packaging of rotavirus genome dsRNAs, which will also lead to a further goal; the establishment of a helper virus-free system to generate infectious rotaviruses entirely from cloned cDNA.

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Executive summary

Overview of the rotavirus life cycle

- All the replication processes occur in the cytoplasm.
- Rotavirus transcription and replication occurs in the double-layered particles and core replication intermediates, respectively.
- The packaging process is still not fully understood.
- Viroplasms appear to be the site for RNA replication and packaging.

Reverse genetics for segmented double-stranded RNA viruses

Bacteriophage Φ6

- A reverse genetics system for bacteriophage ϕ6 was initially developed by the *in vitro* packaging-replication system.
- The first system was devised to cause replication and packaging of the viral genome intracellularly, resulting in the establishment of a new *in vivo* transcript acquisition system.
- Viral mRNAs alone are sufficient to initiate virus replication cycles.

Executive summary

Birnaviruses

- A reverse genetics system to generate infectious birnaviruses entirely from cDNA was developed initially with the use of synthetic transcripts.
- A system was successfully devised to generate viral transcripts intracellularly, allowing the improved production of cDNA-derived transfectant viruses.
- Viral mRNAs alone are sufficient to initiate virus replication cycles.

Reovirus

- A helper virus-dependent reverse genetics system for reovirus, in which transfected viral RNAs are infectious, has been developed. This system also requires the single-stranded (ss)RNA-primed rabbit reticulocyte lysate, helper virus and the transformed cells to express a particular viral protein. Other laboratories have found this system difficult to reproduce.
- Attempts to initiate virus replication cycles by introducing viral mRNAs alone have been unsuccessful.

Rotavirus

- A reverse genetics system for rotavirus with a helper virus-driven procedure was established recently. This system is based on the incorporation of an intracellularly transcribed cDNA-derived ssRNA into the genome of the helper virus. This is much simpler than the reovirus system described previously.
- Attempts to initiate virus replication cycles by introducing viral mRNAs alone have been unsuccessful.

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Evolutionary History and Global Spread of the Emerging G12 Human Rotaviruses[▽]†

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G12 rotaviruses were first detected in diarrheic children in the Philippines in 1987, but no further cases were reported until 1998. However, G12 rotaviruses have been detected all over the world in recent years. Here, we report the worldwide variations of G12 rotaviruses to investigate the evolutionary mechanisms by which they managed to spread globally in a short period of time. We sequenced the complete genomes (11 segments) of nine G12 rotaviruses isolated in Bangladesh, Belgium, Thailand, and the Philippines and compared them with the genomes of other rotavirus strains. Our genetic analyses revealed that after introduction of the VP7 gene of the rare G12 genotype into more common local strains through reassortment, a vast genetic diversity was generated and several new variants with distinct gene constellations emerged. These reassortment events most likely took place in Southeast Asian countries and spread to other parts of the world. The acquirement of gene segments from human-adapted rotaviruses might allow G12 to better propagate in humans and hence to develop into an important emerging human pathogen.

Group A rotaviruses are one of the major causes of severe gastroenteritis in young children and animals. More than 125 million infants and young children develop rotavirus diarrhea globally each year, resulting in 440,000 deaths among children less than 5 years of age, mostly in developing countries (29). This high disease burden motivated major efforts to develop rotavirus vaccines. However, the high degree of genetic and antigenic variation among rotaviruses hinders the vaccine development programs (5, 9, 16, 28, 34, 42).

The rotavirus genome contains 11 double-stranded RNA segments, ranging in size from 664 to 3,302 nucleotides, encoding six structural viral proteins (VP) and six nonstructural proteins (NSP) (8). The viral capsid is formed by three concentric layers: a central core, an inner protein layer, and an outer protein layer (31). The outer protein layer is composed of VP4 and VP7, the two major antigens of the virus, and the middle layer is composed of VP6 molecules arranged as trimers. The central core is composed mainly of VP2 and contains the gene segments and enzyme complexes responsible for the processes of RNA transcription and replication (18).

Rotaviruses are classified into G and P genotypes on the basis of the sequence diversities of the two outer layer proteins VP7 and VP4, respectively, which are the two viral proteins that elicit neutralizing antibody responses. An 89% amino acid cutoff percentage has been used to define different G and P genotypes (11, 18). At least 15 G genotypes and 26 P genotypes have been reported to date in mammals and avian species (8, 21, 33). The segmented nature of the rotavirus genome provides an opportunity for genetic reassortment, which plays an important role in the generation of virus diversity through genetic shift as demonstrated by many investigators (1, 7, 12, 15, 22, 24, 36, 41). In addition, "genogrouping" based on overall genomic RNA homology by hybridization assays has been proposed (25, 26). Using this approach, three genogroups of human rotaviruses have been defined: Wa-like, DS-1-like, and AU-1-like. In this genogrouping system, a strain is considered to belong to a

TABLE 1. Strains sequenced and deposited in GenBank for this study

Strain	Genotype	Country of origin	Yr of isolation	GenBank accession no.
RV161-00	G12P[6]	Bangladesh	2000	DQ490540–DQ490550
RV176-00	G12P[6]	Bangladesh	2000	DQ490551–DQ490561
N26-02	G12P[6]	Bangladesh	2002	DQ146682–DQ146692
Dhaka25-02	G12P[8]	Bangladesh	2002	DQ146649–DQ146659
Dhaka12-03	G12P[6]	Bangladesh	2003	DQ146660–DQ146670
Matlab13-03	G12P[6]	Bangladesh	2003	DQ146671–DQ146681
B4633-03	G12P[8]	Belgium	2003	DQ146638–DQ146648
L26	G12P[4]	Philippines	1987	DQ146693–DQ146698
T152	G12P[9]	Thailand	1998	DQ146699–DQ146706
MV404-02	G12P[6]	United Kingdom	2002	DQ501280–DQ501282

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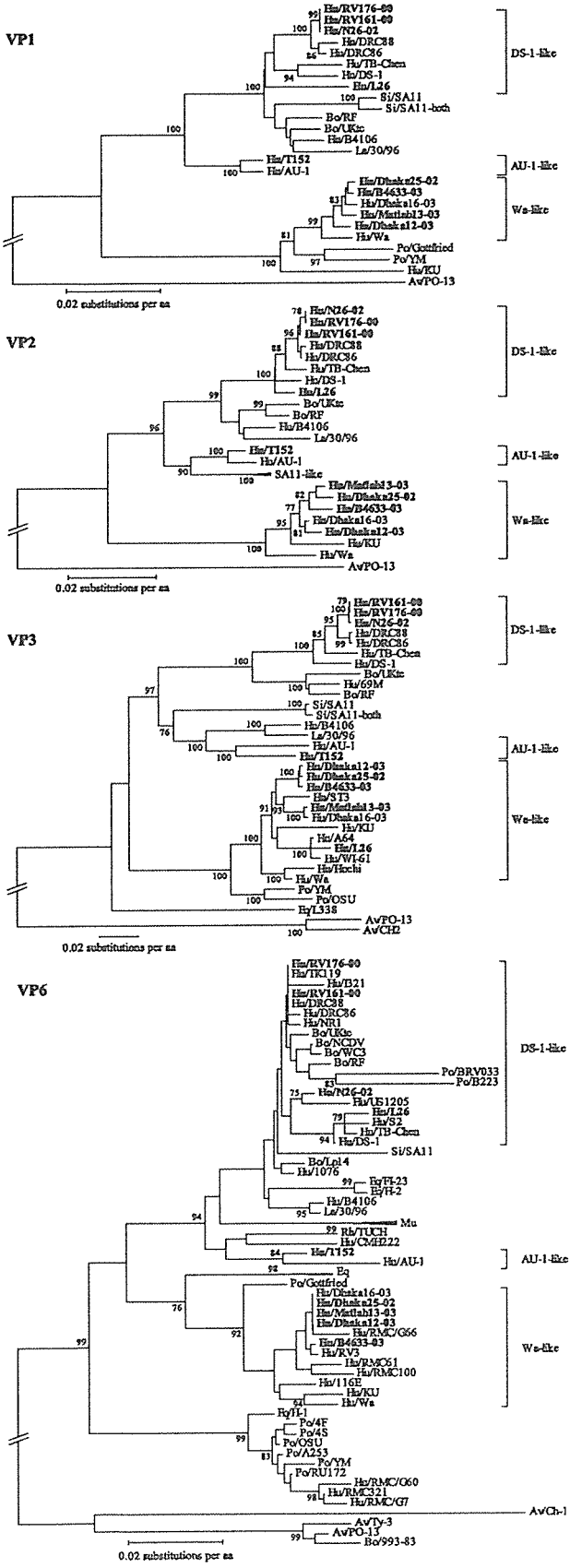


FIG. 1. Phylogenetic dendrograms based on the complete amino acid (aa) sequences of the structural proteins VP1, VP2, VP3, and VP6. Accession numbers can be found in the supplemental material. The numbers adjacent to the nodes represent the percentages of bootstrap support (of 1,000 replicates) for the clusters to the right of the nodes. Bootstrap values lower than 75% are not shown. Hu, human; La, lapine; Bo, bovine; Po, porcine; Si, simian; Eq, equine; Fe, feline; Rh, rhesus; Mu, murine; Av, avian. G12 strains analyzed in this study are in bold.

certain genogroup if it contains at least seven gene segments similar to those in that particular genogroup.

The first G12 strain, L26 (G12P[4]), was detected in children less than 2 years old in 1987 in the Philippines (38). More than 10 years later, G12 strains were isolated in Thailand (1998) and the United States (1999) and subsequently in several Asian countries, such as India (1999 to 2005), Bangladesh (2000 to 2005), Japan (2003), and Korea (2002 and 2003) (6, 14, 17, 30, 35, 37). In Europe, G12 strains were identified in the United Kingdom (2002 and 2006) and Belgium (2003). They were also found in Argentina (1999 to 2003) and Brazil (2004) (3, 4). Using hybridization assays, the recent G12 strains were demonstrated to be distantly related to the prototype strain L26 and belonged to the AU-1-like or DS-1-like genogroup (14, 37, 43).

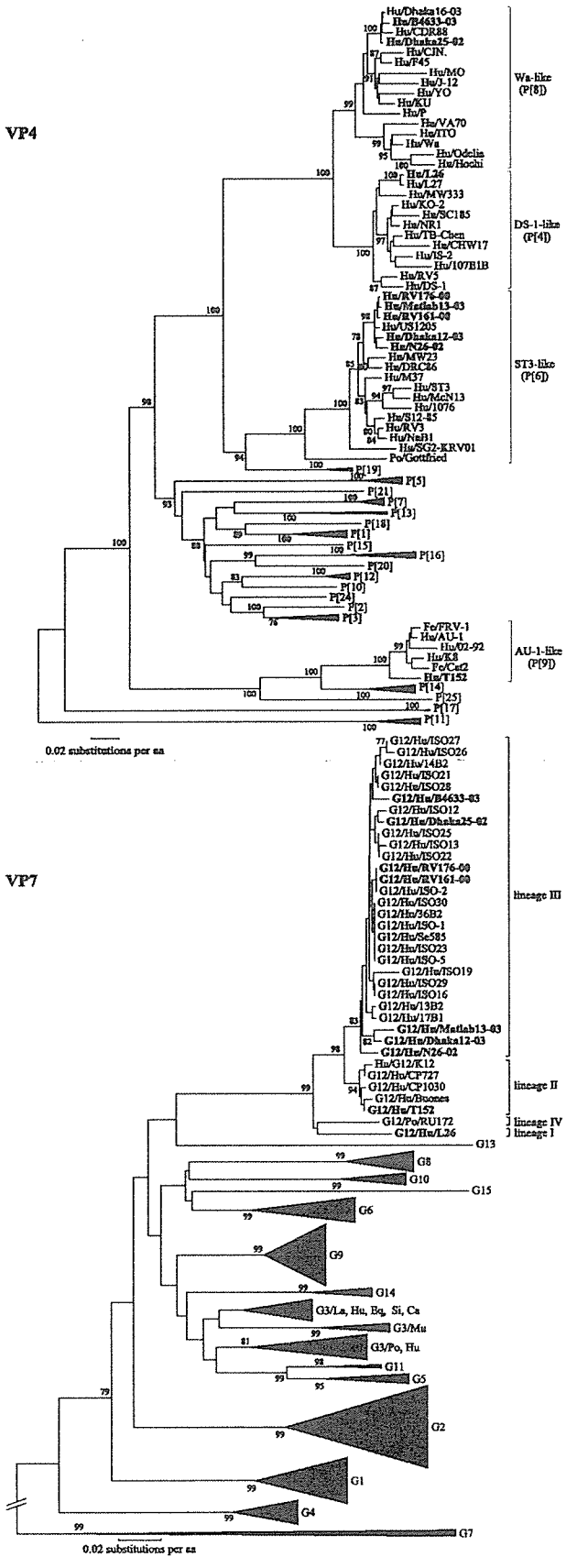
In the present study, G12 rotaviruses isolated in Bangladesh (2000 to 2005) and Belgium (2003) as well as the prototype strains L26 and T152 were analyzed through comparison and phylogenetic analysis of the derived amino acid sequences of all 11 gene segments.

MATERIALS AND METHODS

Sample collection. From 1999 to 2005, a total of 441 rotavirus-positive stool specimens from patients attending the Matlab and Dhaka hospitals of ICDDR,B, Bangladesh, were genotyped, 18 of which contained G12 rotaviruses. In Belgium, three G12 rotaviruses were detected in children admitted to the Gasthuisberg hospital, Leuven, during the 2003-to-2004 rotavirus season (n = 182). The G12 strains were untypeable with our routine multiplex PCR because no G12-specific primer was used. In our routine multiplex reverse transcription-PCR (RT-PCR), six G-genotype-specific primers (G1, G2, G3, G4, G8, and G9) and five P-genotype-specific primers (P[4], P[6], P[8], P[9], and P[11]) were included. For detecting the untypeable G12 strains, the VP7 gene segments were amplified using Beg9 and End9 primers. The amplified products were sequenced in both directions with the same primers. Nucleotide sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI; National Institutes of Health, Bethesda, MD) BLAST (Basic Local Alignment Search Tool) server in GenBank, release 153.0 (2). The tissue culture supernatants of the prototype G12 strain L26, isolated in the Philippines in 1987, and strain T152, isolated in Thailand in 1998, were used.

RNA extraction. Viral RNA was extracted using a QIAamp viral RNA mini kit (QIAGEN/Westburg, Leusden, The Netherlands) according to the manufacturer's instructions.

RT-PCR. The extracted RNA was denatured at 97°C for 5 min, and RT-PCR was carried out using a QIAGEN OneStep RT-PCR kit (QIAGEN/Westburg) as described by Gouvea and colleagues (13). The forward and reverse primers used for the amplification of different gene segments were developed based on alignments of known 5′ and 3′ sequences of the respective gene segments found in GenBank. The reaction was carried out with an initial reverse transcription step at 45°C for 30 min, followed by PCR activation at 95°C for 15 min, 40 cycles of amplification, and a final extension of 7 min at 72°C, in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems Group, Foster City, CA). The cycle conditions for the amplification of VP1, VP2, VP3, and VP4 were 30 s at 94°C, 30 s at 45°C, and 6 min at 70°C; for the other gene segments, the conditions were 30 s at 94°C, 30 s at 45°C, and 2.5 min at 72°C.



Nucleotide sequencing. The PCR products were purified with a QIAquick PCR purification kit (QIAGEN/Westburg) and sequenced using the dideoxynucleotide chain termination method with an ABI PRISM BigDye Terminator cycle sequencing reaction kit (Applied Biosystems Group) on an ABI PRISM 3100 automated sequencer (Applied Biosystems Group). The sequencing was performed with the forward and reverse primers used for the RT-PCR. Primer walking sequencing was performed to cover the complete sequences of the respective fragments on both strands.

Determination of the 5′- and 3′-terminal sequences. To obtain the complete nucleotide sequences, the 5′- and 3′-terminal sequences of the 11 gene segments were determined as previously described (23).

Nucleotide and protein sequence analysis. The chromatogram sequencing files were analyzed using Chromas 2.23 (Technelysium, Queensland, Australia), and contigs were prepared using SeqMan II (DNASTAR, Madison, WI). Multiple sequence alignments were calculated using ClustalX 1.81 (39). Sequences were manually edited by the GeneDoc version 2.6.002 alignment editor (27).

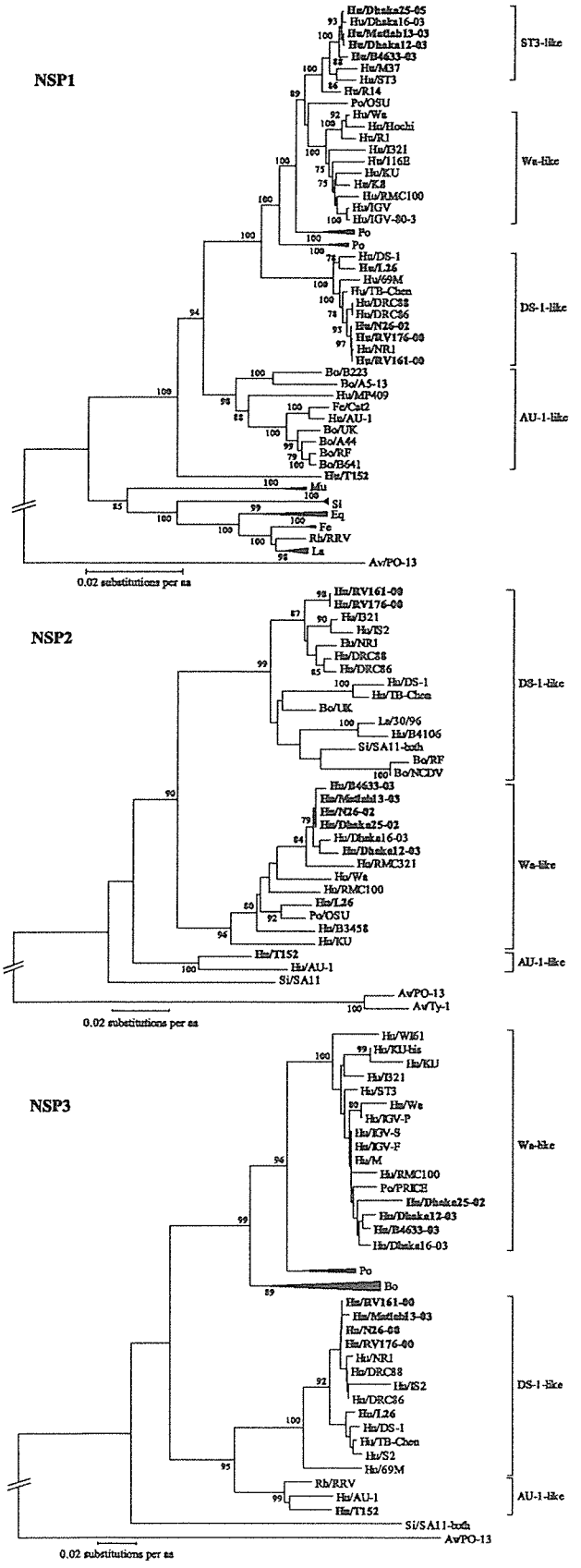
Phylogenetic analysis. The dendrograms were constructed using the neighbor-joining method with MEGA version 3.1 software (19). The similarity percentages between amino acid sequences were calculated by using the Poisson correction distance model.

Genogrouping strategy. Initially, the 11 gene segments (the VP1 to VP4, VP6, VP7, and NSP1 to NSP5 gene segments) for all G12 strains isolated in our study (n = 21) were amplified with a forward and a reverse primer described by Matthijnssens and colleagues (23). Sequencing of these RT-PCR products by using the forward primers produced a nucleotide sequence of at least 600 bp from the 5′ end of each gene segment. The corresponding partial sequences of all gene segments of G12 strains were compared to each other. Based on the nucleotide similarity, at least six different gene combinations were detected among the Bangladeshi G12 strains. A single G12 strain from each unique gene constellation (strains RV161-00, RV176-00, N26-02, Dhaka25-02, Dhaka12-03, and Matlab13-03) was selected for sequencing of its entire genomic complement. All three Belgian G12 strains were found to be nearly identical based on the partial sequences, and the complete genome of one representative Belgian G12 strain, B4633-03, was sequenced. In practical terms, each of the partial gene segments of the strains which were excluded from the analysis was virtually identical to one of the representative strains which were sequenced completely. Several gene segments of the prototype G12 strains L26 (the VP1, VP2, VP6, NSP2, NSP3, and NSP5 gene segments) and T152 (the VP1, VP2, VP3, VP6, NSP2, NSP3, NSP4, and NSP5 gene segments) which were not available in GenBank were also sequenced.

In order to establish the interrelationships among the different G12 isolates described in this study and their relationship with other human rotavirus strains, pairwise comparisons on the amino acid level were conducted between all the G12s and the human reference strains Wa, DS-1, and AU-1. In addition, phylogenetic dendrograms were constructed to compare these strains with each other and with other human and animal rotavirus strains. Both of these data sets were used to deduce and/or speculate about the possibilities that certain differences in the homologues gene sequences were due to genetic drift or due to reassortments. These deductions/speculations were made, keeping in mind that all the different rotavirus proteins, and their respective gene segments, are subjected to different selective pressures from the environment and the host immune systems, resulting in different levels of nucleotide and amino acid conservation.

Nucleotide sequence accession numbers. The nucleotide sequence data for complete genomes of rotavirus strains reported in this paper were submitted to GenBank under the accession numbers included in Table 1.

FIG. 2. Phylogenetic dendrograms based on the complete amino acid (aa) sequences of the outer capsid proteins VP4 and VP7. Accession numbers can be found in the supplemental material. The sizes of the triangles are indications of the numbers of sequences that they represent. The numbers adjacent to the nodes represent the percentages of bootstrap support (of 1,000 replicates) for the clusters to the right of the nodes. Bootstrap values lower than 75% are not shown. Hu, human; Po, porcine; Fe, feline. G12 strains analyzed in this study are in bold.



RESULTS

Complete nucleotide sequences for the 11 gene segments encoding VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4, and NSP5 of the representative G12 rotavirus strains isolated in our study were determined. Phylogenetic trees for each gene segment, which included the deduced amino acid sequences of the G12 strains together with the corresponding gene sequences of the rotavirus strains available in GenBank, were constructed (Fig. 1 to 4). Additionally, multiple sequence alignments for all gene segments were conducted and similarity matrices were constructed (Fig. 5 and 6).

L26. The prototype G12 strain L26 (G12P[4]) was isolated in the Philippines during 1987. Our pairwise comparisons and phylogenetic analyses revealed that seven gene segments of strain L26 (the VP1, VP2, VP4, VP6, NSP1, NSP3, and NSP4 gene segments) were very closely related to the corresponding gene segments of human DS-1-like rotavirus strains (Fig. 1 to 6). The VP3, NSP2, and NSP5 gene segments of strain L26 were closely related to the human strain Wa and recent Wa-like strains (strains RMC100 and B4633-03, etc.). It is remarkable that next to these close genetic relationships, also very high similarities (>96% for NSP5 and >98% for NSP2 [data not shown]) and a close phylogenetic clustering were found between L26 and porcine rotavirus strains OSU, YM, and RU172. These genomic characteristics indicated that the prototype G12 rotavirus L26 most likely contained an assortment of Wa- and DS-1-like gene segments in combination with a novel VP7 (G12) specificity which belonged to lineage I of the G12 branch (Fig. 2). Although there are only very few complete gene sequences available for porcine rotaviruses, the above-mentioned data indicate that porcine rotaviruses might also be involved in the reassortment events leading to the occurrence of L26.

T152. More than 10 years after the isolation of strain L26, the G12P[9] strain T152 was isolated in Thailand. Eight gene segments (the VP1 to VP4, VP6, and NSP2 to NSP4 gene segments) of strain T152 were unrelated to the prototype G12 strain L26 but were closely related to strain AU-1 (Fig. 1 to 6). The VP7 gene segment clustered in lineage II of the G12 branch (Fig. 2), and the NSP1 gene was placed in a unique branch that was not related to any other known rotavirus (Fig. 3). The NSP5 segment of strain T152 clustered with rhesus strain RRV, indicating that this gene segment might be of animal origin (Fig. 4).

RV161-00, RV176-00, and N26-02. The first generation of Bangladeshi G12 strains (n = 4), represented by strains RV161-00, RV176-00, and N26-02, was isolated between 2000 and 2002. They all clustered in the G12 lineage III branch of

FIG. 3. Phylogenetic dendrograms based on the complete amino acid (aa) sequences of the nonstructural proteins NSP1, NSP2, and NSP3. Accession numbers can be found in the supplemental material. The numbers adjacent to the nodes represent the percentages of bootstrap support (of 1,000 replicates) for the clusters to the right of the nodes. Bootstrap values lower than 75% are not shown. Hu, human; La, lapine; Bo, bovine; Po, porcine; Si, simian; Eq, equine; Fe, feline; Rh, rhesus; Mu, murine; Av, avian. G12 strains analyzed in this study are in bold.

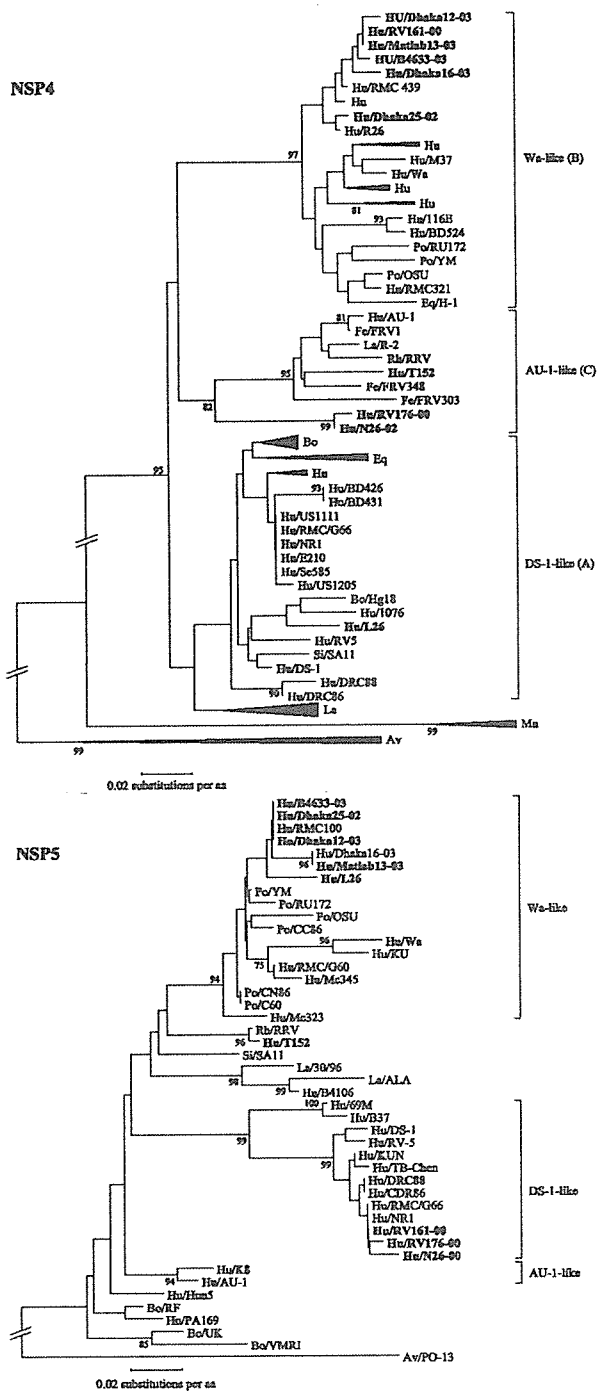


FIG. 4. Phylogenetic dendrograms based on the complete amino acid (aa) sequences of the nonstructural proteins NSP4 and NSP5. Accession numbers can be found in the supplemental material. For NSP4, the three established genogroups are shown. The numbers adjacent to the nodes represent the percentages of bootstrap support (of 1,000 replicates) for the clusters to the right of the nodes. Bootstrap values lower than 75% are not shown. Hu, human; La, lapine; Bo, bovine; Po, porcine; Si, simian; Eq, equine; Fe, feline; Rh, rhesus; Mu, murine; Av, avian. G12 strains analyzed in this study are in bold.

the VP7 tree and possessed the P[6] (ST3-like) VP4 specificity (Fig. 2). The VP1 to VP3, VP6, NSP1, NSP3, and NSP5 gene segments of these three G12P[6] strains were very closely related to each other and to DS-1-like rotavirus strains (Fig. 1 to 5). The NSP2 gene segments of strains RV161-00 and RV176-00 were also closely related to DS-1-like rotaviruses, while the NSP2 gene of strain N26-02 was closely related to Wa-like strains (Fig. 3). The NSP4 gene segment of strain RV161-00 was closely related to Wa-like rotavirus strains, whereas the NSP4 genes of strains RV176-00 and N26-02 were distantly related to AU-1-like strains (Fig. 4). These data showed that strains RV161-00, RV176-00, and N26-02 were closely related and had a common ancestor. They were most likely generated after several reassortment events between DS-1-like strains and strains donating their VP7 (G12), VP4 (P[6]), NSP2, and NSP4 gene segments.

Dhaka25-02, Dhaka12-03, and Matlab13-03. The second generation of Bangladeshi G12 strains ($n = 14$), represented by strains Dhaka25-02, Dhaka12-03, and Matlab13-03, was isolated between 2002 and 2005. Their VP7 gene segments clustered very closely in the lineage III branch of the VP7 phylogenetic tree, together with the other first-generation Bangladeshi G12 strains (Fig. 2). Strains Dhaka12-03 and Matlab13-03 contained a VP4 gene segment with the P[6] (ST3-like) specificity, whereas strain Dhaka25-02 contained the P[8] (Wa-like) specificity (Fig. 2). Seven other gene segments (the VP1 to VP3, VP6, NSP2, NSP4, and NSP5 gene segments) of strains Dhaka25-02, Dhaka12-03, and Matlab13-03 were very closely related to each other and to Wa-like strains (Fig. 1 and 3 to 5). The NSP3 genes of strains Dhaka25-02 and Dhaka12-03 were also Wa-like, whereas NSP3 of strain Matlab13-03 was DS-1-like. The NSP1 gene segments of all three strains were closely related to ST3-like strains. These data suggested that strains Dhaka25-02, Dhaka12-03, and Matlab13-03 were very closely related and had a common ancestor. They were most likely generated after several reassortment events between Wa-like strains and strains donating their VP7 (G12), VP4 (P[6]), NSP1, and NSP3 gene segments.

B4633-03. For the three nearly identical Belgian G12 rotavirus strains, strain B4633-03 was sequenced completely as a representative. This strain clustered very closely with strain Dhaka25-02 and showed very high similarities, ranging from 96.5% to 100% on the amino acid level with the Wa-like Bangladeshi G12 strain Dhaka25-02 for all 11 gene segments (Fig. 1 to 6), indicating a common origin for both strains.

MV404-02. From the United Kingdom G12P[6] rotavirus strain MV404-02, isolated in 2002, only partial sequences of the VP7-, VP4-, and VP6-encoding gene segments could be determined. All three partial sequences showed close genetic relationships with the G12P[6] rotavirus strains Dhaka12-03 and Matlab13-03 (data not shown). No more genetic material was left to determine the nature of the remaining eight gene segments.

Remaining G12s. The U.S. G12P[6] strain Se585 was isolated in 1999 (14). Analysis of the VP4 and VP7 genes of strain Se585 revealed very close relationships (98.0% to 100% amino acid similarities) with the G12P[6] strains isolated in Bangladesh (strains RV161-00 and RV176-00) and India (strains ISO-1, ISO-2, and ISO-5) (Fig. 2). The NSP4 gene was closely

VP1

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	89.4										
AU-1	3	91.0	94.6									
L26	4	89.2	98.7	94.2								
T152	5	90.8	94.3	98.3	94.1							
RV161-00	6	89.5	97.8	95.1	97.2	95.0						
RV176-00	7	89.5	97.8	95.1	97.2	95.0	100.0					
N26-01	8	89.5	97.6	95.1	97.2	95.0	100.0	100.0				
Dhaka25-02	9	98.7	89.3	91.1	89.1	90.7	89.6	89.6	89.6			
Dhaka12-03	10	98.6	89.4	91.1	89.1	90.9	89.5	89.5	89.5	99.2		
Matlab13-03	11	98.7	89.2	90.9	89.0	90.7	89.5	89.5	89.5	99.4	99.2	
B4633-03	12	98.8	89.4	91.0	89.0	90.8	89.5	89.5	89.5	99.7	99.3	99.5

VP2

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	91.3										
AU-1	3	93.0	95.3									
L26	4	91.4	99.0	95.7								
T152	5	93.5	95.5	99.1	96.0							
RV161-00	6	90.9	98.8	95.2	99.0	95.4						
RV176-00	7	91.0	98.7	95.1	98.8	95.3	99.9					
N26-01	8	91.0	98.7	95.1	98.8	95.3	99.9	100.0				
Dhaka25-02	9	97.7	91.0	92.6	91.0	93.0	90.6	90.8	90.8			
Dhaka12-03	10	97.7	91.1	93.1	91.1	93.4	90.8	90.9	90.9	98.7		
Matlab13-03	11	97.8	91.1	92.9	91.1	93.1	90.8	90.9	90.9	99.5	99.0	
B4633-03	12	97.6	90.9	92.8	90.9	93.0	90.5	90.6	90.6	99.0	98.8	99.2

VP3

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	79.9										
AU-1	3	81.8	81.9									
L26	4	95.1	77.2	80.3								
T152	5	82.7	82.5	92.9	80.8							
RV161-00	6	79.9	95.5	81.8	77.8	82.5						
RV176-00	7	79.9	95.5	81.8	77.8	82.5	100.0					
N26-01	8	79.9	95.3	81.8	77.8	82.5	99.9	99.9				
Dhaka25-02	9	98.3	79.2	81.8	95.2	82.9	79.2	79.2	79.2			
Dhaka12-03	10	98.3	79.3	82.1	95.2	82.9	79.3	79.3	79.3	99.8		
Matlab13-03	11	98.0	78.9	80.8	95.7	82.2	79.0	79.0	79.0	97.0	97.0	
B4633-03	12	98.5	79.3	81.9	95.2	83.1	79.3	79.3	79.3	99.6	99.6	99.8

VP4

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	88.8										
AU-1	3	56.1	57.5									
L26	4	88.7	95.1	56.9								
T152	5	55.3	56.7	95.5	55.9							
RV161-00	6	76.0	73.9	58.9	74.7	58.3						
RV176-00	7	75.9	74.0	59.1	74.9	58.5	99.6					
N26-01	8	75.2	73.5	58.7	74.0	57.9	98.3	98.7				
Dhaka25-02	9	94.1	90.5	57.7	90.7	58.3	75.4	75.2	74.7			
Dhaka12-03	10	75.5	73.9	59.1	74.4	58.5	99.0	99.4	98.6	75.0		
Matlab13-03	11	75.9	74.0	58.9	74.9	58.3	99.6	99.7	98.4	75.0	99.1	
B4633-03	12	93.6	90.1	57.1	90.2	55.7	74.9	74.7	74.2	99.2	74.5	74.5

VP6

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	91.9										
AU-1	3	91.3	93.5									
L26	4	91.3	99.2	93.0								
T152	5	91.3	95.1	97.4	94.8							
RV161-00	6	91.6	98.7	94.6	98.0	96.1						
RV176-00	7	91.6	98.7	94.6	98.0	96.1	100.0					
N26-01	8	90.8	98.5	93.8	97.7	95.4	99.2	99.2				
Dhaka25-02	9	97.7	92.4	91.9	91.9	91.3	92.4	92.4	91.6			
Dhaka12-03	10	97.7	92.4	91.9	91.9	91.3	92.4	92.4	91.6	100.0		
Matlab13-03	11	97.7	92.4	91.9	91.9	91.3	92.4	92.4	91.6	100.0	100.0	
B4633-03	12	98.0	92.1	91.9	91.6	91.3	92.1	92.1	91.3	99.7	99.7	99.7

NSP1

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	62.1										
AU-1	3	43.6	44.7									
L26	4	63.0	93.6	44.7								
T152	5	37.7	32.9	39.2	33.7							
RV161-00	6	62.4	93.2	47.5	90.9	34.5						
RV176-00	7	62.4	93.0	47.5	90.7	34.1	99.8					
N26-01	8	62.1	93.0	47.5	90.7	34.5	99.8	99.6				
Dhaka25-02	9	82.0	84.5	44.7	83.0	34.9	83.9	83.9	84.2			
Dhaka12-03	10	81.5	83.9	44.3	82.4	34.5	83.3	83.3	83.6	99.4		
Matlab13-03	11	81.7	84.2	44.7	82.7	34.9	83.6	83.6	83.9	99.6	99.8	
B4633-03	12	80.7	83.0	45.8	81.8	34.9	83.6	83.6	83.9	97.5	97.3	97.5

NSP2

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	87.9										
AU-1	3	88.3	84.7									
L26	4	95.5	88.3	89.0								
T152	5	89.7	86.5	94.8	90.1							
RV161-00	6	90.4	93.8	87.6	90.1	88.3						
RV176-00	7	90.4	93.8	87.6	90.1	88.3	100.0					
N26-01	8	96.8	87.6	87.9	96.1	89.0	89.7	89.7				
Dhaka25-02	9	96.8	87.6	87.9	96.1	89.0	89.7	89.7	100.0			
Dhaka12-03	10	96.5	87.6	87.2	95.8	88.3	89.0	89.0	99.0	99.0		
Matlab13-03	11	96.8	87.6	87.9	96.1	89.0	89.7	89.7	100.0	100.0	99.0	
B4633-03	12	96.5	87.6	87.6	95.8	88.7	89.4	89.4	99.7	99.7	98.7	99.7

NSP3

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	79.3										
AU-1	3	81.3	88.0									
L26	4	79.7	99.0	88.4								
T152	5	81.3	88.7	95.4	89.1							
RV161-00	6	80.5	97.7	88.0	97.4	88.7						
RV176-00	7	80.5	97.7	88.0	97.4	88.7	100.0					
N26-01	8	80.5	97.7	88.0	97.4	88.7	100.0	100.0				
Dhaka25-02	9	95.4	79.7	81.6	80.1	80.5	81.6	81.6	81.6			
Dhaka12-03	10	97.1	80.1	82.0	80.5	81.6	81.3	81.3	81.3	97.1		
Matlab13-03	11	80.1	97.4	87.7	97.1	88.4	99.7	99.7	99.7	81.3	80.9	
B4633-03	12	97.1	79.7	81.6	80.1	81.3	80.9	80.9	80.9	97.1	99.0	80.5

NSP4

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	81.9										
AU-1	3	82.6	82.6									
L26	4	81.9	94.7	83.2								
T152	5	83.2	81.9	94.1	83.2							
RV161-00	6	95.3	84.6	82.6	83.2	83.2						
RV176-00	7	83.9	81.9	89.1	83.2	87.2	83.9					
N26-01	8	84.6	82.6	89.8	83.9	87.9	84.6	99.4				
Dhaka25-02	9	95.9	84.6	83.2	83.2	83.9	97.7	84.6	85.2			
Dhaka12-03	10	94.7	83.9	81.9	82.6	82.6	99.4	83.2	83.9	97.1		
Matlab13-03	11	95.3	84.6	82.6	83.2	83.2	100.0	83.9	84.6	97.7	99.4	
B4633-03	12	95.3	83.2	81.2	83.2	81.9	97.7	83.2	83.9	96.5	97.1	97.7

NSP5

		1	2	3	4	5	6	7	8	9	10	11
Wa	1											
DS-1	2	79.7										
AU-1	3	88.7	85.8									
L26	4	92.0	82.2	88.7								
T152	5	88.7	83.4	92.0	90.4							
RV161-00	6	80.9	95.8	86.4	82.2	85.8						
RV176-00	7	79.7	95.8	85.2	80.9	84.6	99.0					
N26-01	8	79.7	94.8	85.2	80.9	84.6	99.0	97.9				
Dhaka25-02	9	94.2	82.8	90.9	97.9	91.5	84.0	82.8	82.8			
Dhaka12-03	10	94.2	82.8	90.9	97.9	91.5	84.0	82.8	82.8	100.0		
Matlab13-03	11	93.1	82.8	90.9	96.4	90.9	84.0	82.8	82.8	98.5	98.5	
B4633-03	12	94.2	82.8	90.9	97.9	91.5	84.0	82.8	82.8	100.0	100.0	98.5

FIG. 5. Percentages of amino acid identity among different proteins (VP1, VP2, VP3, VP4, VP6, NSP1, NSP2, NSP3, NSP4, and NSP5) of G12 rotavirus strains and three reference strains from the major human rotavirus genogroups.

related to Bangladeshi strain BD426 and Indian strain RMC/G66 (Fig. 4).

The Indian human G12 strains were found in combination with the P[4], P[6], and P[8] specificities (35). The VP7 gene segments of these strains were most closely related to the Bangladeshi G12 strains belonging to lineage III (Fig. 2).

The VP7 and VP4 amino acid sequences of the Japanese G12P[9] rotaviruses isolated between 2003 and 2004 (strains CP727, CP1030, and K12) (37) were almost identical to those of the Thai strain T152 and clustered together in G12 lineage II (Fig. 2).

The G12P[9] strains HC91 and Buenos (named after the place of isolation, since no name was assigned) were isolated during 2003 and 2004 in Brazil and Argentina, respectively (4, 36). Both their VP7 and their VP4 gene segments were very similar to each other and to the Asian AU-1-like G12 strains in lineage II (Fig. 2).

The first nonhuman G12 strain, RU172 (G12P[7]), was iso-

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Wa	G1	1																																
DS-1	G2	2	70.9																															
AU-1	G3	3	80.0	71.3																														
ST3	G4	4	74.2	67.1	72.6																													
OSU	G5	5	75.4	70.1	82.9	72.2																												
NSDV	G6	6	78.8	71.3	80.3	73.4	79.2																											
PO-13†	G7	7	43.7	44.2	48.4	46.3	47.9	46.3																										
B37	G8	8	71.3	71.7	80.0	68.4	76.1	77.3	44.7																									
Wi61	G9	9	77.7	74.2	83.7	77.3	80.3	81.1	48.9	78.1																								
B223	G10	10	75.7	68.8	80.0	66.8	76.1	78.8	46.3	74.6	78.8																							
YM	G11	11	76.1	72.6	85.8	72.6	88.6	80.3	47.4	80.0	84.4	77.3																						
PHI/L26/1987	G12	12	73.4	72.6	78.1	69.7	78.1	75.4	49.4	74.6	79.6	74.2	78.1																					
IND/RU172/2004	G12	13	75.7	74.2	78.5	70.6	76.5	75.4	46.3	72.6	80.7	73.0	77.3	93.6																				
THA/T152/1998	G12	14	76.5	73.8	80.0	71.7	78.5	76.9	47.4	74.6	80.0	74.2	79.6	94.3	94.3																			
JAP/CP1030/2003	G12	15	76.1	73.8	80.3	72.2	78.8	76.5	47.9	75.0	79.6	74.6	80.0	94.6	94.0	99.7																		
JAP/CP727/2003	G12	16	76.5	73.8	80.0	71.7	78.5	76.9	47.4	74.6	80.0	74.2	79.6	94.3	94.0	99.7	99.4																	
JAP/K12/2004	G12	17	76.1	73.4	80.0	71.3	78.1	76.5	46.8	74.2	79.6	73.8	79.2	94.0	94.3	99.7	99.4	99.1	99.7															
ARG/Buenos/2003‡	G12	18	76.1	73.8	79.6	71.3	78.1	76.5	47.4	74.2	79.6	73.8	79.2	94.0	94.3	99.7	99.4	99.4	99.1															
BRA/HC91/2004*	G12	19	76.5	73.8	80.0	71.7	78.5	76.9	47.4	74.6	80.0	74.2	79.6	94.3	94.3	100.0	99.7	99.7	99.4	99.7														
BEL/B4633-03/2003	G12	20	75.7	73.4	78.5	70.9	76.5	75.7	45.8	73.0	79.2	73.0	78.5	92.3	93.6	97.5	97.2	97.2	98.9	97.2	97.5													
IND/ISO-1/2001	G12	21	76.1	73.4	79.2	71.7	77.3	76.5	46.3	73.4	80.0	73.8	79.2	93.0	94.0	98.4	98.1	98.1	97.8	98.1	98.4	99.1												
IND/ISO-2/2001	G12	22	76.1	73.4	79.2	71.7	77.3	76.1	45.8	73.4	80.0	73.4	79.2	93.0	94.0	98.1	97.8	97.8	97.5	97.8	98.1	98.8	99.7											
IND/ISO-5/2001	G12	23	76.1	73.4	79.2	71.7	77.3	76.5	46.3	73.4	80.0	73.8	79.2	93.0	94.0	98.4	98.1	98.1	97.8	98.1	98.4	99.1	100.0	99.7										
BAN/RV161-00/2000	G12	24	76.1	73.4	79.2	71.7	77.3	76.1	45.8	73.4	80.0	73.4	79.2	93.0	94.0	98.1	97.8	97.8	97.5	97.8	98.1	98.8	99.7	100.0	99.7									
BAN/RV176-00/2000	G12	25	76.1	73.4	79.2	71.7	77.3	76.1	45.8	73.4	80.0	73.4	79.2	93.0	94.0	98.1	97.8	97.8	97.5	97.8	98.1	98.8	99.7	100.0	99.7	100.0								
BAN/N26-02/2002	G12	26	75.7	73.4	79.6	71.7	78.1	75.7	46.8	73.8	79.6	73.8	79.6	93.6	94.0	97.5	97.8	97.2	96.9	97.2	97.5	98.1	99.1	99.4	99.1	99.4	99.4							
USA/Se585/1999**	G12	27	76.1	73.4	79.2	71.7	77.3	76.5	46.3	73.4	80.0	73.8	79.2	93.0	94.0	98.4	98.1	98.1	97.8	98.1	98.4	99.1	100.0	99.7	100.0	99.7	99.7	99.1						
BAN/Dhaka25-02/2002	G12	28	76.5	73.8	79.2	70.9	77.3	75.7	45.8	73.4	79.2	74.2	79.2	93.0	93.3	98.1	97.8	97.8	97.5	97.8	98.1	98.4	99.4	99.1	99.4	99.1	99.1	98.4	99.4					
BAN/Dhaka12-03/2003	G12	29	76.1	74.2	79.2	70.9	78.1	77.3	45.8	73.4	79.8	73.8	78.1	92.7	93.6	97.5	97.2	97.2	96.9	97.2	97.5	98.1	99.1	98.8	99.1	98.8	98.8	98.1	99.1	98.4				
BAN/Matlab13-03/2003	G12	30	75.4	73.4	78.1	69.7	76.1	76.9	45.3	73.0	78.8	72.6	77.3	92.0	93.0	96.9	96.6	96.6	96.2	96.6	96.9	97.5	98.4	98.1	98.4	98.1	98.1	97.5	98.4	97.8	98.8			
L338	G13	31	70.9	68.0	77.7	70.5	75.0	75.0	45.8	71.7	75.7	70.1	76.1	75.4	75.7	76.1	76.1	76.1	75.7	75.7	76.1	75.7	76.1	75.7	76.1	75.7	75.7	76.1	76.1	75.4	76.5	76.1		
FI23	G14	32	76.1	70.1	83.7	74.6	76.9	75.7	49.4	75.7	84.0	72.2	82.2	76.1	76.9	77.3	77.7	77.7	77.3	76.9	77.3	77.3	76.5	77.3	77.3	77.3	77.3	77.3	77.7	77.3	77.3	76.5	75.7	74.6
Hg18	G15	33	73.6	64.6	76.5	69.7	75.7	74.2	44.2	70.9	76.1	72.2	75.7	71.7	70.1	71.7	72.2	71.7	71.3	71.3	71.7	69.3	70.1	70.1	70.1	70.1	70.1	70.5	70.1	69.7	70.5	70.5	69.3	72.2

* partial sequence amino acid 9 to 322.
** partial sequence amino acid 3 to 325.
† three amino acids were deleted
‡ the strain name was unavailable and was named after the origin of isolation (Buenos Aires, Argentina)

FIG. 6. Percentages of amino acid identity among VP7 proteins of G12 rotavirus strains and other reference strains from 15 G genotypes. ARG, Argentina; BAN, Bangladesh; BEL, Belgium; BRA, Brazil; IND, India; JAP, Japan; PHI, Philippines; THA, Thailand.

lated recently from a pig in India (10). The VP7 gene of strain RU172 showed considerable sequence diversity at the amino acid level (5.4% to 7.0%) compared with the cognate genes of human G12 strains and was placed in lineage IV (Fig. 2 and 6). The VP6, NSP4, and NSP5 gene segments of this strain were shown to be closely related to the porcine rotavirus strains OSU and YM (Fig. 1 and 4).

DISCUSSION

Most rotavirus genotyping studies have focused mainly on G (VP7) and P (VP4) genotyping. Currently, there is a paucity of information regarding the other gene segments (e.g., the VP1, VP2, VP3, NSP2, and NSP3 gene segments, etc.) and their role in the pathogenicity, severity, propagation, and spread of the virus is largely unknown. All the strains discussed in this study were initially called G12 rotaviruses based on one gene segment (the VP7 gene segment); however, they were shown to be very different when all the 11 gene segments were considered. By characterizing several complete genomes, we have shown that rotaviruses are basically a population of reassortants where unpredictable variations in gene combinations are very common. Thus, our study underscores the need for complete genome-based "genogrouping" to illustrate the whole story regarding the diversity, evolution, and origin of viruses with segmented genomes.

G12 rotaviruses have until recently received little attention. Since 1998, a decade after their first detection in the Philippines, G12 strains have been isolated in Asia, Europe, South America, and North America, suggesting their possible emergence worldwide. The strong increase in the frequency of detection of these G12 rotavirus strains raises questions concerning their origin and evolution and how

TABLE 2. Gene-protein assignments

G12 rotavirus strain	Strain similar to indicated protein encoded by indicated RNA segment (% amino acid similarity)										
	VP1	VP2	VP3	VP4	NSP1	VP6	NSP3	NSP2	VP7	NSP4	NSP5
	1	2	3	4	5	6	7[a]	8[a]	9[a]	10	11
L26	DS-1 (96.7)	DS-1 (99.0)	Wa (95.1)	DS-1 P[4] (95.1)	DS-1 (93.6)	DS-1 (95.1)	DS-1 (99.0)	Wa (95.5)	G12-I	DS-1 (94.7)	Wa (92.0)
T152	AU-1 (98.3)	AU-1 (99.1)	AU-1 (92.9)	AU-1 P[9] (95.5)	None	AU-1 (97.4)	AU-1 (95.4)	AU-1 (94.8)	G12-II	AU-1 (94.1)	RRV (99.0)
RV161-00	DS-1 (97.6)	DS-1 (98.8)	DS-1 (95.5)	ST3 P[6] (96.0)	DS-1 (93.2)	DS-1 (98.7)	DS-1 (97.7)	DS-1 (93.8)	G12-III	Wa (95.3)	DS-1 (95.8)
RV176-00	DS-1 (97.6)	DS-1 (98.7)	DS-1 (95.5)	ST3 P[6] (96.0)	DS-1 (93.0)	DS-1 (98.7)	DS-1 (97.7)	DS-1 (93.8)	G12-III	AU-1[b] (89.8)	DS-1 (95.8)
N26-02	DS-1 (97.6)	DS-1 (98.7)	DS-1 (95.3)	ST3 P[6] (95.0)	DS-1 (93.0)	DS-1 (98.5)	DS-1 (97.7)	Wa (96.8)	G12-III	AU-1[b] (89.1)	DS-1 (94.8)
Dhaka25-02	Wa (98.7)	Wa (97.7)	Wa (96.3)	Wa P[8] (94.1)	ST3 (93.0)	Wa (97.7)	Wa (95.4)	Wa (96.8)	G12-III	Wa (95.9)	Wa (94.2)
Dhaka12-03	Wa (98.6)	Wa (97.7)	Wa (96.3)	ST3 P[6] (93.6)	ST3 (93.0)	Wa (97.7)	Wa (97.1)	Wa (96.5)	G12-III	Wa (94.7)	Wa (94.2)
Matlab13-03	Wa (98.7)	Wa (97.8)	Wa (96.0)	ST3 P[6] (95.0)	ST3 (93.0)	Wa (97.7)	DS-1 (97.4)	Wa (96.8)	G12-III	Wa (95.3)	Wa (93.1)
B4633-03	Wa (98.8)	Wa (97.6)	Wa (96.5)	Wa P[8] (93.6)	ST3 (91.0)	Wa (98.0)	Wa (97.1)	Wa (96.5)	G12-III	Wa (95.3)	Wa (94.2)

[a] The gene-protein assignment shown here is identical to that for the SA11 rotavirus strain. Depending on the strain, the gene-protein assignments for RNA segments 7 to 9 differ; however, the RNA triplet always encodes VP7, NSP2, and NSP3.
[b] The NSP4 gene segments of RV176-00 and N26-02 are only distantly related to the NSP4 gene segment of AU-1.

they were able to spread all over the world. A summary of our attempts to genotype the different gene segments of G12 rotaviruses included in this study is given in Table 2, which allows the following conclusions regarding their evolution, origin, and spread.

The vast majority of G12 rotavirus strains have been isolated in Asia and, more specifically, in Southeast Asia. Our study has shown that a very large genetic diversity is present in the G12 population, caused mainly by genetic reassortments. This geographical region might be the main origin of all the different G12 strains isolated all over the world. This possibility is strengthened by the observation that from 25 G12 rotavirus strains isolated in India between 2003 and 2005, three different G- and P-genotype combinations were found (G12P[8], G12P[6], and G12P[4]) (35). Even more variation might be found when the other nine gene segments are also analyzed. A similar observation was recently made in Nepal, where 29 G12 strains showed at least five different electropherotypes, suggesting the existence of at least five different gene constellations, similar to the situation found in India and Bangladesh (40). From Southeast Asia, they might be transported across the globe by the increasing mobility of humans and animals. This might have happened to the Belgian strain B4633-03, which was nearly identical to strain Dhaka25-02 and to the United Kingdom strain MV404-02, which was closely related to strain Dhaka12-03. Although only a very limited number of sequences from G12P[9] strains isolated in Japan, Brazil, and Argentina are available, they seem to show high resemblances to the Thai AU-1-like G12 reference strain T152. Further analysis and comparison of these strains could reveal the full story of their relatedness.

The origin of the G12 moiety remains obscure, but the recent isolation of a G12 rotavirus from a pig (strain RU172) and the observation that the first G12 rotavirus (strain L26) has OSU-like NSP2 and YM/OSU-like NSP5 gene segments (Fig. 3 and 4) might be indications that G12 has an animal, more specifically porcine, origin. The further investigation of this possibility is hampered by the very small amount of sequence data available for porcine rotavirus strains, which underscores the need for more (sequence) data on animal rotaviruses.

The most successful among the current reassortant human strains is the human G9 rotavirus. G9 was first detected in 1983, and after about 12 years of being detected only very sporadically, it became one of the most predominant rotavirus strains (32, 36). Similar to the G12 rotaviruses, G9 also exhibited a high reassortment activity (20, 41). G12 might be mirroring the pattern of evolutionary events that led to the worldwide emergence of G9 as a major human rotavirus genotype. This possibility is strengthened by the observation that G12 rotaviruses are being detected regularly in recent surveys in Argentina (6% from 1999 to 2003), Nepal (20% in 2003 and 2004), and India (30% in 2005) (4, 35, 40) and other unpublished data from Australia, Iran, Saudi Arabia, Slovenia, Hungary, and South Africa.

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A rotavirus strain isolated from pig-tailed macaque (*Macaca nemestrina*) with diarrhea bears a P6[1]:G8 specificity

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Abstract

A distinct rotavirus strain (PTRV) was isolated in cell cultures from a stool sample obtained from a diarrheic 3-year-old female pig-tailed macaque (*Macaca nemestrina*) that was born at the breeding colony of the University of Washington in Seattle. Unlike other known simian rotavirus strains including vervet monkey rotavirus SA11 which bears P5B[2]:G3 or P6[1]:G3 specificity, rhesus monkey rotavirus MMU18006 with P5B[3]:G3 specificity, pig-tailed macaque rotavirus YK-1 with P[3]:G3 specificity and rhesus monkey rotavirus TUCH with P[24]:G3 specificity, the cell-culture-grown PTRV strain was shown to bear P6[1]:G8 specificity as determined by VP4 (P)- and VP7 (G)-specific neutralization assays as well as gene sequence analyses. The virus in the original diarrhea stool was also shown to bear genotypes P[1] and G8. In addition, the PTRV strain exhibited a "long" electropherotype, subgroup I specificity and NSP4 genotype A specificity. The PTRV probe formed (i) 8–9 hybrid bands with genomic RNAs of various bovine rotavirus strains and (ii) only 2–3 hybrid bands with simian rotavirus RNAs as demonstrated by RNA–RNA hybridization, suggesting a possible bovine origin of the virus. Serologic analysis of serum samples obtained from selected pig-tailed macaques in the colony suggested that a rotavirus bearing P[1]:G8 specificity was endemic among macaques for at least 8 years (1987–1994). This is the first report describing an isolation of a simian rotavirus bearing a non-G3 VP7 and possibly a P6[1] specificities. Because of its unique simian serotype, this virus may prove to be valuable in challenge studies in a non-human primate model in studies of rotavirus immunity.

Keywords: Rotavirus; Simian rotavirus; Pig-tailed macaque rotavirus

Introduction

Group A rotaviruses are the single most important etiologic agents of severe diarrhea of infants and young children worldwide and are estimated to be responsible for a median of approximately 440,000 deaths each year among children <5 years of age, primarily in the developing countries (Kapikian et al., 2001; Parashar et al., 2003). Thus, the development and implementation of a safe and effective rotavirus vaccine remain important global public health goals.

Group A rotaviruses are ubiquitous in almost all mammalian and avian species with some rare exceptions such as guinea pigs and rats which do not undergo natural group A rotavirus infection (Kapikian et al., 2001). Since humans and non-human primates share various similar genetic and physiologic characteristics, rotavirus strains isolated from non-human primates have received special attention, being used extensively in both basic and applied rotavirus research. For example, (i) strain SA11 isolated from a healthy vervet monkey in South Africa in 1958 and cultivated efficiently in cell culture (Malherbe and Strickland-Cholmley, 1967) has been a prototype strain of

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group A rotaviruses and played a central role in our understanding of molecular biology of rotaviruses (Estes, 2001) and (ii) strain MMU18006 isolated from a diarrheic rhesus monkey in the United States in 1979 (Stuker et al., 1980) is the serotype 3 component of a quadrivalent (serotype 1, 2, 3 and 4) vaccine (Midthun et al., 1985, 1986), which was the first licensed human rotavirus vaccine in the US (*RotaShield*, Wyeth-Lederle Vaccines and Pediatrics, Philadelphia, PA; Biovirx, USA) (Advisory Committee on Immunization Practices, 1999; Spiegel, 2003).

A complete rotavirus particle possesses a triple-capsid architecture: outer, intermediate and inner capsid or core. The core encases 11 segments of double-stranded (ds) RNA that encode 6 structural (VP1–4, 6 and 7) and 6 nonstructural (NSP1–6) proteins. Outer capsid proteins VP4 (spike protein) and VP7 (major surface glycoprotein) are independent neutralization (Greenberg et al., 1985; Hoshino et al., 1985; Offit and Blavat, 1986) and protective (Hoshino et al., 1988; Matsui et al., 1989; Offit et al., 1986) antigens. Thus, rotavirus has a binary system for serotype classification and nomenclature: VP7 or G (VP7 is a glycoprotein) serotype and VP4 or P (VP4 is protease sensitive) serotype (Estes, 2001; Kapikian et al., 2001). By definition, "serotype" is a type determined by serological methods with neutralization assay as the keystone, whereas "genotype" is a type determined by non-serological methods such as RT-PCR, hybridization or gene sequencing. Fourteen G serotypes (15 G genotypes) and 14 P serotypes (25 P genotypes) have been established thus far (Liprandi et al., 2003; Martella et al., 2003; McNeal et al., 2005; Rahman et al., 2005). The number of rotavirus G and P types detected varies markedly from one animal species to another. For example, eleven G types and 12 P types have been detected in humans, whereas only one G and 2 P types have been detected in mice. Among the established 15 G types, G3 presents unique features: (i) it has the broadest animal host range which includes human, rhesus monkey, vervet monkey, pig-tailed macaque, horse, cow, goat, pig, dog, cat, rabbit and mouse; and (ii) in some animal species (e.g., mouse, rabbit, cat, dog and simians), the G3 has been the only type detected.

Although various species of non-human primates have played an indispensable role in basic research as well as in vaccine development of many infectious diseases of humans such as HIV and viral hepatitis, only a few simian rotavirus strains have been isolated and characterized. Only five simian rotavirus strains have been reported; (i) the vervet monkey rotavirus SA11 strain (Malherbe and Strickland-Cholmley, 1967; Estes et al., 1982; Mattion and Estes, 1991); (ii) the rhesus monkey rotavirus MMU18006 and MMU17959 strains (Stuker et al., 1980); (iii) pig-tailed macaque rotavirus YK-1 strain (Westerman et al., 2005a); and (iv) rhesus monkey rotavirus TUCH strain (McNeal et al., 2005). A major reason why we have so few simian rotavirus strains may be because of a lack of surveillance, as all simian rotaviruses are thought to belong to G3, which may have restricted the development of a homologous simian rotavirus model, which would be important if a challenge model with a different serotype were available. In this paper, we report the isolation in cell cultures of a rotavirus strain (PTRV) derived from a diarrheic pig-tailed macaque and the determination of (i) the G and P specificities of the isolate by VP4- and VP7-specific neutralization and nucleotide sequence analyses, (ii) selected basic characteristics including electropherotype, subgroup specificity and NSP4 genotype, (iii) genetic characteristics as determined by whole genome RNA–RNA hybridization and (iv) prevalence of this virus among pig-tailed macaques in the colony.

Results and discussion

Isolation and characterization of pig-tailed macaque rotavirus PTRV strain

A rotavirus strain was isolated from a female pig-tailed macaque born in 1987 at the Medical Lake breeding facility of the Washington Regional (now National) Primate Center, the University of Washington, Seattle, Washington. Approximately 2 months after being put into group housing at 3 years of age, the monkey developed diarrhea, and a diarrheal stool sample was collected for examination. According to the animal records, this specific pig-tailed macaque had had diarrhea episodes often and various diarrhea-causing bacteria and/or parasites had been detected. However, on the date the animal was admitted to the treatment room in 1990, no such bacteria and/or parasites were detected. A rotavirus (designated as the PTRV strain) was isolated in MA104 cell cultures from this stool specimen. The virus isolate was passaged serially twice in MA104 cells and sent to the National Institutes of Health (NIH), Bethesda, Maryland for further characterization. At the NIH, the virus was passaged once in MA104 cells and then plaque-purified three times. The plaque-purified PTRV virus was used for analysis of various phenotypic and genotypic characteristics of the virus as well as for immunization of guinea pigs. The plaque-purified PTRV stain exhibited a "long" electropherotype as determined by polyacrylamide gel electrophoresis (PAGE), which was distinct from that of selected human and animal rotavirus strains including those of simian (strains SA11 [G3] and MMU18006 [G3]), human (strains 69M [G8], 1290 [G8] and HAL1166 [G8]) and bovine (strains Cody [G8], 678 [G8], NCDV [G6] and UK [G6]) origin (Fig. 1), indicating that the PTRV virus was not a laboratory contaminant introduced at the NIH. The electropherotype of viruses present in the first passage MA104 cell lysate was identical to that of the plaque-purified PTRV strain (Fig. 2), indicating that the PTRV virus was not a laboratory contaminant introduced at the University of Washington. In addition, when the virus was isolated in cell cultures from a diarrheal stool sample, no bovine rotaviruses existed in the laboratory. Analysis of RNA extracted from the approximately 20% original diarrheal stool suspension that was kept at −70 °C failed to produce a visible pattern in a silver-stained PAGE gel, indicating that the virus was not shed in large number in the stool. The PTRV strain was shown to bear subgroup I specificity (data not shown).

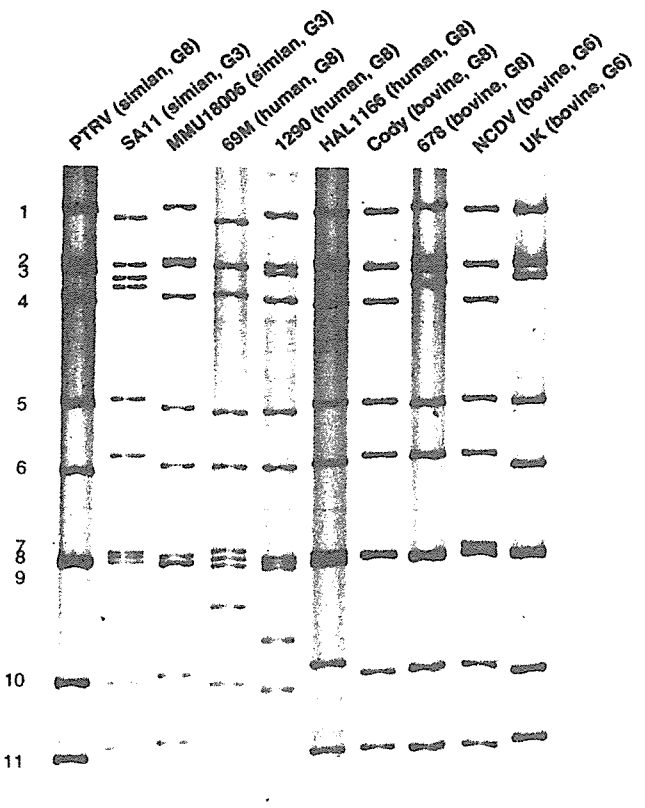

Fig. 1. Electrophoretic migration patterns of genomic RNAs of selected simian, human and bovine rotavirus strains (listed at the top) in 10% polyacrylamide gel. Genomic RNAs were electophoresed at 13 mA for 15 h, and the resulting migration patterns were visualized by staining of gel with silver nitrate.

Determination of G serotype/genotype and P serotype/genotype of the PTRV strain

Tables 1, 2 and 3 summarize the neutralization characteristics of outer capsid proteins VP7 and VP4 of the PTRV strain. First, the PTRV virus was tested against a battery of antisera raised to selected reference rotavirus strains belonging to G1–G14. As shown in Table 1, the PTRV strain was: (i) neutralized to a high titer (1:10,240–1:20,480) which was well within 20 antibody units of the homologous titer by guinea pig hyperimmune antiserum raised to each of 3 G8 strains with differing VP4 specificities (69M, Cody and 678); (ii) neutralized by antiserum to NCDV (P6[1]:G6) to a moderate titer (1:640) which was 16-fold lower than the homologous titer (1:10,240); and (iii) neutralized to a low titer (1:80–1:160) by antiserum to P, SA11 or L338, which was more than 20 antibody units beyond its homologous titer. These results suggested from this one-way test that the PTRV strain belonged to serotype G8 and P6.

Next, we performed reciprocal neutralization assays in which two different antisera raised to the PTRV were tested against various reference rotavirus strains representing G1–G14 (Table 2) to determine if there was a two-way "G8, P6" relationship. Antisera to PTRV (i) neutralized each of five G8 rotavirus strains (69M, 1290, HAL1166, Cody and 678) to a titer similar or identical to that of the PTRV strain (1:10,240–1:20,480), (ii) neutralized the G6 NCDV strain to a 4- to 8-fold lower titer which was within 20 antibody units of its homologous titer and (iii) neutralized the G3, G5, G11, G13 and G14 viruses to a low titer of 1:160–1:320 which was not within 20 antibody units of its homologous titer. These results indicated that in this one-way test the PTRV strain shared (i) high levels of neutralization specificity with G8 viruses which was most likely via VP7 because of the different VP4 specificities of the G8 strains and (ii) high levels of neutralization specificity with the NCDV strain which may have been via VP4.

In order to clarify the VP4 serotype specificity of the PTRV virus, we generated several reassortant rotaviruses and their antisera in an attempt to dissociate the VP7–VP4 specificities. As shown in Table 3, the PTRV virus was neutralized to a high titer by antiserum to reassortant DS-1 × 69M which bore a single VP7 gene of human rotavirus 69M with G8 specificity and the remaining 10 genes of human rotavirus DS-1 strain. Moreover, in a reciprocal reassortant with regard to VP4 and VP7, the virus was not neutralized significantly by antiserum to reassortant 69M × DS-1 (P4[10]:G2) which bore only the 69M VP4 and the remaining genes from DS-1. In addition, the PTRV was not neutralized by antiserum to reassortant HAL1166 × DS-1 (P11[14]:G2) which bore only the HAL1166 VP4 and the remaining genes from DS-1, indicating again that the PTRV VP7 bore G8 specificity because of the high degree of neutralization of the HAL1166 strain by antiserum to the PTRV as shown previously in Table 2. Furthermore, antiserum to PTRV × DS-1 (P?:G2) neutralized the PTRV strain to a titer similar or identical to that of antiserum raised to G2 reassortant NCDV × DS-1 (P6[1]:G2) or Cody × DS-1 (P6[1]:G2), indicating that the PTRV, NCDV and Cody strains shared the same P6 specificity. The PTRV

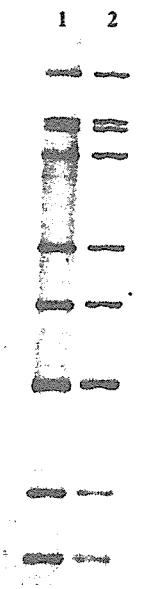

Fig. 2. Electrophoretic migration patterns of genomic RNAs of triply plaque-purified PTRV strain (lane 1) and the virus isolated in MA104 cell cultures from stool sample obtained from pig-tailed macaque with diarrhea (the first cell culture passage material) (lane 2).

Table 1
Neutralization profile of guinea pig hyperimmune antiserum raised to selected prototype rotavirus strains belonging to G1–G14 against pig-tailed macaque rotavirus PTRV strain

Rotavirus			Antibody titer[a] of guinea pig hyperimmune antiserum raised to indicated rotavirus strain								
Strain	G type	P type	Wa (P1A[8]:G1)	DS-1 (P1B[4]:G2)	P (P1A[8]:G3)	SA11 (P5B[2]:G3)	ST3 (P2A[6]:G4)	OSU (P9[7]:G5)	UK (P7[5]:G6)	NCDV (P6[1]:G6)	Ch2 (P?:G7)
PTRV	?	?	<80	<80	80	160	<80	<80	<80	640	<80
			69M (P4[10]:G8)	Cody (P6[1]:G8)	678 (P7[5]:G8)	WI61 (P1A[8]:G9)	B223 (P8[11]:G10)	YM (P9[7]:G11)	L26 (P1B[4]:G12)	L338 (P[18]:G13)	FI23 (P4[12]:G14)
PTRV	?	?	20480	10240	10240	<80	<80	<80	<80	80	<80

[a] Reciprocal of 60% plaque reduction neutralization antibody titer. The homologous 60% PRN antibody titers ranged from 10,240 to >81,920 in previous test, in this laboratory.

virus was neutralized to a low titer (1:160–1:640), which was not within 20 antibody units of its homologous titer by antiserum raised to each of three G3 reassortants DS-1 × P (P1B[4]:G3), DS-1 × RRV (P1B[4]:G3) and DS-1 × SA11 (P1B[4]:G3) with identical VP4 specificity, indicating that low levels of cross-reactivity between the G8 PTRV virus and G3 viruses (Table 1) were due to shared low levels of VP7 antigenic specificity because, as shown in Table 1, antisera to a P1B virus failed to neutralized the PTRV. Low levels (i.e., less than 20 antibody units) of neutralization cross-reactivity between G8 and G3 viruses were not unexpected since they share high level of amino acid identity in antigenic region C (VR8) of the VP7 protein (Green et al., 1989). In addition, a VP7-specific monoclonal antibody that neutralizes both G3 and G8 viruses has been generated (Kobayashi et al., 1991).

Although antiserum to SA11 × DS-1 (P5B[2]:G2) did not neutralize the PTRV virus, antiserum to RRV × DS-1 (P5B[3]:G2) neutralized the virus to a low titer (1:320) (Table 3) which was not within 20 antibody units of its homologous titer, indicating that the PTRV and RRV shared low levels of neutralization cross-reactivity not only on VP7 but also on VP4. Low levels of VP4 neutralization cross-

Table 2
Neutralization profile of guinea pig hyperimmune antisera raised to pig-tailed macaque rotavirus PTRV strain against selected prototype rotavirus strains belonging to G1–G14

Rotaviruses against which guinea pig PTRV antisera were tested						Antibody titer[a] of guinea pig hyperimmune antiserum to PTRV versus indicated virus		Reference
Strain	Host	Country of origin	Year collected	G type	P type [genotype]	serum #1	serum #2	
Wa	Human	USA	1974	1	1A[8]	<80	<80	Wyatt et al., 1982
DS-1	Human	USA	1976	2	1B[4]	<80	<80	Wyatt et al., 1982
P	Human	USA	1974	3	1A[8]	160	160	Wyatt et al., 1982
AU-1	Human	Japan	1982	3	3[9]	160	160	Kitaoka et al., 1987
Ro1845	Human	Israel	1985	3	5A[3]	320	160	Aboudy et al., 1988
MMU18006	Simian	USA	1979	3	5B[3]	320	320	Stuker et al., 1980
SA11	Simian	S. Africa	1958	3	5B[2]	320	160	Malherbe and Strickland-Cholmley, 1967
ST3	Human	UK	1975	4	2A[6]	<80	<80	Wyatt et al., 1983
OSU	Porcine	USA	1976	5	9[7]	320	320	Bohl et al., 1984
UK	Bovine	UK	1973	6	7[5]	<80	<80	Woode et al., 1975
NCDV	Bovine	USA	1967	6	6[1]	2560	2560	Mebus et al., 1971
Ch2	Chicken	UK	1980[b]	7	?	<80	<80	McNulty et al., 1980
69M	Human	Indonesia	1979–81	8	4[10]	20,480	20,480	Matsuno et al., 1985
1290	Human	Kenya	1991–94	8	1B[4]	20,480	10,240	Nakata et al., 1999
HAL1166	Human	Finland	1986	8	11[14]	10,240	10,240	Gerna et al., 1990
Cody	Bovine	USA	1995[b]	8	6[1]	10,240	10,240	Lu et al., 1995
678	Bovine	UK	1984[b]	8	7[5]	20,480	10,240	Ojeh et al., 1984
WI61	Human	USA	1983	9	1A[8]	<80	<80	Clark et al., 1987
B223	Bovine	USA	1983[b]	10	8[11]	<80	<80	Woode et al., 1983
YM	Porcine	Mexico	1983	11	9[7]	320	160	Ruiz et al., 1989
L26	Human	Philippines	1987–88	12	1B[4]	<80	<80	Urasawa et al., 1990
L338	Equine	UK	1991[b]	13	[18]	160	160	Browning et al., 1991a
FI23	Equine	UK	1991[b]	14	4[12]	320	160	Browning et al., 1991b
PTRV	Simian	USA	1990	?	?	20,480[c]	10,240	This study

[a] Reciprocal of 60% plaque reduction neutralization antibody titer. The homologous 60% PRN antibody titers ranged from 10,240 to > 81,920.
[b] Year published.
[c] Homologous values are underlined.

Table 3
Neutralization profile of guinea pig hyperimmune antiserum raise to selected reassortant rotaviruses against pig-tailed macaque rotavirus PTRV strain

Rotavirus			Antibody titer[a] of guinea pig hyperimmune antiserum raised to indicated reassortant						
Strain	G type	P type	DS-1 × 69M (P1B[4]:G8)	69M × DS-1 (P4[10]:G2)	HAL1166 × DS-1 (P11[14]:G2)	NCDV × DS-1 (P6[1]:G2)	Cody × DS-1 (P6[1]:G2)	PTRV × DS-1 (P?:G2)	DS-1 × P (P1B[4]:G3)
PTRV	?	?	40,960	80	<80	10,240	5120	5120	160
			DS-1 × RRV (P1B[4]:G3)	DS-1 × SA11 (P1B[4]:G3)	SA11 × DS-1 (P5B[2]:G2)	RRV × DS-1 (P5B[3]:G2)	OSU × DS-1 (P9[7]:G2)	YM × DS-1 (P9[7]:G2)	L338 × DS-1 (P[18]:G2)
PTRV	?	?	640	160	<80	320	160	320	160

[a] Reciprocal of 60% plaque reduction neutralization antibody titer. The homologous 60% PRN antibody titers ranged from 10,240 to 40,960 in previous tests in this laboratory.

reactivity between viruses bearing P5B[3] (RRV) and P6[1] (NCDV) specificities were reported previously (Midthun et al., 1985).

Antiserum to each of three G2 reassortants OSU × DS-1 (P9[7]:G2), YM × DS-1 (P9[7]:G2) or L338 × DS-1 (P[18]:G2) neutralized the PTRV virus to a low titer (1:160–1:320), which was not within 20 antibody units of its homologous titer, indicating that the cross-reactivity observed between the PTRV and OSU, YM or L338 (Table 1) was due to shared low levels of VP4 cross-reactivity. Thus, these neutralization results demonstrated that the PTRV strain bore a P6:G8 specificity.

The virus in the original stool obtained from diarrheal pig-tailed macaque was shown by PCR typing assay to bear genotypes P[1] and G8, confirming that the PTRV strain was not a laboratory contaminant.

Sequence analysis of the gene encoding VP7, VP8 or NSP4 of the PTRV strain*

The deduced amino acid (aa) sequence identity of the PRTV VP7 for selected G8 strains ranged from 93.9% (human rotavirus MW23 strain) to 98.6% (bovine rotavirus Tokushima 9503 strain and guanaco rotavirus GRV Rio Negro strain). Consistent with this finding, phylogenetic analysis of the VP7 gene indicated that the PTRV VP7 gene was more closely related to that of Tokushima9503 or GRV Rio Negro than that of MW23 (Fig. 3).

The PTRV VP8* gene demonstrated the highest nucleotide (nt) identity with that of P[1] viruses (e.g., 97.0% with bovine rotavirus NCDV strain, 96.9% with bovine rotavirus C486 strain and 95.0% with simian rotavirus SA114fM strain), suggesting that the PTRV VP4 gene belonged to P[1] genotype. This finding was in agreement with neutralization characteristics of the PTRV VP4 (Tables 1, 2 and 3). Phylogenetic analysis of the PTRV VP8* gene demonstrated that it clustered with that of other P[1] viruses (Fig. 4).

Analysis of NSP4 gene of the PTRV showed that it belonged to NSP4 genotype A: 96.7% nt identity versus genotype A bovine rotavirus RF strain, 83.7% nt identity versus genotype B human rotavirus Wa strain and 82.7% nt identity versus genotype C human rotavirus AU-1 strain. This finding was confirmed by phylogenetic analysis of selected NSP4 genes belonging to genotype A, B or C which indicated

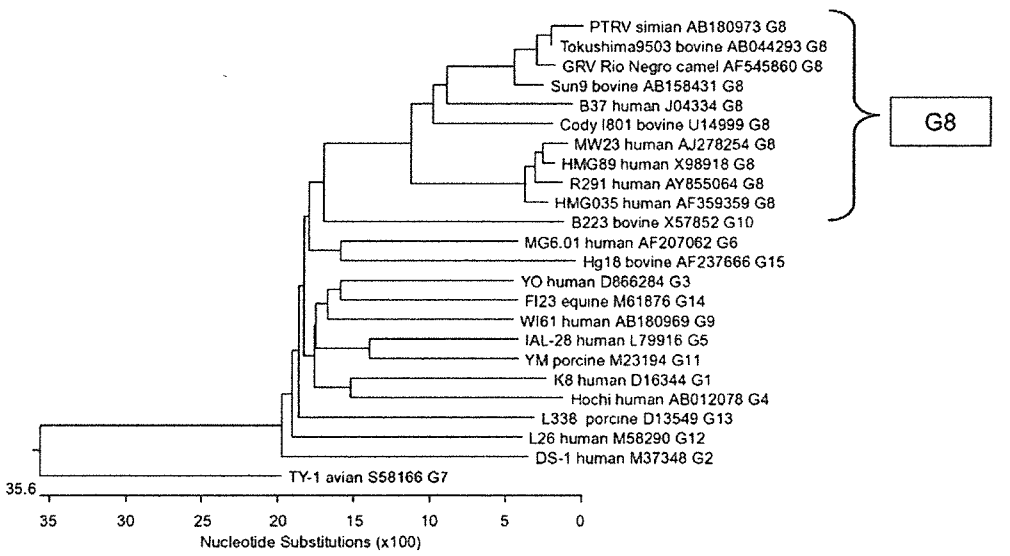

Fig. 3. Phylogenetic analysis of VP7 genes of the PTRV strain and other rotavirus strains representative of the 15 G types indicates that the PTRV VP7 gene belongs to G8 type. Phylogenetic tree was constructed by employing the Clustal W algorithm of the MegAlign program in DNASTAR software package (Madison, WI). The length of each pair of branches represents the distance between sequence pairs.