RNA Extraction

Viral RNA was extracted from 150 μl of 10–20% stool suspension in phosphate-buffered saline, using the Qiagen RNeasy minikit (QIAGEN Ltd., West Sussex, UK).

RT-PCR and Nucleotide Sequencing

Reverse transcription of the genomic RNA was carried out in a 50 μl reaction mixture containing random hexamers. An RT-PCR assay was employed that targeted the ORF1/ORF2 boundary region of the genome, comprising the 3′ terminal region of the polymerase gene and the 5′ terminal region of the capsid protein gene [Kojima et al., 2002]. For the amplification of the GI genome, primers GI-SKF (5′-CTG CCC GAA TTY GTA AAT GA-3′) and GI-SKR (5′-CCA ACC CAR CCA TTR TAC A-3′) were used [Kojima et al., 2002]. For the amplification of the GII genome, a single positive-sense primer GII-SKF (5′-CNT GGG AGG GCG ATC GCA A-3′) and a negative-sense primer GII-SKR (5′-CCR CCN GCATRH CCR TTR TAC AT-3′) were used [Kojima et al., 2002]. For the detection of the Alphatron type norovirus which is controversially grouped into either GII/17 [Okada et al., 2005] or GIV [Zheng et al., 2006], an additional negative-sense primer AL-SKR (5′-CCA CCA GCA TAT GAA TTG TAC AT-3′) was used. The PCR was performed with an initial denaturation step at 94°C for 3 min, followed by 40 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 50°C, an extension step at 72°C for 2 min, followed by final extension of 15 min at 94°C. RT-PCR products were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination. The expected product size for GI strains is 330 bp and that for GII and Alphatron type norovirus is 344 bp. Approximately one-third of RT-PCR products were sequenced, using the same primers used for RT-PCR amplification, for confirmation of the presence of norovirus and for the assignment of genotype after phylogenetic analysis. The RT-PCR products were purified using Amersham Biosciences Microspin Columns (Buckinghamshire, UK). Sequencing was carried out at Lark Technologies Laboratory (Essex, UK).

Phylogenetic Analysis

Phylogenetic relationships were illustrated by aligning sequences spanning the conserved ORF1/ORF2 boundary region [Kageyama et al., 2004] using the ClustalW multiple alignment program (DDBJ, Mishima, Japan). A phylogenetic tree was drawn according to the Neighbor-Joining method. Assignment of norovirus to genotype was made according to the scheme proposed by Okada et al. [2005].

RESULTS

Virus Prevalence, Clinical Features and Age Distribution

Of 260 stool samples examined, 78 (30%) were positive for norovirus. Among the 78 noroviruses identified, 18 (23%) belonged to GI, 58 (74%) belonged to GII, and two (3%) comprised both GI and GII. The median age of children with norovirus infection was 8 months (range 2–40 months), and who had a median duration of diarrhea of 3 days. While fever occurred in only 36% of norovirus-infected children, 82% of children had vomiting and intravenous fluids were administered in 99% of cases.

Comparison of Norovirus and Rotavirus Positive Samples

Among 13 (5%) children from whom both norovirus and rotavirus were detected, all had vomiting and received intravenous fluid replacement, and one child died. Clinical characteristics including fever, vomiting, and requirement for intravenous fluids were compared between 65 children with single norovirus infections and 83 children with single rotavirus infections. Vomiting was significantly more common among rotavirus cases (n = 76, 92%) compared with norovirus cases (n = 54, 83%) [$P = 0.005$], with no significant differences for the other features. When the age distribution of cases was compared (Fig. 1), there was a tendency for rotavirus to infect slightly younger children (mean age, 7 months) than for norovirus (mean age, 8 months) [$P = 0.052$]. There was no difference between the median

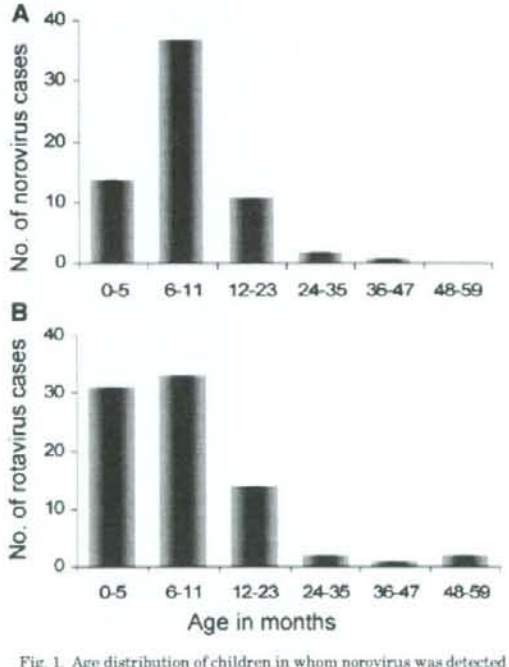

Fig. 1. Age distribution of children in whom norovirus was detected but rotavirus was not detected (**A**) and of children from whom rotavirus was detected but norovirus was not detected (**B**).

duration of diarrhea for norovirus vs. rotavirus infections (3 days for both).

Phylogenetic Analysis

Nucleotide sequences were obtained from 28 norovirus-containing specimens chosen at random from within each genogroup. These comprised 4 out of 18 GI-containing specimens, 23 out of 58 GII-containing specimens and 1 out of 2 specimens that comprised a mixed GI + GII norovirus infection. Based on the nucleotide sequences obtained, a phylogenetic tree was constructed that also included 14 reference GI noroviruses and 17 reference GII noroviruses (Fig. 2). The clustering pattern with one of the reference strains unambiguously identified the genotype (genetic cluster) of each of the Iraqi noroviruses sequenced (Fig. 2, Table I). While there were 3 and 4 discrete genotypes identified among GI and GII noroviruses, respectively, 19 specimens (66%) were shown to belong to GII/4, the commonest norovirus genotype, represented by the Lordsdale strain (UK).

DISCUSSION

Despite increasing recognition that norovirus is an important etiological agent of childhood diarrhea, relatively little is known about its epidemiological significance among children in developing countries, and in the Middle East in particular. This study, the first of its kind in the region, clearly documented the presence of norovirus in approximately one-third of Iraqi children under age 5 years who were admitted to hospital with severe, acute gastroenteritis. The detection rate of norovirus (30%) is only slightly lower than that of rotavirus, which was previously detected by ELISA in 37% of children [Ahmed et al., 2006]. Given that 5% of samples contained mixed infection with both norovirus and rotavirus, these two viruses together accounted for 62% of cases of acute gastroenteritis. Furthermore, it is likely that the number of rotavirus infections would have been further increased by the use of RT-PCR compared with ELISA [Amar et al., 2007]. Two issues deserve mention; first, viral agents

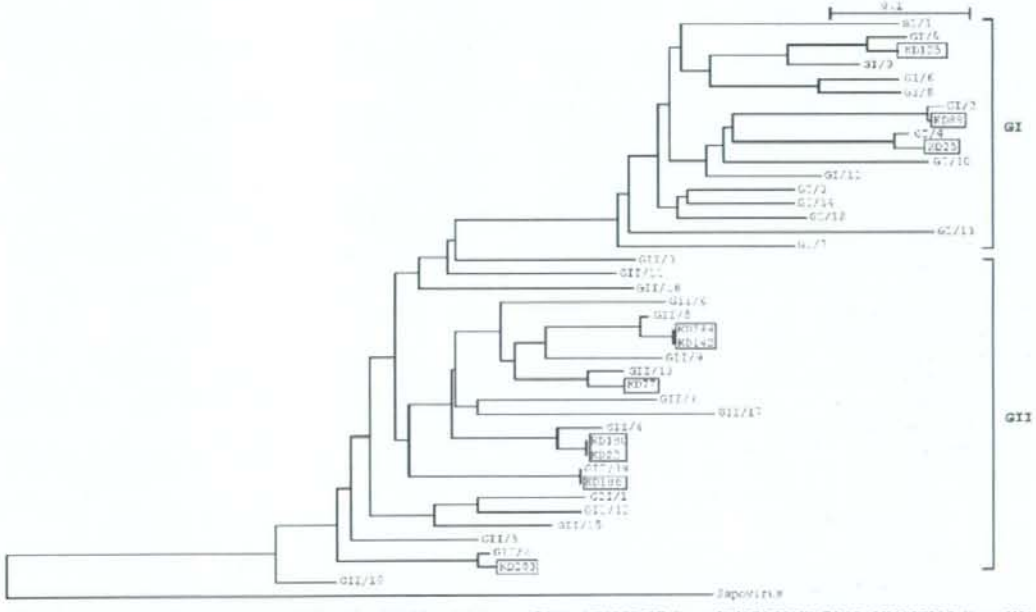

Fig. 2. A phylogenetic tree constructed by the Neighbor-Joining method based on the nucleotide sequences of the ORF1/ORF2 junction region. The Iraqi norovirus strains sequenced in this study are boxed, and are assigned the prefix KD followed by the sample number. The nucleotide sequences have been assigned the following accession numbers: KD22 (AB366006); KD77 (AB366007); KD106 (AB366008); KD142 (AB366009); KD180 (AB366010); KD244 (AB366011); KD203 (AB366012); KD25 (AB366013); KD88 (AB366014); and KD125 (AB366015). The following reference strains were used for each genotype: GI/1_M87661Norwalk/68/US, GI/2_L07418Southampton/91, GI/3_U04469DesertShieldDSV395/90/US, GI/4_AB042808Chiba-407/87/JP, GI/5_AJ277614Musgrove/89/UK, GI/6_AF093797BS5/98/GE, GI/7_AJ277609Winchester/94/UK, GI/8_AB081723WUG1/00/JP, GI/9_AB039774SaitamaSzUG1/99/JP, GI/10_AF538679Boxer/01/US, GI/11_AB058547SaitamaKU8GI/99/JP, GI/12_AB058525SaitamaKU-19aGI/00/JP, GI/13_AB112132SaitamaT35aGI/01/JP, GI/14_AB112-100SaitamaT25GI/01/JP, GII/1_U07611Hawaii/71/US, GII/2_X81879-Melksham/89/UK, GII/3_U22498Mexico/89/MX, GII/4_X86557Lords-dale/93/UK, GII/5_AJ277607Hillingdon/90/UK, GII/6_AB039776Sai-tamaU3/97/JP, GII/7_AJ277608Leeds/90/UK, GII/8_AB067543-SaitamaU25/98/JP, GII/9_AY054299IdahoFalls/378/96/US, GII/10_A-Y237415Mc37/99/Thai, GII/11_AB112221SaitamaT29GII/01/JP, GII/12_AB039775SaitamaU1/97/JP, GII/13_AY130761M7/99/US, GII/14_AB078334Kashiwa47/00/JP, GII/15_AB058582SaitamaKU80aGII/99/JP, GII/16_AB112260SaitamaT53GII/02/JP, GII/17_AF195847Alphatron/98/NE. As an outgroup, the Manchester strain of *Sapovirus* was used (X86560).

TABLE I. Relative Frequencies of Norovirus Genotypes Identified in 28 Fecal Specimens from Iraqi Kurdistan Children

Genotype	Number	Percentage
GI/2	1	3.4
GI/4[a]	3	10.4
GI/5	1	3.4
GII/2	1	3.4
GII/4[a]	19	65.6
GII/8	2	7
GII/13	1	3.4
GII/14	1	3.4
	29	100

[a]Specimen KD125 contained two sequences (GI/4 and GII/4), thus the total number of genotypes is 29; the number of norovirus-containing specimens is 28.

account for the majority of cases of severe diarrhea in this population. Second, norovirus has the potential to replace rotavirus in importance as rotavirus vaccines enter childhood immunization schedules over the next decade, with resulting expected decrease in the numbers of severe rotavirus gastroenteritis cases [Nakagomi and Cunliffe, 2007].

In keeping with many previous studies conducted elsewhere, noroviruses belonging to genogroup GII were predominant in this short survey [Moreno-Espinosa et al., 2004]. However, while amplifying and sequencing a short region of the highly conserved ORF1/ORF2 junction, we successfully grouped 28 noroviruses into 7 existing genotypes that were detected during a short period of 2 months in a defined area of northern Iraq. We demonstrated similarly extensive genetic diversity among rotaviruses in the same population, for which there were 7 combinations of G and P genotypes [Ahmed et al., 2006]. Noroviruses belonging to GII/4 accounted for the majority of noroviruses circulating in this region, and indeed noroviruses of this genotype have recently predominated in various parts of the world including in Europe [Lopman et al., 2004]. Whether Iraqi GII/4 noroviruses are the variant strain dominating in Europe is not known, since the polymerase region where the signature mutations for the variant are accumulated was not sequenced in the current study.

This study, which adds to the growing literature on norovirus infections among children in developing countries [Papaventis et al., 2007], is limited by its short duration, and included only hospitalized children. Nevertheless, the high norovirus detection rate and extensive genetic diversity identified should encourage further studies of this emerging pathogen among children in the region.

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Generation of Recombinant Rotavirus with an Antigenic Mosaic of Cross-Reactive Neutralization Epitopes on VP4[▽]

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Recombinant rotavirus (RV) with cDNA-derived chimeric VP4 was generated using recently developed reverse genetics for RV. The rescued virus, KU//rVP4(SA11)-II(DS-1), contains SA11 (simian RV strain, G3P[2])-based VP4, in which a cross-reactive neutralization epitope (amino acids 381 to 401) on VP5* is replaced by the corresponding sequence of a different P-type DS-1 (human RV strain, G2P[4]). Serological analyses with a panel of anti-VP4- and -VP7-neutralizing monoclonal antibodies revealed that the rescued virus carries a novel antigenic mosaic of cross-reactive neutralization epitopes on its VP4 surface. This is the first report of the generation of a recombinant RV with artificial amino acid substitutions.

Rotavirus (RV), a member of the family *Reoviridae*, is the leading etiological agent of severe gastroenteritis in infants and young children worldwide (15, 16). RV particles possess three concentric capsid layers that enclose an 11-segmented genome of double-stranded RNA (dsRNA) (6). The outer capsid VP4 is a spike protein essential for viral attachment and entry. It is also the major antigenic component on the surface of this virus. VP4 defines P serotypes, and at least 14 P serotypes have been assigned; however, since it is difficult to differentiate P serotypes serologically, P genotypes based on the VP4 sequence have been proposed and 26 P genotypes (P[1] to P[26]) have been reported (6).

The expression of foreign epitopes on viral surfaces has been reported for several positive- and negative-stranded RNA viruses (reviewed in references 1, 7, 14, 21, and 28). In contrast, *Reoviridae* members such as RV have been refractory to direct genetic manipulation, except for mammalian orthoreovirus (10, 17–19), and therefore could not be used as vectors for the expression of foreign epitopes. Only recently has reverse genetics for RV, which enables one to generate infectious RVs containing a cDNA-derived gene segment, been successful (11). This RV system is based on helper virus-driven reverse genetics, which was originally developed for influenza viruses by Enami and colleagues (4, 5, 12).

In the present study, we used this system to introduce site-specific mutations into one of the three cross-reactive neutralization epitopes of RV VP4, which resulted in the preparation of previously undescribed recombinant RV expressing chimeric VP4 on its surface. The use of this approach to construct chimeric RVs may lead to a new generation of effective vaccine vectors against RV disease, as well as research on the molecular biology of RV.

Generation of recombinant RV carrying chimeric VP4. A previous study revealed cross-reactive neutralization epitopes

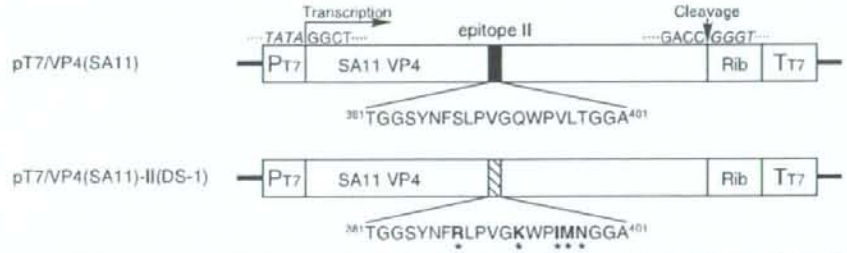

FIG. 1. Schematic representation of SA11 virus-based transcription plasmids encoding the full-length VP4 gene. Plasmid pT7/VP4(SA11) contains the authentic full-length VP4 gene cDNA of SA11, flanked by the T7 RNA polymerase promoter and hepatitis delta virus (HDV) ribozyme, followed by the T7 RNA polymerase terminator. Manipulation of the VP4 gene by means of amino acid mutations (positions are indicated by bold letters and asterisks below the sequences) was carried out in pT7/VP4(SA11); the mutant plasmid pT7/VP4(SA11)-II(DS-1) contains five amino acid mutations within the epitope II sequence. Numbers indicate the amino acid positions in the SA11 VP4 sequence. PT7, Rib, and TT7 denote the T7 RNA polymerase promoter, the HDV ribozyme, and the T7 RNA polymerase terminator, respectively.

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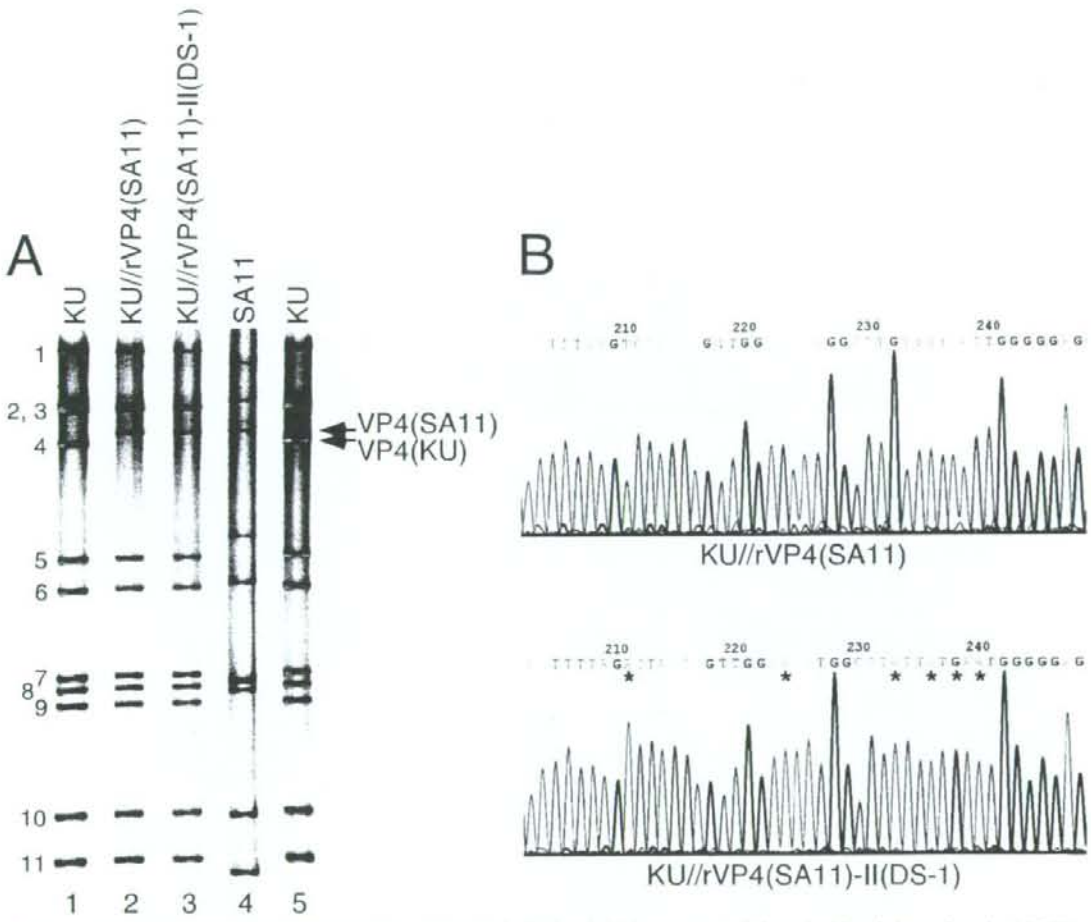

FIG. 2. Rescue of recombinant viruses containing a cDNA-derived chimeric VP4 genome. (A) Polyacrylamide gel electrophoresis of dsRNAs extracted from the rescued VP4 gene transfectants. Lanes 1 and 5, dsRNAs from the KU helper virus; lanes 2 and 3, dsRNAs from the recombinant KU//rVP4(SA11) virus containing the cDNA-derived authentic VP4 gene (lane 2) and the rescued KU//rVP4(SA11)-II(DS-1) virus (lane 3); lane 4, dsRNAs of SA11 used for VP4 gene cloning. The numbers on the left indicate the order of the genomic dsRNA segments of the KU helper virus. (B) Site-specific mutations introduced into the dsRNA genome of the rescued VP4 gene transfectant. The full-length VP4 genes of the recombinant KU//rVP4(SA11) and KU//rVP4(SA11)-II(DS-1) viruses were amplified by reverse transcription-PCR to yield a 2,386-bp product. The 2,386-bp fragments were directly sequenced, which demonstrated the site-specific mutations introduced within the VP4 genome of the rescued KU//rVP4(SA11)-II(DS-1) virus (positions are indicated by asterisks below the sequence).

I, II, and III on VP5* of RV (26). We chose epitope II for expression in the chimeric VP4 molecules because its sequence is highly hydrophobic and it is thought to be especially immunodominant (13, 26). To replace the epitope II sequence in VP4 of the SA11 virus (simian RV strain, G3P[2]), genetic manipulation was carried out in a pX8dT-based (20) T7 RNA polymerase-driven plasmid, pT7/VP4(SA11), encoding the full-length VP4 gene of SA11 (Fig. 1) (11). In the mutated plasmid, pT7/VP4(SA11)-II(DS-1) (Fig. 1), the amino acid sequence (amino acids 381 to 401) of epitope II to be expressed was replaced by the corresponding one from a different P-type virus, DS-1 (human RV strain, G2P[4]), by using a QuikChange II site-directed mutagenesis kit (Stratagene) with primers (+) 5'-AGaCTACCAGTTGGAaAATGGCCTaTTa TgAaTGGGGGGAG-3' and (−) 5'-CAtTcAtAAtAGGCCATT tTCCAACTGGTAGtCTAAAATT-3'. The nucleotides shown in lowercase are mutated to modify the amino acid sequence of epitope II from that of SA11. As shown in Fig. 1, the sequence differences between the SA11 and DS-1 viruses in epitope II are found at five amino acids. To generate recombinant RVs carrying chimeric VP4, reverse genetics for RV (11) was performed. Briefly, a monolayer of COS-7 cells, which had been infected beforehand with a recombinant vaccinia virus expressing T7 RNA polymerase (rDIs-T7pol) (9), was transfected

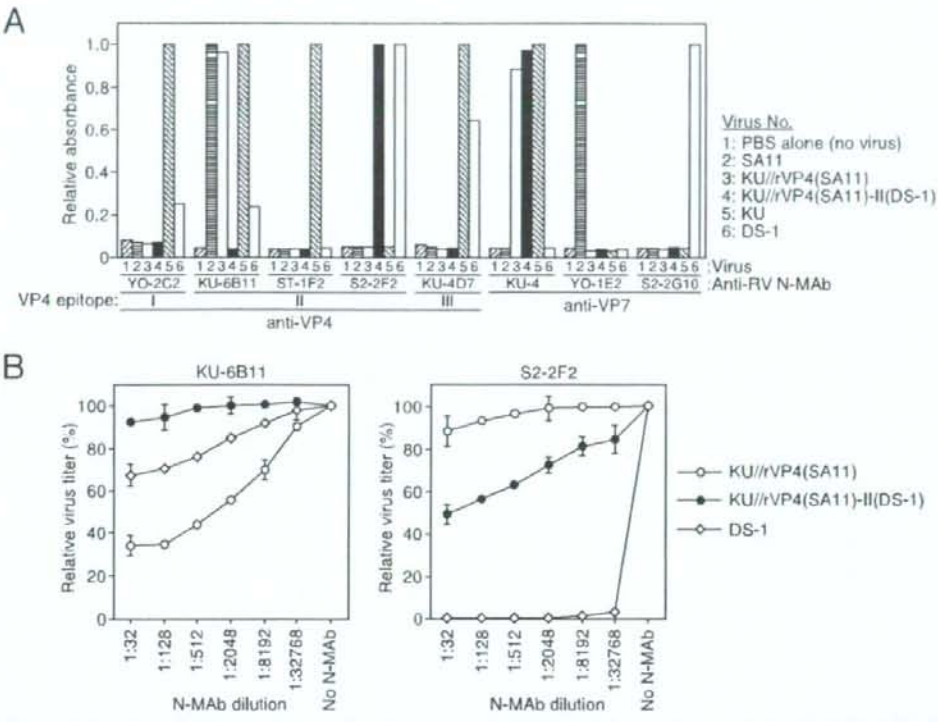

FIG. 3. Serological analyses of rescued viruses. (A) Reactivity patterns of the rescued viruses with a panel of anti-VP4 (YO-2C2, KU-6B11, ST-1F2, S2-2F2, and KU-4D7) and anti-VP7 (KU-4, YO-1E2, and S2-2G10) N-MAbs by antigen capture ELISA: phosphate-buffered saline (PBS) alone (no virus) (virus 1), SA11 (virus 2), KU//rVP4(SA11) (virus 3), KU//rVP4(SA11)-II(DS-1) (virus 4), KU (virus 5), and DS-1 (virus 6). Values are expressed as relative absorbances, with values for KU (in cases of YO-2C2, KU-6B11, ST-1F2, KU-4D7, and KU-4), DS-1 (S2-2F2 and S2-2G10), and SA11 (YO-1E2) normalized to 1.0. The experiment was repeated three times with similar results, and representative results are shown. (B) Neutralization of the rescued viruses by anti-VP4 N-MAbs (KU-6B11 and S2-2F2). For the plaque neutralization assay, CV-1 cells per well of a six-well plate were infected with approximately 100 PFU of virus which had been preincubated with different N-MAb dilutions at 37°C for 1 h. After 1 h of adsorption, the virus-antibody mixtures were removed prior to the addition of the primary overlay medium. At 2 days postinfection, the cell monolayers were stained with secondary overlay medium containing 0.7% agarose and 0.005% neutral red. Values are expressed as relative virus titers, with the value for no N-MAb normalized to 100%. The data shown are the mean viral titers and standard deviations for three independent cell cultures.

with the constructed pT7/VP4(SA11)-II(DS-1) plasmid and then infected with the KU helper virus (human RV strain, G1P[8]). When cultures of transfected cells were passaged in fresh MA104 cells in the presence of two neutralizing monoclonal antibodies (N-MAbs), YO-2C2 (22) and ST-1F2 (23), that specifically neutralize the KU helper virus, an RV-induced cytopathic effect was detected. The rescued virus, named KU//rVP4(SA11)-II(DS-1), was plaque purified three times.

RNA analysis of the rescued virus. Virion dsRNAs from the rescued KU//rVP4(SA11)-II(DS-1) virus were extracted and then analyzed by polyacrylamide gel electrophoresis (Fig. 2A) as described previously (11). As expected, the VP4 dsRNA of the rescued KU//rVP4(SA11)-II(DS-1) virus (Fig. 2A, lane 3) migrated to almost the same position as the corresponding segments of the SA11 virus (lane 4) and the recombinant KU//rVP4(SA11) virus possessing a cDNA-derived authentic SA11 VP4 genome with a KU backbone (lane 2) (11), the mobility being slower than that of the VP4 segment of the KU helper virus (lanes 1 and 5). Direct sequencing of the rescued virus also indicated that the designed six nucleotides mutations in the epitope II sequence were stably introduced into the VP4 dsRNA segment of the infectious RV (Fig. 2B). These results confirmed that the rescued KU//rVP4(SA11)-II(DS-1) virus is a KU-based recombinant virus carrying a chimeric VP4 gene and proved that substitutions at this particular site are compatible with the preparation of a fully functional virus.

Serological characterization of the rescued virus. The antigenic properties of the rescued KU//rVP4(SA11)-II(DS-1) virus were investigated by an enzyme-linked immunosorbent assay (ELISA) and a plaque neutralization assay, using a panel of anti-VP4 and -VP7 N-MAbs (22–26). Different anti-VP4 N-MAbs are found to recognize each of the three cross-reactive neutralization epitopes (26): epitope I (defined by YO-2C2), epitope II (defined by KU-6B11, ST-1F2, and S2-2F2), and epitope III (defined by KU-4D7). Notably, KU-6B11 and ST-1F2 recognize the epitope II sequences of at least the P[2]

and P[8] types, and S2-2F2 is directed to that of the P[4] type. First, antigen capture ELISA was performed using these anti-VP4 (YO-2C2, KU-6B11, ST-1F2, S2-2F2, and KU-4D7) and anti-VP7 (KU-4, YO-1E2, and S2-2G10) N-MAbs, as described previously (11) (Fig. 3A). The SA11 (G3P[2]), recombinant KU//rVP4(SA11) (G1P[2]), and KU (G1P[8]) viruses reacted with KU-6B11 (directed to epitope II), which is found to react commonly with the P[2] and P[8] types, but did not react with S2-2F2 (epitope II), which recognizes the P[4] type, such as the DS-1 virus (G2P[4]). The KU helper virus also showed reactivity with ST-1F2 (epitope II), which selectively reacts with the P[8] type. On the other hand, the rescued KU//rVP4(SA11)-II(DS-1) virus lost reactivity with KU-6B11 and ST-1F2 but showed reactivity with S2-2F2, whereas the DS-1 virus reacted with KU-6B11 moderately. In addition, this rescued KU//rVP4(SA11)-II(DS-1) virus showed no reactivity with YO-2C2 (epitope I) or KU-4D7 (epitope III), as found for SA11 and KU//rVP4(SA11) viruses containing authentic SA11 VP4. Further, the KU//rVP4(SA11)-II(DS-1), KU//rVP4(SA11), and KU helper viruses reacted with anti-VP7 KU-4, specific for the G1 type, but not with YO-1E2 or S2-2G10, specific for G3 or G2, respectively. These results demonstrated that the rescued KU//rVP4(SA11)-II(DS-1) virus is a KU-based recombinant virus expressing both the DS-1- and SA11-derived epitopes on its VP4 surface, that is, this chimeric virus carries the designed antigenic mosaic of cross-reactive neutralization epitopes on its VP4 surface.

Next, a plaque neutralization test involving two representative anti-VP4 N-MAbs (KU-6B11 and S2-2F2) was performed. As shown in Fig. 3B, the KU//rVP4(SA11) virus (G1P[2]) was neutralized by KU-6B11, which recognizes the P[2] type, but not by S2-2F2, which recognizes the P[4] type. Conversely, the rescued KU//rVP4(SA11)-II(DS-1) virus was neutralized by S2-2F2 but not by KU-6B11, although S2-2F2 neutralized this chimeric virus less efficiently than it neutralized DS-1 (G2P[4]). The less efficient neutralization of this chimeric virus by S2-2F2 suggests that although the replacement of the epitope II sequence did not affect the property of VP4 as far as binding with this N-MAb is concerned, it may have caused a slight conformational change around the domain that resulted in less-efficient neutralization by this N-MAb. These results may reflect the finding that epitope II appears to be conformational (26). Also, the observation that DS-1 but not chimeric KU//rVP4(SA11)-II(DS-1) was moderately recognized (Fig. 3A) and neutralized (Fig. 3B) by KU-6B11 is possibly due to a slight conformational change around epitope II in the chimeric virus with the genetic manipulations. In any case, these results revealed that the antigenic properties of the rescued KU//rVP4(SA11)-II(DS-1) virus changed from those of the parental KU//rVP4(SA11) virus due to substitutions within the epitope II sequence, leading to the expression of the replaced DS-1-derived epitope II on its VP4 surface.

Infectivity of the rescued virus. The VP4 spike protein possesses a single internal hydrophobic domain (amino acids 384 to 401) that exhibits sequence similarity with the internal fusogenic domain of the E1 glycoprotein of some alphaviruses (13) and that is involved in the permeabilization of the recombinant VP5* model and cellular membranes (2, 3, 8). Interestingly, this putative fusion sequence significantly overlaps with the epitope II domain (amino acids 381 to 401) studied above.

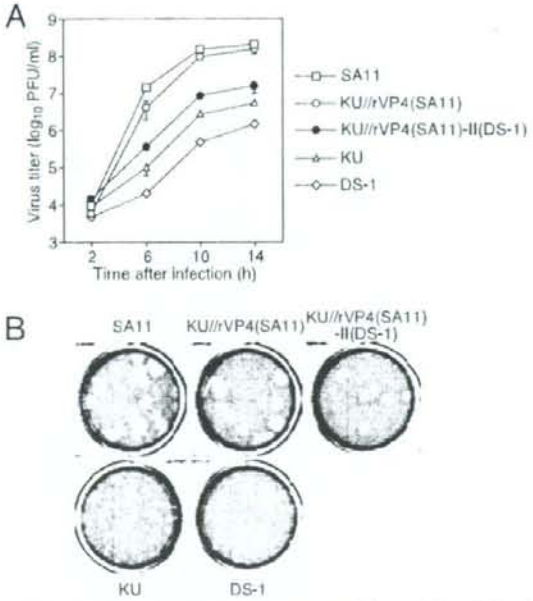

FIG. 4. Infectivity of rescued viruses containing a cDNA-derived VP4 genome. (A) Single-step growth curves of the rescued viruses. A monolayer of MA104 cells was infected with the SA11, KU//rVP4(SA11), KU//rVP4(SA11)-II(DS-1), KU, and DS-1 viruses at a multiplicity of infection of 5, incubated for the intervals shown, and then lysed by freeze-thaw cycles. Virus titers were determined by a plaque assay with CV-1 cells. The data shown are the mean viral titers and standard deviations for three independent cell cultures. (B) Plaques of the rescued viruses. The viruses were directly plated on CV-1 cells for plaque formation. After 2 days, infected cells were stained with secondary overlay medium containing 0.7% agarose and 0.005% neutral red. The experiment was repeated three times with similar results, and representative results are shown.

As this potent fusogenic domain is likely to be critical for viral infection, the chimeric KU//rVP4(SA11)-II(DS-1) virus may have altered biological characteristics (infectivity) compared with the parental KU//rVP4(SA11) virus, owing to the five amino acids mutations in the epitope II domain. To assess the infectivity of the recombinant virus, SA11, KU//rVP4(SA11), KU//rVP4(SA11)-II(DS-1), KU, and DS-1 were used for the infection of MA104 cells (Fig. 4A). As observed in a previous study with the parental KU//rVP4(SA11) virus carrying authentic SA11 VP4 (11), titers similar to those of SA11 were attained. In contrast, the rescued KU//rVP4(SA11)-II(DS-1) virus, which expresses chimeric VP4 possessing the DS-1-derived epitope II sequence (hydrophobic domain), displayed impaired growth (<10-fold-lower titer) compared to KU//rVP4(SA11). Human RVs showed significantly lower growth than the others carrying the SA11-based VP4. We then examined the plaque sizes in CV-1 cells for these viruses by measuring the mean diameters of each of 20 plaques from two independent assays (Fig. 4B). The virus growth titers correlated well with the sizes of the plaques formed. That is, the viruses with high infectivity [SA11 and KU//rVP4(SA11)]

formed large plaques (diameters, 4.99 ± 0.50 and 5.44 ± 0.60 mm, respectively), as has been demonstrated by SA11 VP4-carrying viruses (27), while the human viruses with low infectivity (KU and DS-1) formed small plaques (diameters, 2.46 ± 0.48 and 0.96 ± 0.23 mm, respectively). On the other hand, the chimeric KU/rVP4(SA11)-II(DS-1) virus formed smaller-sized plaques than the SA11 and KU//rVP4(SA11) viruses but larger ones than the KU and DS-1 viruses (diameter, 3.80 ± 0.36 mm). Therefore, these results indicate that the amino acid substitutions in the epitope II (hydrophobic domain) sequence and/or a slight conformational change in and around the domain replaced with that of the slow-growing human DS-1 virus may significantly affect the infectivity of the fast-growing parental KU//rVP4(SA11) virus, leading to attenuation. Further mutational studies involving reverse genetics will provide information on which amino acid residues within this domain are involved in the control of the virus growth rate.

In summary, a recombinant RV with an antigenic mosaic of cross-reactive neutralization epitopes on its VP4 surface could be engineered by means of reverse genetics, which indicates that RVs may be used as expression vectors for foreign epitopes. It is expected that this approach will be valuable for the development of novel RV vaccines and vaccine vectors, as well as for an understanding of the molecular basis of RV pathogenesis, although the efficiency of this system is not optimal yet.

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ARTICLE

Analysis of Rotavirus Antigenemia and Extraintestinal Manifestations in Children With Rotavirus Gastroenteritis

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What's Known on This Subject

Rotavirus antigen is detected in acute-phase serum collected from patients with rotavirus gastroenteritis.

What This Study Adds

An association between rotavirus antigenemia and clinical features, particularly extraintestinal manifestations, and the association between serum cytokine levels and rotavirus antigen quantity are clarified.

ABSTRACT

OBJECTIVE. This study was conducted to examine the association between rotavirus antigenemia and clinical features, particularly extraintestinal manifestations, and the association between serum cytokine levels and rotavirus antigen quantity.

METHODS. Sixty hospitalized children who received a diagnosis of acute rotavirus gastroenteritis were enrolled in this study. Paired serum samples were collected from the 60 children when admitted to and discharged from the hospital. Associations among viral antigen levels and fever, elevated transaminase levels, and seizures were evaluated to determine whether antigenemia correlated with disease severity. Viral antigen was measured by using an in-house enzyme-linked immunosorbent assay that detected VP6 antigen. A flow-cytometric bead array was used to measure serum cytokine levels.

RESULTS. Rotavirus antigen levels were significantly higher in serum collected at the time of hospital admission than at the time of discharge. Serum rotavirus antigen levels peaked on day 2 of the illness (2.02 ± 0.73), followed by a gradual decrease in antigen levels to nearly undetectable levels by day 6. The quantity of rotavirus antigen was significantly higher in serum collected from patients with fever than those without fever. The presence or absence of elevated transaminase levels and seizures was not associated with serum rotavirus antigen levels. A weak but significantly positive association was observed between interleukin 8 levels and antigenemia. A weak but significantly negative association was observed between interleukin 10 levels and antigenemia.

CONCLUSIONS. Rotavirus antigenemia is frequently observed in a patient's serum during the acute phase, and viral antigen levels change dramatically during the acute phase of the illness. Because patients with fever had higher rotavirus antigen levels, antigenemia severity might contribute to fever. The host immune response plays an important role in controlling antigenemia levels. *Pediatrics* 2008;122:392–397

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Key Words
rotavirus, antigenemia, cytokine

Abbreviations
ELISA—enzyme-linked immunosorbent assay
PBST—phosphate-buffered saline that contains Tween 20
CBA—cytometric bead array
OD—optical density
IL—interleukin

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ROTAVIRUS IS THE major cause of gastroenteritis in young children worldwide. Severe dehydration caused by rotavirus-induced diarrhea and vomiting can be fatal in developing countries. Meanwhile, gastroenteritis induced by rotavirus infection causes a large economic burden in developed countries. Initially, rotavirus replication was thought to be limited to the gastrointestinal tract in patients with gastroenteritis; however, it is widely known that rotavirus gastroenteritis is sometimes complicated by high fever, elevated transaminase levels,[1,2] seizures,[3–5] and encephalitis,[6,7] which may be caused by systemic viral infection. Rotavirus RNA has been detected in the cerebrospinal fluid of patients with convulsions[8]; however, it is unclear whether the RNA was really there. Rotavirus antigens and RNA were detected in the serum of children with rotavirus infection.[9–11] In addition, several investigators have demonstrated that rotavirus antigen is detected not only in serum but also in multiple organs, including the stomach, intestine, liver, lung, spleen, kidney, pancreas, thymus, and bladder, in rotavirus-infected animals.[12]

These findings suggest that rotavirus spreads beyond the intestine in children with rotavirus gastroenteritis, resulting in systemic viral infection.

We have demonstrated that viremia generally correlates with illness severity in children with varicella-zoster virus infection[13] and human herpesvirus 6 infection.[14] Although several lines of evidence suggest that systemic rotavirus infection occurs in infected children,[8–11] it is not well understood whether rotavirus antigenemia levels correlate with disease severity. Only 1 report has postulated that rotavirus RNAemia is associated with high fever, but this study did not present statistical analyses on these findings.[15] Moreover, it has been suggested that cytokines may play an important role in rotavirus gastroenteritis pathogenesis[16–21]; however, no clinical studies have investigated the correlation between levels of rotavirus antigenemia and serum cytokines to date. We hypothesized that the grade of systemic rotavirus replication and cytokine levels induced by viral infection play an important role in extraintestinal manifestations such as fever, elevated transaminase levels, and seizures; therefore, in this study, we examined the association between rotavirus antigenemia and clinical features, particularly extraintestinal manifestations. Moreover, because it has been suggested that cytokines are involved in the pathogenesis of rotavirus gastroenteritis, we also examined the association between serum cytokine levels and rotavirus antigen levels.

METHODS

Patient Characteristics and Sample Collection

Sixty hospitalized children with a diagnosis of acute rotavirus gastroenteritis were enrolled in this study. All patients were admitted to 1 of 4 pediatric departments (Fujita Health University, Kariya Toyota General Hospital, Toyokawa City Hospital, or Showa Hospital) between December 2004 and March 2006. The patients' guardians consented to their participation in this study. This study was approved by the review boards of all 4 institutes. Diagnosis of rotavirus gastroenteritis was confirmed by detection of rotavirus antigen in stool samples by using an immunochromato assay (Dipstick [Eiken, Tokyo, Japan]). Paired serum samples were collected from the 60 children (age: 1.4 ± 1.4 years; gender: 40 boys and 20 girls) at the time of hospital admission (days 1–5; day 1 was defined as the date of onset of symptoms [eg, fever, vomiting, diarrhea]) and discharge (days 4–13). In addition, 20 serum samples were collected from age-matched control children whose stool samples were negative for rotavirus antigen by the immunochromato assay.

Clinical features of the children were examined retrospectively by using medical charts. To assess whether serum rotavirus antigen levels correlated with disease severity, we examined the association between viral antigen levels and fever (>37.5°C), elevated transaminase levels (alanine aminotransferase: >50 IU/L), and seizures, symptoms that are caused by systemic viral infection. The presence or absence of these clinical manifestations was evaluated from the data collected at the time of hospital admission.

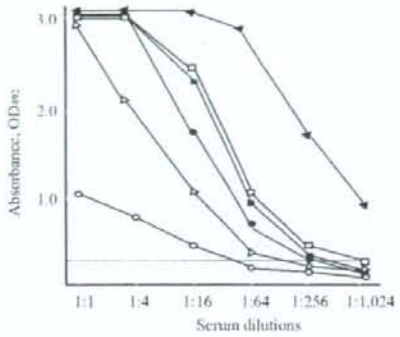

FIGURE 1

ODs of serially diluted serum samples that were collected from patients with rotavirus gastroenteritis. The dotted line indicates the baseline value.

Rotavirus Antigen Detection

Rotavirus antigen was measured using an in-house enzyme-linked immunosorbent assay (ELISA) that detects VP6 antigen of the virus. Fifty microliters of diluted (1:16) serum was used to detect rotavirus antigen. The dilution ratio was determined from preliminary studies by using several different serum samples that were collected from patients with rotavirus gastroenteritis. As shown in Fig 1, a 1:16 dilution was appropriate to measure quantitatively viral antigen in patients' serum. Ninety-six–well plates (Nalgen Nunc International, Rochester, NY) coated with a monoclonal antibody against the VP6 antigen of rotavirus (YO-156)[22] were used for the ELISA.[21] The antibody YO-156 (immunoglobulin G2a subclass) is highly reactive with a common epitope of group A rotaviruses, and the antibody can detect all of the group A rotavirus strains that have been examined. Specificity of the antibody was confirmed by immunoprecipitation assay, immune electron microscopy, and Western blotting analysis.[22] After blocking with 1% bovine serum albumin in phosphate-buffered saline that contains Tween 20 (PBST), the plate was washed with PBST. The plate was then incubated with 50 μL of diluted patients' serum at 4°C overnight. After washing the plate with PBST, 50 μL of antihuman rotavirus hyperimmune rabbit serum diluted 1:5000 with PBST that contained 2.5% skim milk was added to each well. The plate was incubated at 37°C for 1.5 hours. After the plate was washed with PBST, it was incubated with a 1:5000 dilution of peroxidase-conjugated donkey antirabbit immunoglobulin G (Jackson ImmunoResearch Laboratory, Inc, West Grove, PA) at 37°C for 1.5 hours. The quantity of monoclonal antibody bound to rotavirus VP6 antigen was assessed after addition of the substrate. The optical density (OD) was read by spectrophotometry at a 492-nm wavelength. To establish an appropriate cutoff value to distinguish between rotavirus-positive and -negative samples, we tested 20 serum samples that were collected from control subjects. Be-

cause the mean OD of the control samples was 0.084 ± 0.014, we defined 0.13 (mean + 3 SD) as the baseline value in this study.

Cytokine Measurements

The flow-cytometric bead array (CBA) was used according to the manufacturer's protocol (Becton Dickinson, San Diego, CA).[21] CBA measured the following cytokines: interleukin (IL)-8, IL-1β, IL-6, IL-10, tumor necrosis factor α, and IL-12. Fifty microliters of sample (standards or test) were added to 50 μL of a cocktail of capture beads and detector antibodies, and the mixture was incubated for 1.5 hours at room temperature in the dark. Excess unbound detector antibody was removed by washing, and 50 μL of reagent was added before data acquisition. Two-color flow-cytometric analysis was performed using a flow cytometer (FACScan [Becton Dickinson, Franklin Lakes, NJ]). A total of 1800 events were acquired by following the protocol supplied. Analysis was performed using CBA dedicated analysis software (CellQuest [Becton Dickinson]). All samples for which the calculated cytokine concentration was below the given sensitivity were treated as undetectable.

Statistical Analysis

Statistical analyses were performed by using Stat View 5.0 (SAS Institute, Cary, NC). Patient gender was compared between the 2 groups by using a χ^2 test. Unpaired comparisons between the patient's age and days of sampling were performed by using a Student's t test. Mean peak absorption levels were compared between the 2 groups by using either a Wilcoxon signed-ranks test or Mann-Whitney U test. Spearman's rank correlation coefficients were used to measure the strength of the association between cytokine levels and rotavirus antigen quantity.

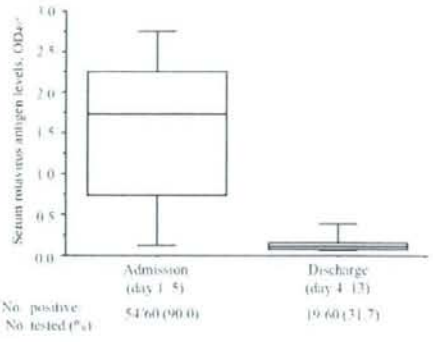

FIGURE 2

Rotavirus antigen levels as assayed by EIA in paired serum samples (0.13 selected as cut-off) collected at the time of admission (1–5 days) and discharge (4–13 days) from the hospital.

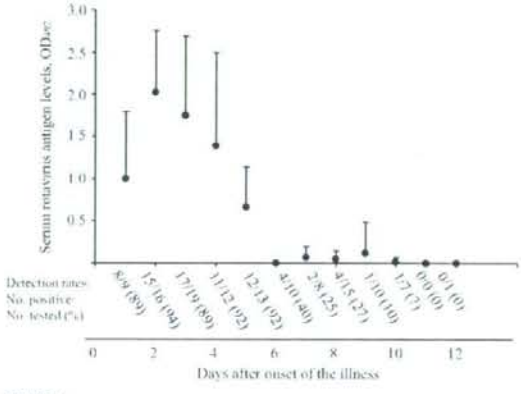

FIGURE 3

Kinetics of rotavirus antigen load and detection rate (Elisa antigen).

RESULTS

Kinetics of Rotavirus Antigenemia

First, we compared rotavirus antigen levels at the time of hospital admission with levels at the time of discharge. Serum samples that were collected at the time of admission were between days 1 and 5 of the illness, and those that were collected at the time of discharge were between days 4 and 13 of the illness. Rotavirus antigen was significantly higher in serum that was collected at the time of admission (days 1–5) than in those collected at the time of discharge (days 4–13; $P < .0001$; Fig 2). In addition to the antigen levels, detection rate of serum rotavirus antigen was also high in serum that was collected at the time of admission (54 of 60 [90%]) in comparison with the samples that were collected at the time of discharge (19 of 60 [31.7%]). To determine rotavirus antigen kinetics after the onset of illness, we monitored the optical densities in all 120 serum samples. As shown in Fig 3, serum rotavirus antigen levels peaked on day 2 (2.02 ± 0.73), followed by a gradual decrease to nearly undetectable levels by day 6. High detection rates (ranging between 89% and 94%) were also observed in serum samples that were collected until day 5.

Association Between Rotavirus Antigenemia Levels and Clinical Symptoms

OD values in serum samples that were collected at the time of admission were compared between patients with and without each clinical manifestation (Fig 4). Although patients' backgrounds were not different between the groups with (gender: 28 boys and 16 girls; age: 1.4 ± 1.3 years; sampling time: 2.7 ± 1.2 days) and without fever (gender: 11 boys and 5 girls; age: 1.4 ± 1.5 years; sampling time: 3.2 ± 1.3 days), the quantity of rotavirus antigen was significantly higher in the serum that was collected from the patients with fever (1.70 ± 0.87) than those without fever (1.07 ± 0.98; $P = .0273$). No febrile episode was observed during admission period in patients without fever at the time of admission to the hospital. Rotavirus antigen levels in patients with (alanine aminotransferase: 84.8 ± 61.8 IU/L) and without elevated transaminase levels were 1.16 ± 0.96 and 1.60 ± 0.92, respectively, with no statistical difference in the antigenemia levels between the 2 groups ($P = .2351$). Finally, we tested whether the level of rotavirus antigenemia was correlated with seizures. The number

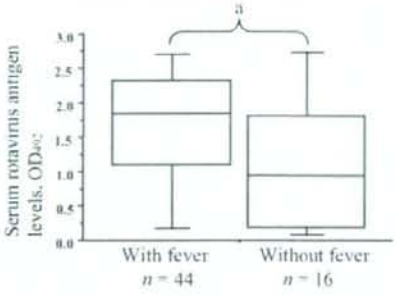

FIGURE 4

of patients who experienced seizures was small ($n = 7$). Moreover, 4 of the 7 patients had febrile seizures, whereas the remaining 3 patients had afebrile seizures, which were diagnosed as benign convulsions associated with mild gastroenteritis. No statistical difference was observed in rotavirus antigenemia levels between patients with (1.38 ± 1.04) and without seizures (1.56 ± 0.93; $P = .6451$).

Association Between Levels of Rotavirus Antigenemia and Cytokines

The associations between the levels of 6 cytokines and the quantity of rotavirus antigen in acute serum samples were examined by Spearman's rank correlation coefficients (Fig 5). We identified a weak ($r = 0.36$) but significantly ($P = .0041$) positive association between IL-8 levels and the severity of rotavirus antigenemia. Although a similar correlation was demonstrated between IL-6 levels and quantity of viral antigen ($r = 0.23$), it was not statistically significant ($P = .0697$). Meanwhile, there was a weak ($r = -0.258$) but significantly ($P = .0464$) negative association between IL-10 levels and rotavirus antigenemia. No statistical correlation was observed between the other 3 cytokines levels and antigen levels.

DISCUSSION

An in-house ELISA that used a monoclonal antibody YO-156 directed to group A common epitope on VP6 was used to measure serum rotavirus antigen in this study. VP6 is an inner capsid protein in rotavirus particle and is the most abundant protein detected in rotavirus particle and in the cells that are infected with rotavirus. The VP6 carries group-specific and subgroup-specific antigens. Our monoclonal antibody can detect both of the 2 subgroups (I or II). VP4 and VP7 are the outer capsid proteins, and they are associated with P-type and G-type specificities, respectively. In human rotavirus, the presence of at least 10 P types and 10 G types has been reported. Thus, to detect rotavirus antigen efficiently, it is reasonable to use monoclonal antibody that commonly is reactive to VP6 of any human rotavirus strain. To determine the appropriate serum dilution to clarify differences in viral antigen levels, we measured OD values in serially diluted serum samples and found that serum diluted 16-fold was the best to quantify viral antigen (Fig 1). As shown in Fig 3, the detection rate of rotavirus antigen in patients' serum ranged between 89% and 94% until 5 days after illness onset, suggesting a high frequency of rotavirus antigenemia during the acute phase of rotavirus gastroenteritis. This result supports previous studies that demonstrated frequent detection of rotavirus antigen in acute-phase serum; however, the detection rate of rotavirus antigen was higher in our study than in 2 previous studies.[10,11] The previous studies used undiluted serum to measure viral antigen, whereas we used serum diluted 16-fold, suggesting that our ELISA system has greater sensitivity than previously published ELISA methods. Indeed, in our comparison of the sensitivity between our in-house ELISA and a commercial kit (Rotaclone [Meridian Bioscience, Inc, Cincinnati, OH]) by using same serum samples, our in-house ELISA exhibited clearly higher sensitivity than the commercial kit (data not shown).

Previous reports demonstrated that rotavirus antigen levels decreased to baseline levels during a 3- to 4-week interval by testing paired serum samples[10]; however, this study demonstrated that rotavirus antigen levels were significantly lower at the time of discharge from the

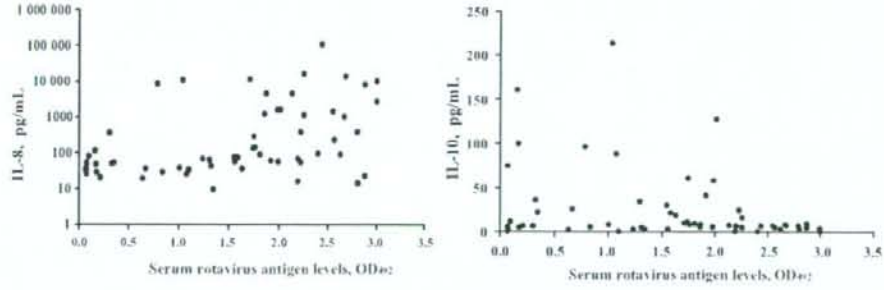

FIGURE 5

hospital than at the time of hospital admission ($P < .0001$). In addition, the viral antigen kinetic analysis showed that viral antigen peaked on day 2 of the illness, and antigen levels quickly returned to almost undetectable levels by day 6. Fischer et al[10] also examined viral antigen levels after disease onset but demonstrated unclear viral antigen kinetics. Thus, to our knowledge, this is the first study to demonstrate the kinetics of rotavirus antigenemia during the acute phase of rotavirus gastroenteritis. The high sensitivity of our EIISA system and sampling schedule allowed us to clarify such a dynamic change of viral antigen during acute illness. Because both innate and adaptive immunities play an important role in viral clearance, an analysis of the antirotavirus host immune response and rotavirus antigenemia would clarify the pathogenesis of rotavirus gastroenteritis.

Although 3 extraintestinal manifestations were evaluated in this study, only fever was statistically associated with viral antigen levels. It has been demonstrated that younger patients generally have higher viral antigen levels than older patients, because viral antigen levels are higher in patients with primary rotavirus infections than those reinfected with the virus.[11] Moreover, sampling time affects rotavirus antigen detection in serum, as shown in Fig 3. When we compared the mean age and sampling time between patients with and without fever, no significant difference was observed between these 2 groups. Thus, we believe that serum rotavirus antigen levels are associated with the occurrence of fever in patients with rotavirus gastroenteritis. There was no correlation between rotavirus antigen levels and 2 additional manifestations: elevated transaminase levels and seizures. The elevated transaminase levels that occurred in these children was mild, as demonstrated previously.[2] It seems that systemic spread of rotavirus antigen is not involved in mild elevated transaminase levels in patients with rotavirus gastroenteritis. It is widely known that rotavirus can cause both febrile seizures[5] and benign convulsions associated with mild gastroenteritis.[3,4] Because the number of patients with seizures was small and the patients with seizures had 2 different types of convulsions, it is difficult to analyze conclusively the correlation between rotavirus antigenemia and seizures. A large number of patients with rotavirus gastroenteritis should be analyzed to determine an association between central nervous system complications, such as seizures and encephalopathy, and viral antigen levels. Moreover, rotavirus antigen should be measured in the cerebrospinal fluid to determine whether the virus can directly invade the central nervous system, as was previously suggested by reverse transcriptase–polymerase chain reaction analysis.[6] Furthermore, because other variables, including genotype, primary or secondary infection, and coinfection with other pathogens, were not evaluated in this study, an association between these factors and antigenemia levels should be analyzed in future study.

It is widely known that cytokines play an important role in the pathogenesis of viral infections. Several in vivo and in vitro studies have suggested that cytokines are involved in the pathogenesis of rotavirus infection.[16–21] Although Jiang et al[20] demonstrated that several cytokines are associated with the severity of rotavirus gastroenteritis, a correlation between cytokine production and rotavirus antigen levels has not been studied. Six cytokines were measured in serum samples that were collected at the time of hospital admission by CBA, a method that can simultaneously measure multiple cytokine levels in a single reaction and therefore requires only small amounts of serum. Two (IL-8 and IL-10) of the 6 cytokines were statistically correlated with rotavirus antigen levels in this study. It has been demonstrated that rotavirus infection can induce the production of several chemokines, including IL-8, in intestinal cell lines and small animal models.[18,21] IL-8 may play an important role in generating the mucosal immune response to rotavirus infection. This study showed that significantly higher IL-8 levels were observed in patients with higher levels of rotavirus antigenemia. The results of this study together with previous in vitro and in vivo studies[18,21] suggest that increased systemic spread of the virus could trigger a strong immune response in the host.

Note that IL-10 levels were negatively correlated with rotavirus antigen levels. This cytokine has modulatory effects on both T-helper 1 and 2 cytokines and plays a regulatory role in inflammatory processes. It has been demonstrated that plasma IL-10 levels increase in the acute phase of rotavirus gastroenteritis[17–20]; however, the role of this cytokine in disease pathogenesis remains obscure.

CONCLUSIONS

The results of this study demonstrate that IL-10 levels were significantly lower in patients with high rotavirus antigen levels, which suggests that this cytokine plays an important role in the immune response against systemic rotavirus infection.

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"Births, not immigration, now account for most of the growth in the nation's Hispanic population, a distinct reversal of trends of the past 30 years. The Hispanic baby boom is transforming the demographics of small-town America in a dramatic way. Some rural counties where the population had been shrinking and aging are growing because of Hispanic immigration and births and now must provide services for the young. 'In all of the uproar over immigration, this is getting missed,' says Kenneth Johnson, demographer at the University of New Hampshire's Carsey Institute. 'All the focus is on immigration, immigration, immigration. At some point, it's not. It's natural increase.' This natural increase—more births than deaths—is accelerating among Hispanics in the USA because they are younger than the US population as a whole. Their median age is 27.4, compared with 37.9 overall, 40.8 for whites, 35.4 for Asians and 31.1 for blacks. Because they are younger and likely to have more children, Hispanics are having an impact that far outlasts their initial entry into the country."

El Nasser H. *USA Today*. June 30, 2008

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Based on nucleotide sequence and phylogenetic analysis of the partial VP6 genes, group A rotaviruses can be mainly differentiated into two genogroups. In this study, a method employing reverse transcription-PCR (RT-PCR) and degenerate primers was established to assign the VP6 genogroup. VP6 genogroup I and genogroup II could be determined according to the sizes of the amplicons: 380 and 780 bp, respectively. The VP6 genogroup of human reference strains of G1 to G4 and G9 types and RotaTeq vaccine strains could be properly assigned by RT-PCR. Eighty rotavirus-positive fecal samples were subjected to enzyme-linked immunosorbent assay (ELISA), RT-PCR, and sequencing of the partial VP6 gene for subgroup and genogroup determination. The results correlated well among these three methods, except for seven samples whose subgroups could not be determined by ELISA. VP6 genogroups of another 150 rotavirus strains recovered between 1981 and 2005 were determined by RT-PCR and sequencing, and the same results were obtained by these two methods. Furthermore, an additional 524 rotavirus-positive fecal samples were tested by RT-PCR, and the VP6 genogroups could be easily determined. The RT-PCR assay developed here provided a reliable and convenient method for assigning the VP6 genogroups of human rotaviruses with a wide range of genetic variation.

Rotaviruses belong to the family of *Reoviridae*, and complete viral particles have a triple-layered icosahedral protein capsid that surrounds the genome of 11 segments of double-stranded RNA, which encode six structural proteins and six nonstructural proteins (5). VP7 and VP4, two outer capsid proteins, induce neutralizing antibodies and are responsible for the serotype specificity (7, 33). VP7 is a major glycoprotein encoded by gene 7, 8, or 9, and VP7-specific types are abbreviated as G serotypes or G genotypes (6, 7, 27). VP4 is a protease-sensitive protein encoded by gene 4, and VP4-specific types are abbreviated as P serotypes or P genotypes (6, 7, 27). Group A rotaviruses have been classified as 16 G serotypes and 27 P genotypes (12, 22). NSP4, a glycoprotein encoded by gene 10, serves as an intracellular receptor for double-layered rotavirus particles and interacts with viral capsid proteins during viral morphogenesis (1, 32), and it has been proposed as a possible viral enterotoxin capable of inducing diarrhea in young mice (2). According to NSP4 gene sequence analysis, five genotypes (A to E) have been identified among human and animal rotaviruses with different G and P types (3, 4, 13, 28).

VP6, a trimeric protein, encoded by gene 6, forms the middle-layer capsid and interacts with both outer capsid proteins VP4 and VP7 and the core protein VP2 (26). According to the antigenic epitopes present on VP6, rotaviruses can be classified into groups (A to G) and subgroups (5). Group A rotaviruses can be differentiated into subgroups I, II, I+II, and non-I non-II, depending on the presence or absence of two distinct epitopes which react with one, both, or neither of the monoclonal antibodies (MAbs) 255/60 and 631/9 (7, 11). Previous studies have mapped amino acid position 305 and the region from positions 296 to 299 to subgroup I specificity, and amino acid position 315 to subgroup II specificity (30), and showed that subgroup epitopes are conformational and appear to be located on the trimeric but not monomeric structures (9). At present, enzyme-linked immunosorbent assay (ELISA) incorporating subgroup-specific MAbs is widely used as an epidemiological tool to monitor rotavirus strains. However, antigenic drift through the accumulation of point mutations may result in poor reactivity in ELISA subgrouping. In the study by Iturriza-Gomara et al. (18), based on nucleotide sequencing and phylogenetic analysis, it was found that samples were clustered into two genogroups—genogroup I and genogroup II. Genogroup I comprised samples serologically determined as subgroup I, and genogroup II comprised samples serologically determined as subgroup II, subgroup I+II, and subgroup non-I non-II. These authors suggested that there were no true human subgroup I+II or subgroup non-I non-II strains, but this misclassification might have been due to the poor reactivity between subgroup II strains and the MAb used (18). In addition, subgroup-specific MAbs are not easily available for most laboratories. For these reasons, a convenient molecular genogrouping method is still needed.

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TABLE 1. Determination of the subgroup and VP6 genogroup by ELISA, RT-PCR, or sequence analysis for rotavirus strains with different G types and electropherotypes recovered between 2000 and 2002[a]

G type	P type	No. of RNA patterns	No. of samples	ELISA			Sequence analysis		RT-PCR	
				Subgroup I	Subgroup II	?[b]	Genogroup I	Genogroup II	Genogroup I	Genogroup II
G1	P[8]	6	16	0	15	1	0	16	0	16
	P[4]	1	1	1	0	0	1	0	1	0
G2	P[4]	5	15	15	0	0	15	0	15	0
	P[8]	1	1	0	1	0	0	1	0	1
G3	P[8]	4	22	0	20	2	0	22	0	22
G4	P[8]	1	3	0	3	0	0	3	0	3
G9	P[8]	6	22	0	18	4	0	22	0	22
Total		24	80	16	57	7	16	64	16	64

[a] The rotavirus samples were selected based on RNA pattern; for each RNA pattern, two or three samples were selected for each year. The genogroups determined by RT-PCR and sequence analysis were in good agreement (100%), and the genogroup and subgroup determined by RT-PCR and ELISA, respectively, were in 91.3% agreement.

[b] The "?" indicates that the sample could not be assigned to either subgroup.

acid sequencing was performed with a BigDye Terminator cycle sequencing kit (v3.1; Applied Biosystems). The primers used for VP6 gene sequencing are VP6-F and VP6-R; for NSP4 gene sequencing, 10BEG.16, 10END.722c, 10.374, and 10.394c (24) were used; for the plasmid with VP6 gene insert sequencing, T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) was used. The labeled products were purified by ethanol precipitation. The pellet was resuspended in the Hi-Di formamide (Applied Biosystems) and then run on an autosequencer (3100-Avant Genetic Analyzer; Applied Biosystems).

Analysis of sequences. The sequence data were analyzed by using GeneWorks software (IntelliGenetics, Mountain View, CA). The phylogenetic relationships among strains were analyzed by the neighbor-joining method and the Tamura-Nei distance matrix listed in the MEGA analytical package (23). The robustness of the neighbor-joining trees was statistically evaluated by bootstrap analysis.

Nucleotide sequence accession numbers. The nucleotide sequences of the partial VP6 genes of the Taiwanese genogroups I and II strains have been submitted to the GenBank sequence database. Accession numbers EU487535 to EU487561 were assigned for P[8]G1 strains with genogroup II specificity in the following order: 81TW5, 85TW516, 86TW569, 87TW914, 88TW1201, 89TW1495, 92TW3580, 92TW3672, 94TW36a, 94TW1164, 94TW1213, 95TW35, 95TW182, 95TW203a, 95TW418, 95TW481, 95TW511, 95TW803, 95TW859, 00TW124, 01TW588, 01TW863, 03TW269, 03TW1284, 04TW577, 04TW628, and 05TW2483. EU487562 was used for strain 01TW564 with P[4]G1 and genogroup I specificity. Accession numbers EU487563 to EU487575 were assigned for P[4]G2 strains with genogroup I specificity in the following order: 81TW6, 83TW278, 92TW59, 93TW111, 93TW114, 94TW133, 00TW3, 00TW469, 00TW532, 01TW499, 02TW376, 04TW360, and 05TW395. EU487576 was used for strain 01TW557 with P[8]G2 and genogroup II specificity. Accession numbers EU487577 to EU487587 were assigned for P[8]G3 strains with genogroup II specificity in the following order: 86TW606, 89TW1532, 00TW668, 01TW1274, 02TW63, 03TW270, 03TW277, 03TW596, 04TW13, 04TW151, and 05TW1320. EU487588 was used for strain 03TW322 with P[4]G3 and genogroup I specificity. Accession numbers EU833479, EU833480, DQ898131, and EU833481 were assigned for P[8]G4 strains with genogroup II specificity in the following order: 85TW425, 85TW432, 01TW964, and 03TW1902. Accession numbers DQ898123 and EU487589 to EU487594 were assigned for P[8]G9 strains with genogroup II specificity in the following order: 99TW1866, 00TW470, 01TW1306, 02TW140, 02TW240, 02TW431, and 02TW641. Additional partial VP6 sequences were assigned accession numbers EU487595 to EU487600 for reference strains in the following order: AU32 (P[8]G9, genogroup II), 95H115 (P[8]G9, genogroup II), P (P[8]G3, genogroup II), ST3 (P[8]G4, genogroup II), WI61 (P[8]G9, genogroup II), and RotaTeq. For each Taiwanese (TW) strain, the first two digits represent the year in which the sample was collected, and the last up to four digits indicate the sample number in the corresponding year.

RESULTS

Assignment of VP6 genogroup by RT-PCR. To establish the RT-PCR assay for determination of rotavirus VP6 genogroup, reference strains, subgroup I strains including DS-1 (P[4]G2), S2 (P[4]G2), SA11 (P[2]G3), and bovine-human reassortant vaccine strains (RotaTeq), and subgroup II strains including Wa (P[8]G1), KU (P[8]G1), P (P[8]G3), YO (P[8]G3), ST3 (P[8]G4), E210 (P[4]G2), WI61 (P[8]G9), 95H115 (P[8]G9), and AU32 (P[8]G9) were subjected to RT-PCR amplification. In the first amplification, the primers, 6BEG.303 and VP6-R, were used to yield DNA products of 824 bp. In the second amplification, primer 6BEG.303 and genogroup I-specific primer 6END.682c and genogroup II-specific primer 6END.1082c were incorporated. Genogroup I and genogroup II could be distinguished according to the sizes of amplicons, 380 bp for genogroup I and 780 bp for genogroup II.

Comparison of RT-PCR, sequencing, and ELISA for determination of VP6 genogroup or subgroup specificity. In order to understand the performance of the RT-PCR method for the determination of VP6 genogroup, 80 rotavirus strains with 24 different RNA patterns, including G1 to G4 and G9 strains recovered between 2000 and 2002 were tested, and they were also tested by nucleotide sequence analysis of the partial VP6 gene. The results obtained by these two methods were identical and 100% in agreement. Sixteen of the 80 strains belonged to genogroup I, and 64 strains belonged to genogroup II (Table 1). The correlation of VP6 genogroup determined by RT-PCR with subgroup determined by ELISA was also analyzed. Using ELISA, we found that 16 of the strains tested belonged to subgroup I and 57 of the strains belonged to subgroup II, although 7 of the strains could not be assigned to either subgroup (Table 1). The subgroup specificity correlated well with VP6 genogroup for 73 of the strains (91.3% agreement).

In order to understand whether the test was suitable for rotavirus strains with a wide range of genetic variation, 150 rotavirus strains recovered over two periods, 1981 to 1999 and 2003 to 2005, were chosen to determine VP6 genogroup by RT-PCR and sequence analysis. The results of these two methods were identical and 100% in agreement for the 150 samples (Table 2).

Altogether, 230 Taiwanese rotavirus strains had been subjected to sequence analysis of the partial VP6 gene. Excluding the primer sequences, 341-bp nucleotide sequences were aligned and compared, and a phylogenetic tree was constructed. Because the tree is too enormous to be shown here,

TABLE 2. Comparison of the VP6 genogroup determined by RT-PCR and sequence analysis of the rotavirus strains with different G types and electropherotypes recovered from 1981 to 1999 and from 2003 to 2005[a]

G type	P type	No. of RNA patterns	Total no. of samples	No. of samples evaluated by:			
				Sequence analysis		RT-PCR	
				Genogroup I	Genogroup II	Genogroup I	Genogroup II
G1	P[8]	19	43	0	43	0	43
	P[6] + P[8]	1	1	0	1	0	1
G2	P[4]	9	45	45	0	45	0
	P[4] + P[6]	1	1	1	0	1	0
G3	P[8]	7	55	0	55	0	55
	P[4]	1	1	1	0	1	0
G4	P[8]	2	3	3	0	3	0
G9	P[8]	1	1	0	1	0	1
Total		41	150	50	100	50	100

[a] The rotavirus samples were selected based on RNA pattern; for each RNA pattern, two or three samples were selected for each year. These samples included G1 strains recovered from 1981 to 1989, 1992 to 1995, 1997, 1999, and 2003 to 2005; G2 strains from 1981, 1983, 1992 to 1995, 1997 to 1999, and 2003 to 2005; G3 strains from 1985, 1986, 1988 to 1991, 1995, 1997, 1999, and 2003 to 2005; G4 strains from 1985 and 2003; and a G9 strain from 1999.

65 rotavirus strains with various genotypes and 52 RNA patterns and covering a broad range of genetic variation were selected for presentation in Fig. 2. Genogroup I could be distinguished into two genetic lineages, and genogroup II could be distinguished into five genetic lineages. The nucleotide sequence identities were 93.3 to 100% for the strains within each lineage, 83 to 91.8% between different lineages within genogroup I, 86.5 to 92.1% between different lineages within genogroup II, and 74.8 to 81.2% between the two different genogroups.

Associations of rotavirus VP6 genogroups and G types and P types. It has been assumed that subgroups usually segregate with P types; that is, P[4] associates with subgroup I, and P[8] associates with subgroup II (17). Therefore, in addition to the VP6 genogroup determined by RT-PCR, the G and P types of the rotavirus strains were also determined, and the associations of the genotypes of different genes were analyzed. An additional 524 rotavirus-positive fecal samples were included for this analysis. Of these, 103 strains belonged to genogroup I, 416 strains belonged to genogroup II, and 5 strains were determined as genogroup I+II. For samples with common G- and P-type combinations and most of the samples with uncommon G- and P-type combinations, the associations between VP6 genogroups and P types were that P[4] associated with genogroup I and P[8] associated with genogroup II (Tables 3 and 4). It has been suggested that there is a 100% linkage between subgroup and NSP4 genotype among human common and reassortant strains (16). For the 28 samples with uncommon G and P type combinations, the NSP4 genes were sequenced, and the NSP4 genotypes were determined by phylogenetic analysis. Except for the five samples with genogroup I+II, NSP4 genotype A associated with genogroup I, and NSP4 genotype B associated with genogroup II (Table 4).

Analysis of the PCR amplicons for the rotavirus samples determined as VP6 genogroup I+II. It was not clear whether the genogroup I+II determined by RT-PCR reflected the VP6 gene molecules containing both of the regions specific for genogroup I and genogroup II or simply resulted from a mixture of genogroup I VP6 molecules and genogroup II VP6 molecules. Therefore, for the five rotavirus samples determined as genogroup I+II, the PCR amplicons were analyzed by cloning and sequencing. Twenty clones were selected for each sample. The genogroup of the cloned VP6 gene was determined by sequence analysis. The sequences of all of these different clones belonged to either genogroup I or genogroup II, and not a single clone was found with sequences containing both of the regions specific for genogroup I and genogroup II.

DISCUSSION

Although subgrouping by ELISA has been widely used, in some cases, a subgroup determined by ELISA seems to be imprecise because of the poor reactivity between MAbs (e.g., MAb 631/9) and subgroup II rotavirus strains (18). A similar situation has been noted to occur despite the use of different MAbs. Taniguchi et al. also found that some rotavirus strains react weakly with one subgroup II-specific MAb, YO-5 (31). In addition to the problem of the poor reactivity of the monoclonal antibodies, which results in untypeable strains due to antigenic drift, the problem of limited supplies of the VP6 subgroup-specific antibodies and that of antibody titer changes during storage are also troublesome. Therefore, development of a conventional and reliable molecular method is necessary. Molecular methods, namely, sequence analysis of the VP6 gene (18) and restriction fragment length polymorphism analysis (19), have been developed to prevent the possible misclassification by ELISA. However, sequence analysis is a technique requiring sophisticated equipment and technical knowledge that might not be available at most laboratories, and the usage of restriction fragment length polymorphism might be hampered by the point mutations that frequently occur in rotavirus. In the present study, RT-PCR, another molecular method that has been extensively used in rotavirus G and P typing (8, 10), was established for assignment of VP6 genogroups. The results showed that the VP6 genogroup I and genogroup II determined by RT-PCR were identical to those determined by sequence analysis. Genetically identified VP6 genogroup I and genogroup II corresponded well to subgroup I and subgroup II determined serologically by ELISA, respectively, suggesting that RT-PCR could be an alternative method for characteriza-

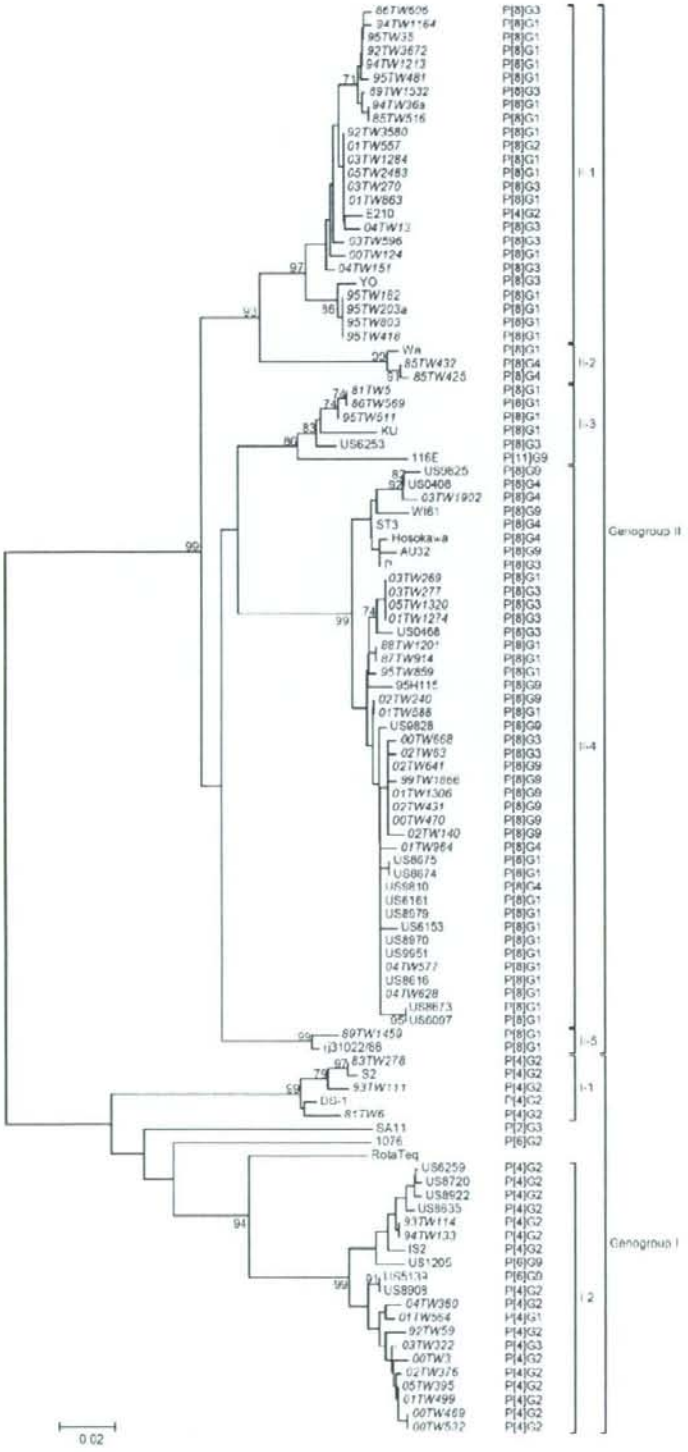

TABLE 3. VP6 genogroups of rotavirus strains with common G and P type combinations[a]

Rotavirus with VP7 and VP4 type	No. of strains	VP6 genogroup
G1P[8]	184	II
G2P[4]	98	I
G3P[8]	4	II
G9P[8]	210	II
Total	496	

[a] VP6 genogroup, G type, and P type were determined by RT-PCR.

TABLE 4. VP6 genogroups and NSP4 genotypes of the rotavirus strains with uncommon G and P type combinations[a]

No. of strains	Rotavirus		VP6 genogroup	No. of strains with NSP4 genotype:	
	G type	P type		A	B
4	G1	P[4]	I	4	0
3	G1	P[4]	I + II	1	2
1	G1	P[4] + P[8]	II	0	1
14	G2	P[8]	II	0	14
1	G3	P[4]	II	0	1
1	G3	P[4] + P[8]	II	0	1
1	G4	P[4]	I	1	0
1	G9	P[4]	II	0	1
2	G9	P[4]	I + II	0	2
28	Total			6	22

[a] VP6 genogroup, G type, and P type were determined by RT-PCR. NSP4 genotype was determined by sequence and phylogenetic analysis (4).

tion of the VP6 genes. Compared to sequence analysis, RT-PCR appeared to be simpler and more rapid.

The seven strains for which the subgroup could not be determined by ELISA were assigned to genogroup II by both sequence analysis and RT-PCR. There is no statistical significance ($P = 0.167$) to the finding that the seven strains whose subgroup could not be determined by ELISA belonged to genogroup II by RT-PCR and sequencing. The absorbances of these seven strains obtained from reactivity with subgroup I MAb S2-37 were near to that of a nonreactive control; however, the low absorbances were obtained either from reactivity with group A-common MAb YO-156 or with subgroup II-specific MAb YO-5. Although the reactivity was greater than that of the nonreactive control, a definite subgroup could not be assigned. These results revealed that containing a higher amount of viral particles in the sample is required for efficient determination of subgroup by ELISA compared to that for the determination of VP6 genogroup by RT-PCR. A relatively high level (4 of 22) of disagreement between ELISA and RT-PCR/sequencing on G9 strains was observed. However, there was no significance ($P = 0.179$) to the finding that rotavirus strains whose subgroup could not be determined by ELISA mostly belonged to the G9 strain. The low absorbances were obtained from reactivity with subgroup II-specific MAb YO-5 in three of the four G9 strains, and only one strain obtained low absorbances from reactivity with both group A-common MAb YO-156 and subgroup II-specific MAb YO-5, which suggested that some G9 strains might have antigenic drift on subgroup II-specific MAb YO-5 recognition region and caused lower reactivity. This further supported that there is a need to develop a molecular method to improve the situation that VP6 subgroup could not be determined by ELISA.

Although the primer sites for RT-PCR genogrouping were not located in the regions considered to have subgroup I or subgroup II specificity, the subgroup-defining regions were included in the partial VP6 gene for sequence analysis, the same region used in the analysis of VP6 genogroups by Iturriza-Gomara et al. (18). The genogrouping results obtained by RT-PCR in the present study were confirmed by sequence analysis of the partial VP6 genes for 230 samples, and the results correlated well and were 100% in agreement, suggesting that the degenerate primers designed for RT-PCR genogrouping were genogroup specific. The genogroup-specific primers were designed based on the VP6 sequences of reference strains from GenBank and those of 80 Taiwanese rotavirus strains with G types of G1 to G4 and G9, P genotypes of P[8] and P[4], and 24 electropherotypes. In order to understand the genogroup specificity of each of the primers, the nucleotide sequence identities within and between the genogroups were analyzed. Comparison of the VP6 gene sequences of the strains listed in Table S1 in the supplemental material, including 23 genogroup I sequences and 73 genogroup II sequences, for the primer regions, revealed that the nucleotide sequence identities of the genogroup I-specific primer within genogroup I and between the two genogroups were 83.3 to 100% and 44.4 to 61.1%, respectively. The identities of the genogroup II-specific primer within genogroup II and between the two genogroups were 89.5 to 100% and 63.2 to 73.7%, respectively.

Recently, 22 VP6 full-length sequences of the U.S. strains collected from 1996 to 2002 were submitted to the GenBank database. Based on phylogenetic analysis, Kerin et al. pointed out that rotavirus VP6 genes have greater variety than was previously suspected (21). Therefore, we included the 22 strains (10 P[8]G1, 5 P[4]G2, 2 P[8]G3, 2 P[8]G4, 1 P[6]G9, and 2 P[8]G9) in the phylogenetic analysis shown in Fig. 2 and realized that the Taiwanese strains analyzed in the present study had more genetic variation. We also compared the nucleotide sequences of these 22 U.S. strains at the regions where the genogroup-specific primers are located (Table S2 in the supplemental material) and found that 4 of the 6 genogroup I strains would be bound by our genogroup I-specific primer and

FIG. 2. Phylogenetic analysis of the nucleotide sequences of the partial VP6 gene (nucleotides 766 to 1106). The 341-bp fragments of the VP6 gene were sequenced and a phylogenetic tree was constructed based on the neighbor-joining method within the MEGA package. Percentage bootstrap values above 70% are shown at branch nodes. Branch length for a 2% nucleotide difference is indicated at the bottom. VP6 genogroup was determined by the clustering with genogroup I or genogroup II reference strains. For each strain, the P and G genotypes are shown. Taiwanese strains are indicated by italic type.