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ORIGINAL ARTICLE

Genetic variations in the gB, UL144 and UL149 genes of human cytomegalovirus strains collected from congenitally and postnatally infected Japanese children

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Abstract Human cytomegalovirus (CMV) is the leading cause of intrauterine viral infection. The association of genetic polymorphisms in some particular genes with the incidence and severity of congenital infection has been controversial. To address this issue, we analyzed the genotypes of the glycoprotein B (gB), UL144 and UL149 genes of CMV clinical strains obtained from 33 congenitally and 31 postnatally infected Japanese children. Our results demonstrated that (1) CMV strains with any combination of genotypes could be vertically transmitted from mother to fetus, potentially causing neurological abnormalities, (2) the gB3 genotype was more prevalent in the congenital cases than in postnatally infected children ($P < 0.05$), particularly in congenital cases with sensorineural hearing loss ($P = 0.009$), (3) there was no relationship between gB genotype and viral load in the urine and dried umbilical cord specimens in the congenital cases, and (4) the UL144 and UL149 genotype distributions had no bias for congenial infection. In future studies, it would be interesting to see whether the gB genotypes serve as a prognostic indicator of CMV-associated diseases.

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Abbreviations

CMV	Cytomegalovirus
PCR	Polymerase chain reaction
SNHL	Sensorineural hearing loss

Introduction

Human herpesvirus 5, also known as human cytomegalovirus (CMV), belongs to the subfamily *Betaherpesvirus* of the family *Herpesviridae*. CMV infects most people during their childhood, usually without any clinical symptoms, and establishes latent infection for the rest of their life. Congenital CMV infection occurs in 0.2–2% of all births worldwide [16, 32] and causes developmental abnormalities in >10% of infected fetuses and/or newborns. In addition to those who are symptomatic at birth, a proportion of asymptomatic newborns face a significant risk of late-onset sequelae, such as sensorineural hearing loss (SNHL) and developmental delay [16, 32]. Through retrospective diagnosis using dried umbilical cord specimens, which are generally preserved in households in Japan [23], we demonstrated that 15% of cases of severe SNHL could be ascribed to congenital CMV infection and that at least half of the CMV-related cases developed SNHL after the age of 6 months [31].

Although the genomic sequences of CMV are generally well conserved among strains, a certain number of genes exhibit a high degree of inter-strain variation [14, 37]. In addition, CMV strains passaged in vitro usually contain deletions and/or mutations in several genes that may influence cell tropism [17, 38]. The nucleotide polymorphisms of

the genes encoding viral envelope glycoproteins, such as glycoprotein B (gB), and the genes encoding cellular homologs of chemokines and chemokine receptors, such as UL144, have been studied extensively due to their potential relevance to cell tropism and virulence in the host [37, 39, 41]. However, the relationship between the genotypes of the gB and UL144 genes and clinical outcome has been controversial [37]. Some of the inconsistent results are due to the demographic heterogeneity of subjects, geographical bias, the lack of a proper control group, and the small number of subjects. The genotypes of the gN and gO genes are useful for the classification of isolates, and gN genotype may be correlated to chronic clinical outcome, but not acute clinical manifestations of congenitally infected cases [27, 36]. Recently, a degree of association between the genotypes of UL149 gene and clinical outcome has been proposed [21]. As we are interested in the association of CMV genotypes with the incidence and severity of congenital infection, we compared genotypes of congenitally infected cases, whose infections were confirmed by clinical and laboratory tests with those of postnatally infected children. Here, we report our analysis of the genetic variations in the gB, UL144 and UL149 genes.

Materials and methods

Study subjects

This study was approved by the Ethical Committee on Human Subjects of National Institute of Infectious Diseases, Asahikawa Medical College, and Fukushima Medical University. Informed consent was obtained from the parents of all children. A total of 72 clinical specimens were collected from 64 Japanese children, including 33 congenitally infected children and 31 postnatally infected children. Clinical specimens included 46 urine, 24 dried umbilical cord, 1 amniotic fluid, and 1 saliva specimen.

Eleven of the congenitally infected cases were identified in a retrospective study on severe SNHL cases [31]. Six of the congenital cases were identified by detection of CMV-specific IgM in maternal or cord blood specimens, and another six by our newborn CMV screening program [19]. The rest of the cases were identified by clinical manifestations at birth ($n = 6$) or by late-onset developmental delay ($n = 4$). All congenital CMV infections were confirmed by the detection of CMV DNA in urine specimens collected within 2 weeks after birth or in dried umbilical cord specimens.

Congenitally infected cases were classified as symptomatic ($n = 11$) or asymptomatic ($n = 22$) at birth. Symptomatic infection was defined as having at least one of the following manifestations: petechial/purpuric rash, jaundice with conjugated hyperbilirubinemia (>2 mg/dl direct bilirubin), hepatosplenomegaly, seizures, thrombocytopenia ($<1 \times 10^5/mm^3$), neuroimaging abnormalities (cerebral calcifications and/or ventriclemegaly), and microcephaly (occipital frontal head circumference at birth, <10th percentile). Premature infants or those with low birth weight (<10th percentile) alone were classified as asymptomatic.

Twenty-two of the 33 congenitally infected cases exhibited one or more of the following neurological abnormalities at birth or during subsequent evaluation in childhood: microcephaly ($n = 1$), cerebral calcifications and/or ventriclemegaly ($n = 4$), seizures ($n = 2$), developmental delay ($n = 16$), and SNHL ($n = 19$). Evaluation of the severity of developmental delay and SNHL was based on the criteria described previously [30, 31].

Postnatally infected children included 23 healthy infants between 12 and 24 months of age, and 8 infant patients between 6 and 29 months of age with one of the following manifestations: hepatic damage ($n = 3$), pneumonitis ($n = 3$), bone marrow transplantation ($n = 1$), and systemic infection due to premature birth ($n = 1$). Postnatally infected healthy children were chosen for the presence of CMV in urine specimens from more than 100 volunteers who attended routine medical check-ups at pediatric hospitals or who inquired regarding their natural infection status. Congenital CMV infection in most of these children was excluded by the absence of CMV DNA in their dried umbilical cord specimens. Dried umbilical cord specimens or dried blood spots were not available in a few cases. However, their congenital infection was unlikely, as the incidence rate of congenital CMV infection in our screening program has been less than 0.5% [19].

DNA preparation and real-time PCR

DNA samples were purified from body fluid specimens and/or dried umbilical cord specimens using commercial kits (QIAampViral RNA mini kit and QIAamp DNA mini kit, QIAGEN), and the CMV copy numbers in the purified DNA samples were determined by real-time PCR assays. The details of the real-time PCR assays were described previously [31].

Genotyping

DNA fragments encoding the variable regions of the gB, UL144, and UL149 genes were amplified by nested PCR using PfuI polymerase (Promega) in a 50-μl reaction volume. For the first-round PCR, 10–100 copies of CMV DNA were used as templates in the reaction, and 2 μl of the first-round PCR products was used for the second-round PCR. Optimized PCR conditions and primer sets are shown in Table 1. The PCR products were separated on

Table 1 Primers and PCR conditions

Genes and primers	Round	Sequence (5′–3′)[b]	Amplicon (bp)[a]	PCR conditions	References
gB					
gBout2	First	GCAGCACCTGGCTCTATCG	974	96C/5 m, [94C/45 s, 65C/45 s, 72C/2 m] × 40, 72C/10 m	[8]
gBout		GCACCTTGACGCTGGTTTGG			
gB1319	Second	GGAAYTSGAACGTTTGGC	304	94C/2 m, [94C/45 s, 60C/45 s, 72C/1 m] × 40, 72C/10 m	[12, modified]
gB1604		GAAACGCGCGGCAATCGG			
UL144					
UL144-F	First	TCTCGTATTACAAACCGCGGAGAGGATG	738	96C/5 m, [94C/45 s, 55C/45 s, 72C/2 m] × 40, 72C/10 m	[31]
UL144-R		ACTCAGACACGGTTCCGTAAAGTGCTTC			
UL144-F2	Second	TTCCGGTAGGAGGCATGAAG	587	94C/2 m, [94C/45 s, 55C/45 s, 72C/1 m20 s] × 40, 72C/10 m	
UL144-R2		GTGACTTCATCGTACCGTGA			
UL149					
UL149-F	First	ACTCCTCTTCCTTCTGCTC	776	96C/5 m, [94C/45 s, 55C/45 s, 72C/90 s] × 40, 72C/10 m	[21]
UL149-R		CCGACGTCTCGTACACTAC			
UL149-F2	Second	GTCTGGTGGCCCTGATGTAC	289	94C/2 m, [94C/45 s, 60C/45 s, 72C/1 m] × 40, 72C/10 m	This study
UL149-R2		CGATTGGGGAGCGACAAGAC			

[a] Based on the Merlin sequence (Accession no: AY446894)

[b] R = A/G, S = G/C, Y = C/T

agarose gels and purified using a DNA extraction kit (QIAEX II, QIAGEN). The purified DNA fragments were sequenced with a BigDye Terminator Cycle Sequencing kit (ver. 3.3, PE Applied Biosystems). The primers for the second-round PCR were used as primers for sequencing.

Obtained sequences were assembled with ATGC software version 4.0 (Genetyx, Tokyo, Japan) and aligned with Genetyx 7.0. Phylogenic analysis was conducted using MEGA software version 3.1 (Biodesign Ins., Tempe, USA [24]). The schema for the genotypes of gB, UL144, and UL149 were based on prior publications [11, 12, 21, 25].

Statistical analysis

Genotype distributions and the relationship between genotype and the outcome of CMV infections were analyzed using the chi-square test. P values of <0.05 were considered significant. Comparison of viral loads among genotypes was performed by the Mann–Whitney U test using SPSS software (ver. 11).

Results

Comparison of strains obtained from different sources of newborns with congenital CMV infection

In this study, sequences of the variable regions of the gB, UL144 and UL149 genes from 72 specimens obtained from 64 children were determined. Infection with multiple strains was not identified at a level detectable in raw sequencing data (>25% in the population). The 64 individuals included one pair of twin newborns with congenital CMV infection. Since the twins had identical sequences for the three genes tested, they were counted as a single entity. Both urine and dried umbilical cord specimens were collected from five congenital CMV cases. The sequences of the gB, UL144 and UL149 variable regions from these five cases were identical regardless of the specimen source. In one of the five cases, the urine specimen was collected 31 months after birth, suggesting the stability of CMV sequences. Urine, amniotic fluid, and dried umbilical cord specimens were collected from one additional case of congenital CMV, and the sequences of the UL144 gene in the three specimens were found to be identical.

Overall distribution of gB, UL144 and UL149 genotypes

The gB, UL144 and UL149 genotypes were analyzed for the 63 strains (the strains from the twins were counted as one). The overall distribution of the 63 gB genotypes was as follows: 43 gB1, no gB2, 16 gB3, no gB4, and 4 not available (na). That of the UL144 genotypes was 24 UL144-A, 25 UL144-B, 10 UL144-C, no UL144-A/B, no UL144-A/C and 4 na; and that of the UL149 genotype was 18 UL149-1, 24 UL149-2, 17 UL149-3, and 4 na (Fig. 1a). Since only one study to date has analyzed the UL149 genotype, the phylogenic tree of the UL149 sequences

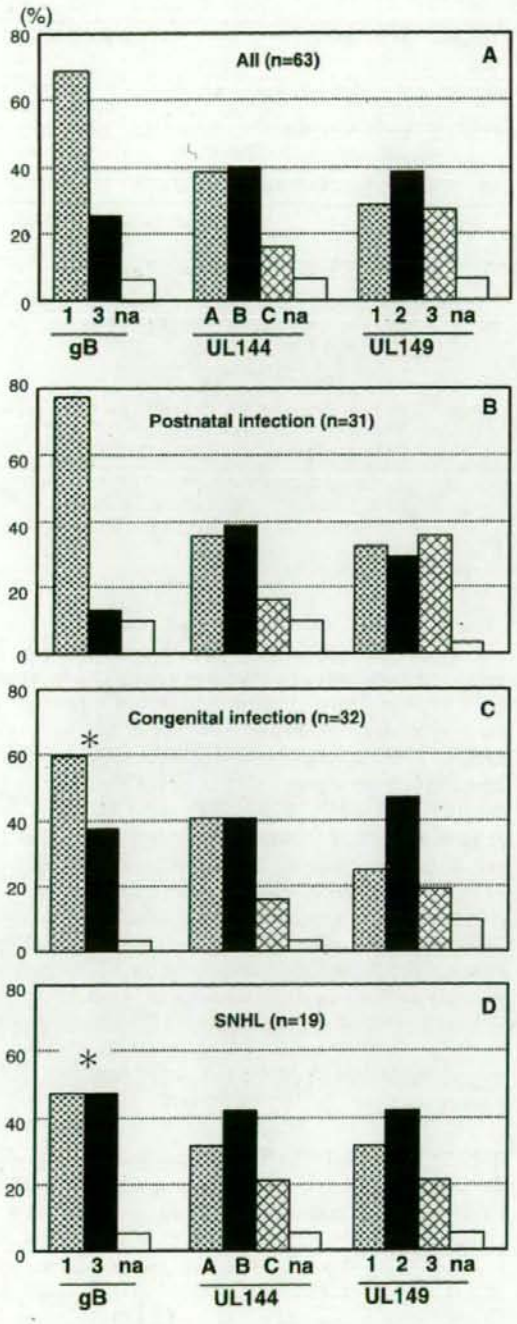

Fig. 1 Distribution of CMV genotypes. *Each panel* shows the genotyping results of the following subjects: **a** all analyzed cases ($n = 63$); **b** postnatally infected children ($n = 31$); **c** congenitally infected cases ($n = 32$); **d** congenital cases with SNHL ($n = 19$). *na* sequence data not available. *Asterisks* indicate a significant difference in the distribution of the genotypes from that of the postnatally infected children

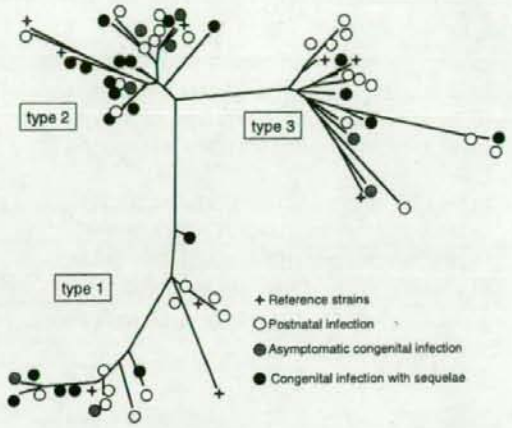

Fig. 2 Unrooted phylogenic dendrogram of CMV UL149 sequences. The tree was generated by the neighbor-joining method based on an approximately 300-bp nucleotide sequence region. *Open circles* postnatal CMV infection ($n = 30$); *shaded circles* congenital CMV infection without clinical symptoms at birth ($n = 8$); *closed circles* congenital CMV infection with neurological abnormalities ($n = 21$); *stars* reference strains ($n = 9$)

obtained from our study is shown in Fig. 2, confirming the previous grouping of CMV strains into three UL149 genotypes.

Comparison of genotype distributions between congenital and postnatal infection

The genotype distributions of each gene were compared between congenitally and postnatally infected children (Fig. 1b, c). The proportion of the gB3 genotype in the congenital group was significantly ($P = 0.035$) higher than that in the postnatally infected group. In contrast, the genotype distributions of the UL144 and UL149 were similar between the two populations.

Relationship between genotype and clinical outcome

A comparison of genotype distribution between strains from the congenital cases with clinical symptoms at birth and those of the cases without symptoms at birth did not reveal any significant differences.

Next, the relationship between genotype and neurological abnormalities was examined (Table 2). Among the 32 congenitally infected cases, 11 cases were identified from the severe SNHL cohort. Among the

Table 2 Relationship between gB genotype and clinical outcome

	No. of collected samples	No. of sequenced samples	Genotype		*P* value[a]
			gB1	gB3	
Postnatally infected	31	28	24 (86%)	4 (14%)	
Healthy	23	20	19 (95%)	1 (5%)	
Others	8	8	5 (63%)	3 (37%)	
Congenitally infected	32[b]	31	19 (61%)	12 (39%)	0.035
Asymptomatic at birth	21	20	13 (65%)	7 (35%)	>0.05
Symptomatic at birth	11	11	6 (55%)	5 (46%)	0.038
Neurologic abnormalities	22	21	12 (57%)	9 (43%)	0.025
SNHL	19	18	9 (50%)	9 (50%)	0.009

[a] Comparison with the postnatally infected children

[b] Twins are counted as one sample

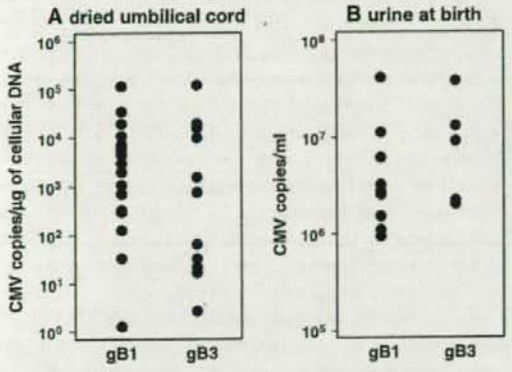

Fig. 3 Relationship between gB genotype and viral load in dried umbilical cord (**a** $n = 30$) and urine specimens (**b** $n = 14$). In (**a**), genotypes of some strains were based on the genotypes determined using urine specimens obtained from the same individuals

remaining 21 cases, the medical records of 11 subjects contained clinical findings that indicated neurological abnormalities, including mental development delay ($n = 9$) and/or SNHL ($n = 8$). Although the remaining ten infants with asymptomatic congenital infection have not yet exhibited any clinical abnormalities, their follow-up periods have not been long enough to observe any late-onset sequelae. The gB genotype distribution of strains from the 21 congenital cases with neurological abnormalities (there was insufficient material from one case for the analysis) indicated a significantly high prevalence of gB3 (43%) compared with the strains from the postnatally infected children ($P = 0.025$). The gB3 genotype was also significantly prevalent among the congenital cases with SNHL ($n = 18$) ($P = 0.009$) (Fig. 1d; Table 2).

In contrast to the gB gene, there were no significant differences in the genotype distributions of the UL144 and UL149 genes in the congenital cases with neurological abnormalities or with SNHL.

Absence of any relationship between viral load and genotype

Since the gB3 genotype was more prevalent in the congenital cases, we investigated the relationship between the gB genotype and viral load (Fig. 3). Copy numbers of CMV DNA in the dried umbilical cord specimens and those in urine specimens collected within a few days after birth were evaluated as viral loads. There was no observable relationship between gB genotype and viral load in the urine or dried umbilical cord specimens (Mann–Whitney *U* test, both $P > 0.05$). Furthermore, no relationship between genotype and viral load was noted for the other genes (data not shown).

Discussion

In this study, we investigated the relationship between the genotypes of the gB, UL144 and UL149 genes and the incidence and severity of congenital infection, and found that (1) CMV strains with any combination of genotypes could be vertically transmitted from mother to fetus, potentially causing neurological abnormalities, (2) the gB3 genotype was more prevalent in the congenital cases than in postnatally infected children ($P < 0.05$), particularly in congenital cases with SNHL ($P = 0.009$), (3) there was no relationship between gB genotype and viral load in congenital cases, and (4) the distribution of UL144 and UL149 genotypes had no bias for congenital infection.

Several studies on immunocompromised patients have identified infections with multiples strains of CMV [1, 13, 15, 18, 20, 40], and a recent study reported a relationship between multiple infection and more severe clinical manifestations in transplantation patients [13]. Using genotype-specific serologic assays, we and others demonstrated that pre-existing immunity did not protect against infection with strains of a different genotype [10, 20]. Multiple infection was observed only in a limited number of

congenitally infected newborns [5, 50], although multiple strains were identified in autopsy tissues from stillborn infants who died of congenital CMV infection [4]. This study did not find any obvious cases of multiple infection, although a more stringent approach, such as sequencing of a significant number of clones, should be performed to detect minor populations. Alternatively, genotype-specific serology would be useful to identify re-infection or multiple infections. In some immunocompromised patients, different strains were isolated from different sources; for example, the ocular fluid and blood, from the same individuals [33]. In this study, urine and dried cord specimens obtained from six congenitally infected newborns had identical sequences for the three genes examined. The urine specimen from one of the cases was collected 31 months after birth, indicating the stability of CMV sequences. Stability of the CMV sequences in the hypervariable regions over time in vivo has also been described [6, 45]. Although one study identified a nucleotide alteration over time [46], analysis of various gene sequences is required to distinguish spontaneous sequence alterations from reinfection with similar strains.

This study found that the gB3 genotype had a significant bias towards congenital infection as well as towards neurological abnormalities, such as SNHL. Differences in both T cell tropism and clinical outcome were reported for the gB genotypes [8, 15, 22, 28, 44, 47, 49]. However, several studies on immunocompromised patients did not find any connection between gB genotype and disease recurrence or the occurrence of invasive diseases [18, 43]. The effects of gB genotype on the clinical outcome of congenital infections have been also controversial [5, 7, 48]. It is unlikely that the inconsistent results are due to differences in the variable region in the gB gene submitted to analysis, because a previous study [42] confirmed an almost complete match, apart from some intragenic recombination, of the gB genotypes between the N-terminal region and the cleavage region. Geographical and demographical differences in the study populations, the lack of a proper control group for comparisons in some studies by others, and small population sizes might in part explain the inconsistent results. Geographic and demographic biases in the distribution of the gB genotypes have been reported in several studies [51], and this and other Japanese studies observed only limited numbers of the gB2 and gB4 strains in pediatric and transplantation populations [29, 46]. As the Japanese society is comprised of people with relatively uniform demographics and socioeconomic status, it is unlikely that selection of study subjects influenced the observations in the current study. In addition, in order to establish a control population, we collected specimens not only from congenital CMV cases but also from postnatally infected, generally healthy children. One critical factor that differentiated this study from others was the inclusion of the congenital cases with SNHL that were identified by a retrospective study on severe SNHL children, as this may engender bias in the make up of the congenital group.

Clinical outcome has been shown to be correlated with viral load in blood specimens [9]. However, the present study did not find any relationship between specific genotype and the viral load in urine or dried umbilical cord specimens from the congenital CMV cases, which is consistent with a previous report describing the absence of any association of specific UL144 and gB genotypes with the viral load in amniotic fluid specimens of congenital cases [2, 35].

We did not find any association between the UL144 and UL149 genotypes and the incidence of congenital infection or clinical outcome. Although Arav-Boger and colleagues reported an association between UL144 genotype and the outcome of congenital disease, the findings have not been supported by other researchers [2–4, 6, 25, 26, 34]. Our results also fail to support the notion of a significant association. Although it was reported recently that particular amino acid sequences of UL149 are associated with microcephaly and Hirschsprung's disease [21], this study did not find any correlation between these particular amino acid sequences and clinical outcomes.

In conclusion, this study indicates that any CMV strain, irrespective of its genotype, can be vertically transmitted from mother to fetus, potentially causing neurological sequelae. In future studies, it would be interesting to clarify whether gB genotype has any value as a prognostic indicator of CMV-associated neurological abnormalities.

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Novel Nonstructural Protein 4 Genetic Group in Rotavirus of Porcine Origin

To the Editor: Infection with group A rotavirus is the main cause of acute gastroenteritis in infants and young children worldwide and in young animals of many species, including piglets. In recent years, several epidemiologic studies designed to monitor the appearance of novel or atypical rotavirus antigenic types have provided evidence for the increasing antigenic diversity of group A rotaviruses (*1–3*). In addition to the 2 rotavirus classification systems, VP7 (G) and VP4 (P) genes, the virus can also be classified on the basis of the nonstructural glycoprotein 4 (NSP4)–encoding gene. Sequence analyses of the NSP4 gene indicated the presence of at least 5 distinct genetic groups among human and animal rotaviruses, termed A to E (*1,4,5*). Among human rotaviruses, the diversity of NSP4 genes has been restricted mainly to genetic groups A and B; only a few human strains possess genetic group C. Conversely, all 5 NSP4 genetic groups (A–E) have been identified in rotaviruses of animal origins. To our knowledge, porcine rotaviruses (PoRVs) have been reported to belong only to NSP4 genetic group B (*1*).

During an epidemiologic survey of PoRV from June 2000 through July 2001, a total of 175 fecal specimens were collected from diarrheic piglets from 6 different farms in Chiang Mai Province, Thailand. Of these, 39 (22.3%) specimens were positive for group A rotavirus (*6*). A novel and unusual PoRV CMP034 strain was isolated from a 7-week-old piglet during this survey. Molecular genetic characterization showed that the CMP034 strain carried a novel P[27] genotype with a new lineage of G2-like rotavirus genotype (*7*). We performed a molecular analysis of the NSP4 gene of this strain in comparison with those of other NSP4 gene sequences available in the GenBank database.

The full-length of NSP4 gene was amplified by NSP4–1a and NSP4–2b primer pairs (*8*). The PCR amplicon was sequenced in both directions by using the BigDye Terminator Cycle Sequencing kit (PerkinElmer-Applied Biosystems, Inc., Foster City, CA, USA) on an automated sequencer (ABI 3100; PerkinElmer-Applied Biosystems, Inc.). The sequence of CMP034 was compared with those of reference strains available in the National Center for Biotechnology Information GenBank database by using BLAST (www.ncbi.nlm.nih.gov/blast). The NSP4 nucleotide sequence of the CMP034 strain was deposited in GenBank under accession no. DQ534017.

The complete NSP4 nucleotide sequence of PoRV CMP034 strain was 750 bp and contained a single long open reading frame coding for a protein of 175 aa. Comparative analysis of the CMP034 NSP4 sequence with those of the 5 representative established genetic groups (A–E) showed the highest sequence identity, at 92.6% nt and 96.9% aa levels, with 1 PoRV strain, P21–5 (*9*). However, CMP034 and P21–5 shared a low degree of sequence identity with other NSP4 genetic groups. The NSP4 sequence identities of the CMPO34 and P21-5 strains ranged from 74% to 78% nt and 75%–79% aa levels with those of genetic group A; 77%–86% nt and 79%–86% aa levels with genetic group B; 69%–73% nt and 75%–78% aa levels with genetic group C; 62%–65% nt and 55%–60% aa levels with genetic group D; and only 43%–50% nt and 29%–33% aa levels with genetic group E. The phylogenetic tree confirmed that PoRV strains CMP034 and P21–5 were located exclusively in a separated branch, which was distantly related to the other 5 known NSP4 genetic groups (Figure). However, a bootstrap support for the separation of

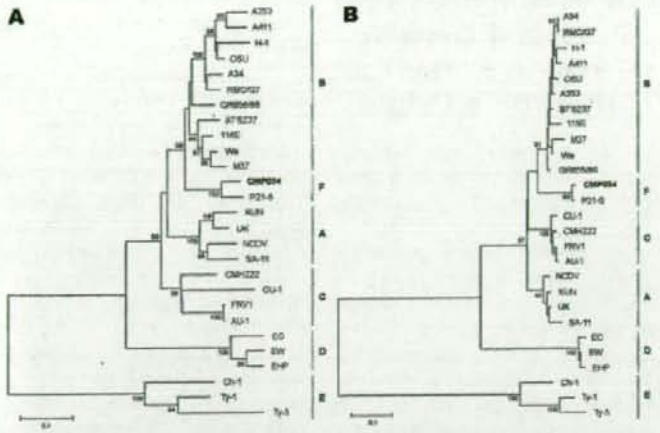

Figure. Phylogenetic analyses of the NSP4 nucleotide (A) and amino acid (B) sequences displaying the relationships between porcine rotavirus strain CMP034 (shown in **boldface**), P21–5, and other 5 known NSP4 genetic groups. Bootstrap values are shown at the branch nodes. Branch length for a 10% nucleotide difference is indicated at the bottom.

the gene into a separate lineage is very strong with nucleotide sequencing but weak by amino acid analysis in this phylogentic tree. Our finding indicates that PoRV strains CMP034 and P21–5 are likely a novel NSP4 genetic group and, therefore, tentatively proposed as a NSP4 genetic group F.

On the basis of the accumulated evidence of transmission of rotaviruses between pigs and other animal species, including humans, pigs are regarded as 1 potential reservoir for the emergence of unusual or novel strains of rotaviruses (*6*,*7*). In our study, the virus carried a novel NSP4 genetic group that has been isolated from a diarrheic piglet in Thailand. The NSP4 sequence analysis of our CMP034 strain revealed a PoRV strain closely related genetically to the NSP4 gene sequence of PoRV strain P21–5 isolated in Slovenia (*9*). PoRV strains CMP034 and P21–5 shared the same VP4 genotype as P[27] with over 90% aa sequence identity. The only difference observed between the 2 strains was that CMP034 belonged to the G2-like genotype whereas P21–5 belonged to G1 genotype. The relatedness between NSP4 sequences of strains CMP034 and P21–5 was confirmed by phylogenetic analysis, which showed that both CMP034 and P21–5 clustered closely together in a branch separated from those of other 5 NSP4 genetic groups. This finding suggests that NSP4 of PoRV strain CMP034 and P21–5 may have derived from the same ancestor. The isolation of 2 strains of rotaviruses with a close genetic relatedness of NSP4 gene from Thailand and Slovenia, 2 countries that are located in different continents, may indicate that this novel NSP4 genetic group has already been introduced into PoRVs worldwide. To verify this hypothesis, extensive epidemiologic surveillance of rotavirus in pigs may need to be conducted in several other regions of the world.

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PorB2/3 Protein Hybrid in *Neisseria meningitidis*

To the Editor: Class 2 and class 3 porin (PorB) proteins are the major proteins found in the outer membrane of *Neisseria meningitidis* (*1*); they function as porins, allowing the passage of small molecules through the outer membrane. PorB outer membrane proteins are transmembrane proteins with 8 predicted surface-exposed loops (I-VIII), which vary in length and in amino acid sequences. Several sequence analyses of these proteins have shown 4 regions with a high level of amino acid variability in loops I, V, VI, and VII (variable regions [VRs] 1–4) (*2*). The extensive antigenic variability of these proteins forms the basis of the *N. meningitidis* serotyping scheme (*3*,*4*). These 2 classes of proteins are mutually exclusive, and they are expressed by alternate alleles (*porB2* and *porB3*) at the *porB* locus (*1*).

All *N. meningitidis* strains received in the Spanish Reference Laboratory for *Neisseria* are routinely serotyped by whole-cell ELISA (*5*) with a set of monoclonal antibodies (MAbs) provided by the National Institute for Biological Standards and Control (South Mimms, UK) that includes the following serotypes: 1 (MN3C6B), 2a (5D4–5), 2b (MN2C3B), 4 (5DC4-C8G8), 14 (MN5C8C), 15 (8B5–5G9), and 21 (6B11F2B5). Those meningococci that appear as nonserotypeable (NT) are analyzed by sequencing the *porB* gene (*6*). In the case discussed here, in the sequencing of a NT strain, the *porB* gene showed an unusual sequence.

This strain, isolated in Spain during 2006, was recovered from the cerebrospinal fluid of a patient with meningococcal disease. The *porB* gene sequence shows VR1–4, which is exclusive of PorB3 protein, and VR2-Eb, VR3–2ab, and VR4-Cc, which are typical of PorB2 (GenBank accession no. EF094023). A comparison of this new sequence with the available *porB* sequences in the *Neisseria*.org database (http://neisseria.org/nm/typing/porB) enabled a more detailed analysis of the fragments corresponding to *porB3* and *porB2* found in this sequence. The fragment from nt 1 to 213 was identical to the *porB3–193* allelic variant (VR1–4, VR2-Aa, VR3–7, VR4–14b), and the second part, with nt 233–972 identical to *porB2–99* (VR1-Dc, VR2-Eb, VR3–2ab, VR4-Cc). The region of 214–232 nt is identical in the 3 variants. Therefore, this is a true hybrid molecule, which appears to have arisen from recombinational events between *porB2–99* and *porB3–193* alleles. In fact, this finding has prompted the inclusion of a new family called porB2/3 hybrid in the *Neisseria*.org database to facilitate the collection of this type of *porB* sequences.

The most likely origin of the *porB2/3* hybrid (4, Eb, 2ab, Cc) is the acquisition of DNA that encodes a VR1–4 sequence by a meningococcus with a *porB2–99* allelic variant. It is less likely that DNA encoding the *porB* VR2-Eb, VR3–2ab, and VR4-Cc sequences was acquired by a meningococcus with the *porB3–193* allelic variant because a longer fragment of DNA would have been transferred.

In spite of the presence of a VR1–4, which should be recognized by the set of MAbs used, this strain appeared as NT. A Western blot assay using MAb type 4 showed a good recognition epitope-MAb. Therefore, the failure of MAbs to identify this strain may have been due to the limited accessibility of the epitope because of the alteration of the PorB protein, which might be affecting its conformation. Once again, genetic characterization should be a preferred method over phenotypic characterization for typing meningococcal strains. Molecular characterization of NT strains in other laboratories might clarify the true frequency of this event.

Intragenic recombination between porin genes of the same allelic family is likely occurring in nature because mosaic gene structure has been reported in *porB* genes. However, *porB2/3* recombinants have never been previously found in the nature. Given the known ability of meningococci to be transformed by DNA from other strains, it is surprising that occurrence of genuine *porB2/3* hybrids has not yet been documented. There is only a report of naturally occurring gonococci expressing a hybrid *porB1a/porB1b* (*7*) (PorB1a and PorB1b gonococcus porins, as in meningococci, are encoded by 2 families of diverged alleles of the *porB* gene [*8*]). Gonoccocal strains expressing the recombinant *por* genes appear to be particularly susceptible to the bactericidal effect of human serum (*9*). A similar situation might happen in *N. meningitidis*, with a selective disadvantage in the invasive process of these hybrid strains, explaining the rarity of naturally occurring hybrids. By contrast, mechanisms like this are frequently used by meningococci to avoid the immune response against ordinary antigens. The balance between advantages and disadvantages at this level would show the true implications of this event.

This finding is relevant regardless of its frequency in nature. This report suggests how frequent the recombination events should occur among the meningococcal population: even theoretical mutually exclusive genes can produce hybrid variants; such knowledge is an important step in the development of future vaccines based on protein formulations.

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Norovirus and Sapovirus Infections among Children with Acute Gastroenteritis in Ho Chi Minh City during 2005–2006

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Summary

A molecular epidemiological study on common diarrheal viruses was conducted in a children's hospital in Ho Chi Minh City between December 2005 and November 2006. Fecal samples were collected from 502 pediatric patients with acute gastroenteritis, and were screened for the presence of norovirus (NoV) and sapovirus (SaV). NoVs GII and SaVs were detected in 6.4% and 1.2% specimens, respectively, while there was no NoV GI found among studied samples. NoVs could be identified through the year, except in April and July, with the peak of detection rate (62.5%) during the rainy season. Conversely, four out of six (66.7%) of the SaV strains were identified during the dry season. Patients aged between 6 and 23 months were found to be more infected by NoVs. The overall mean severity score of norovirus-positive patients was 9.8 ± 3.6, and no significant difference of severity scores among patients belonged to different age groups, gender and place of living. The results of phylogenetic analysis showed the diversity of caliciviruses circulating in the area, and various types of recombination were identified among NoVs and SaVs detected. These results provide important information on calicivirus infections among Vietnamese children.

Key words: norovirus, sapovirus, clinical manifestations, recombinant, Vietnam.

Introduction

Norovirus (NoV) and sapovirus (SaV) are members of the family *Caliciviridae* (other two genera are *Lagovirus* and *Vesivirus*). The NoV and SaV strains are determined as the major causes of non-bacterial acute gastroenteritis in infants and young children [1, 2].

After being discovered through electron microscope in 1972 [3], NoVs were identified widely in epidemiological studies, and were the cause of outbreaks of gastroenteritis in various settings including hospitals [4, 5], schools [6, 7], cruise ships [8, 9], restaurants [10, 11] and day care centers [12, 13]. Sequence analyses of worldwide NoVs revealed that they are classified into seven distinct genogroups (GI to GVII), of these, GI, GII, GIV, GVI and GVII are known to infect humans [14]. NoV contains a positive-sense single-stranded RNA genome surrounded by an icosahedral capsid. The NoV genome contains three open reading frames (ORFs). The ORF1 encodes non-structural proteins, including NTPase, protease and RNA-dependent RNA polymerase (RdRp), OR2 encodes the capsid protein (VP1) and ORF3 encodes a minor structural protein (VP2).

SaV infects both children and adults, and have been found to cause outbreaks of gastroenteritis in kindergarten [15], hospital [16] and mental health care facility [17]. SaV- associated diarrhea is usually mild, compared to that caused by NoVs [18].

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TABLE 1
Primer pairs used to amplify NoVs and SaVs in this study

Primer	Target virus	Polarity	Sequence position (5′ to 3′)/reference strain	Amplicon size	Target region
G1SKF	NoV GI	+	5342-5361 (Norwalk/68)	330bp	Polymerase and capsid junction
G1SKR	NoV GI	−	5653-5671 (Norwalk/68)		
COG2F	NoV GII	+	5003-5028 (Lordsdale)	387bp	Polymerase and capsid junction
G2SKR	NoV GII	−	5367-5389 (Lordsdale)		
SLV5317	SaV	+	5083-5105 (Manchester)	434bp	Polymerase and capsid junction
SLV5719	SaV	−	5494-5516 (Manchester)		

NoV GI, norovirus GI; NoV GII, norovirus GII; SaV, sapovirus.

SaVs can be divided into five genogroups (GI to GV), among which, GI, GII, GIV and GV are identified within humans [19]. The SaV GI, GIV and GV genomes contain three ORFs, whereas the SaV GII genome contains two ORFs. ORF1 encodes all the non-structural proteins, including RdRp, and the major capsid protein (VP1). ORF2 encodes a small protein, and ORF3 encode a protein of unknown function [20].

Normally, in both NoV and SaV, the genogroup/genotypes are generally maintained across the three ORFs. A recombinant NoV or SaV can be defined as one that clusters with two distinct groups of strains when two different regions (normally the capsid and polymerase) of the genome are subjected to phylogenetic analysis. Since the first NoV recombinant, Snow Mountain strain [21], was reported, various naturally occurring recombinants in different types have been identified [22–25]. Likewise, the identification of SaV recombinants have been reported elsewhere [26–28].

In Vietnam, NoVs and SaVs were identified from several epidemiological surveillances, and are considered as the important agents of viral gastroenteritis in the country [29, 30]. The first Vietnamese NoV recombinants were reported from a surveillance during 1999–2000 [30], since then, neither data about calicivirus infections nor recombinant virus has been reported. A hospital-based surveillance was conducted in Ho Chi Minh City during 2005–2006 that investigated the presence of common viral agents causing diarrhea in children, has been described elsewhere [31]. In this study, we reported in details the detection of NoVs and SaVs in the surveillance mentioned above, and described the molecular characteristics of NoV and SaV strains detected. The clinical manifestations and the evaluation of disease severity in patients were also included.

Materials and Methods

Patients

Patients with acute gastroenteritis who either visited the out-patient ward or were admitted to the Department of Gastroenterology, Children's Hospital 1, Ho Chi Minh City from December 2005 to Novemer 2006, were recruited in the surveillance. Patients were examined by pediatricians, and the clinical symptoms of dehydration were assessed based on the WHO guideline [32]. A 20-point Vesikari's score was used to evaluate the disease severity in patients [33].

Fecal samples collection and virus detection

A total of 502 fecal samples were collected from studied patients (one specimen from each patient). The fecal specimens from the outpatients were collected at the out-patient ward or from the inpatients within 24 h after admission and stored at −20°C until use. They were prepared as a 10% suspension in distilled water and the viral RNA genomes were extracted from the fecal suspension with a QIAamp Viral RNA Mini kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instruction. The presence of NoVs and SaVs in fecal specimens was determined by RT-multiplex PCR [34]. Three primer pairs, G1SKR–G1SKF, COG2F–G2SKR and SLV5317–SLV5749 [34] were used to amplify NoVs GI, NoVs GII and SaVs, respectively (Table 1). PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min, and then visualized under ultraviolet light. The results were recorded by photography.

Nucleotide sequencing and phylogenetic analysis

All of NoVs and SaVs detected in this study were subjected to nucleotide sequencing by using the Big Dye Terminator Cycle Sequencing kit version 3.1 and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc.) according to the manufacturer's instruction. Primer pairs mentioned above were used as sequencing primers, generating a partial nucleotide sequence, including both polymerase region and the capsid region [34]. Similarities of the sequenced strains with other strains were assessed by BLAST search using the default options (DNA DataBank of Japan). Multiple sequence alignments were

TABLE 2
Monthly distribution of NoVs and SaVs detected from children with acute gastroenteritis in the Children's Hospital 1, Ho Chi Minh City, during 2005–2006

Seasonality	Dry season					Rainy season						Dry	Total (%)
	Dec 05	Jan 06	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	
No of specimens	20	30	43	57	30	53	32	17	30	32	60	98	502 (100)
No (%) of NoVs	2 (10.0)	1 (3.3)	2 (4.7)	4 (7.0)	0 (0)	3 (5.7)	2 (6.3)	0 (0)	6 (20.0)	4 (12.5)	5 (8.3)	3 (3.1)	32 (6.4)
No (%) of SaVs	0 (0)	1 (3.3)	2 (4.7)	0 (0)	1 (3.3)	1 (1.9)	0 (0)	0 (0)	1 (3.3)	0 (0)	0 (0)	0 (0)	6 (1.2)

NoV, norovirus; SaV, sapovirus. 62.5% of NoVs were identified in the rainy season.

calculated using the CLUSTALX program, and the phylogenetic trees were constructed by the neighbor-joining method with the MEGA 3.1 software package [35], and using different NoVs and SaVs sequences available in GenBank for comparison and as outgroups.

Accession numbers

The selected nucleotide sequences of Vietnamese NoVs and SaVs strains described in this study have been deposited in GenBank under accession numbers EU137732–EU137739.

Results

Detection of NoVs and SaVs

Among 502 fecal specimens collected during the 1-year surveillance, NoVs GII were determined in 32 (6.4%) specimens, and SaVs were detected in six (1.2%) specimens. Fifteen and four specimens showing positive with NoV and SaV, respectively, were found to be in mixed infection with other viral pathogens. There was no NoV GI found in this study. Regarding seasonal pattern, NoVs could be identified through the year, except in April and July. Twenty out of 32 (62.5%) of the NoVs were detected during the rainy season, which usually begins in May and ends in October in the southern part of Vietnam, including Ho Chi Minh City. Conversely, four out of six (66.7%) of SaV strains were identified during the dry season (Table 2).

Characteristics of the NoV and SaV-positive patients

The Table 3 showed the characteristics of positive cases with NoVs and SaVs. Twenty-eight and four patients showing positive with NoVs and SaVs, respectively, had adequate medical records for further analyses. To characterize the age distribution, all patients were classified into five different age groups (<6, 6–11, 12–23, 24–35 and >35 months old). NoV patients were neither found in <6 nor in >35 months of age, while 27 out of 28 NoV patients were between 6 and 23 months of age. Similarly, three out of four SaV cases were classified into either 6–11 or 12–23 months age group. Majority of NoV patients (22/28, 78.6%) were male, however, three out of four SaV patients were female. Although the surveillance was conducted in a children's hospital in Ho Chi Minh City, only 11/28 (39.3%) cases lived in the city, the remaining 17/28 (60.7%) of NoV patients came from various provinces in the southern part of Vietnam.

Clinical signs and symptoms of NoV infections

Seventeen patients showing monoinfection with NoV [31] were selected for analysis of the clinical manifestations, among them 15 medical records were enough data for further analyses. The main clinical signs and symptoms observed in children with NoV infection were diarrhea (100%), watery stool (93.3%), vomiting (66.7%), highest temperature $\geq$38.5°C (33.3%), coughing (26.7%) and coryza (6.7%). The mean duration of diarrhea and vomiting were 4.4 ± 3.9 days and 1.5 ± 1.7 days, respectively, and the maximum episodes of diarrhea and vomiting were 6.5 ± 2.5 times per day and 3.3 ± 2.8 times per day, respectively (Table 4).

Evaluation of severity in patients showing monoinfection with NoV by using a 20-point numerical score showed that the mean severity score of NoV positive patients was 9.8 ± 3.6. The severity scores were analyzed further by age groups, gender, place of living (Ho Chi Minh City and non-Ho Chi Minh City residents), time of collection (during rainy and dry season) and status of patients (hospitalized and non-hospitalized patients) (Table 3). Obviously, the mean severity scores of patients belonging to some groups were observationally lower than those of other groups (e.g., patients who were 12–23 months old, or patients who lived in Ho Chi Minh City); however, the difference was not statistically significant ($p > 0.05$). The only significant difference was observed between inpatients and outpatients, with the mean severity scores in each group being 10.82 ± 3.49 ($N = 11$) and 7.0 ± 2.45 ($N = 4$), respectively ($p < 0.05$). A comparison of the mean severity scores between monoinfection cases and

TABLE 3
Attributes of NoV positive cases[a] and mean severity score of patients in each group

	Distribution of patients by										
	Age (months)					Gender		Place of living		Patient status	
	<6	6–11	12–23	24–35	>35	Male	Female	HCMC[b]	Others	Inpatient	Outpatient
No. (%) of NoV cases	0 (0)	10 (35.7)	17 (60.7)	1 (3.6)	0 (0)	22 (78.6)	6 (21.4)	11 (39.3)	17 (60.7)	21 (75)	7 (25)
Vesikari's score[c]		11.4 ± 4.28 (*N* = 5)	8.67 ± 3.16 (*N* = 9)	12.0 (*N* = 1)		9.7 ± 3.97 (*N* = 10)	10.0 ± 3.16 (*N* = 5)	8.2 ± 1.92 (*N* = 5)	10.6 ± 4.06 (*N* = 10)	10.82 ± 3.49[d] (*N* = 11)	7.0 ± 2.45[d] (*N* = 4)

[a]Data based on 28 complete medical records.
[b]HCMC, Ho Chi Minh City.
[c]Data based on 15 medical records of patients who showed monoinfection with NoV.
[d]$P < 0.05$.

TABLE 4
Clinical signs and symptoms of patients who showed monoinfection with NoV during a one-year surveillance in Ho Chi Minh City, 2005–2006

Signs and symptoms	NoV infection cases
Diarrhea	100%
Watery stool	93.3%
Vomiting	66.7%
Temperature ≥ 38.5°C	33.3%
Coughing	26.7%
Coryza	6.7%
Mean duration of diarrhea	4.4 ± 3.9 days
Mean duration of vomiting	1.5 ± 1.7 days
Maximum episodes of diarrhea/day	6.5 ± 2.5 times per day
Maximum episodes of vomit/day	3.3 ± 2.8 times per day

mixed infection cases was also performed, however, the difference was not statistically significant (data not shown).

Only one medical record from two patients showing monoinfection with SaV was available, therefore, description of the clinical features of SaV infection in this study was not performed.

Phylogenetic analysis of NoV strains and identification of various recombinations

All of the 32 NoV strains detected in this study were successfully determined nucleotide sequence with the amplified fragments, which included both polymerase and capsid region. Phylogenetic analysis based on the capsid region revealed that 16/32 (50%) NoV strains clustered within the GII.4, and 13/32 (40.6%) strains belonged to the GII.3b cluster, according to the classification reported by Phan *et al.* [14]. One strain, HCMC91, clustered together with GII.12 NoV strains (96% nucleotide identity with the Chitta strain), and other two strains, HCMC204 and HCMC311, belonged to the GII.6 cluster (95% nucleotide identity with the SaitamaU17 strain). Interestingly, these two Vietnamese GII.6 strains did not group with any GII.6 NoV strains from sublineage a to d, therefore, clustered into a novel sublineage, tentatively called GII.6e (Fig. 1).

To verify the sequence identities of the GII strains, an additional phylogenetic analysis of Vietnamese NoV strains and other reference strains based on the polymerase region was performed (Fig. 2). All of the 16 capsid-based GII.4 NoV strains maintained their genotype in the polymerase region, however, other strains bored a different either genotype or subgenotype when polymerase-based grouping was carried out. Twelve out of the 13 capsid-based GII.3b NoV strains clustered into the GII.3a lineage,

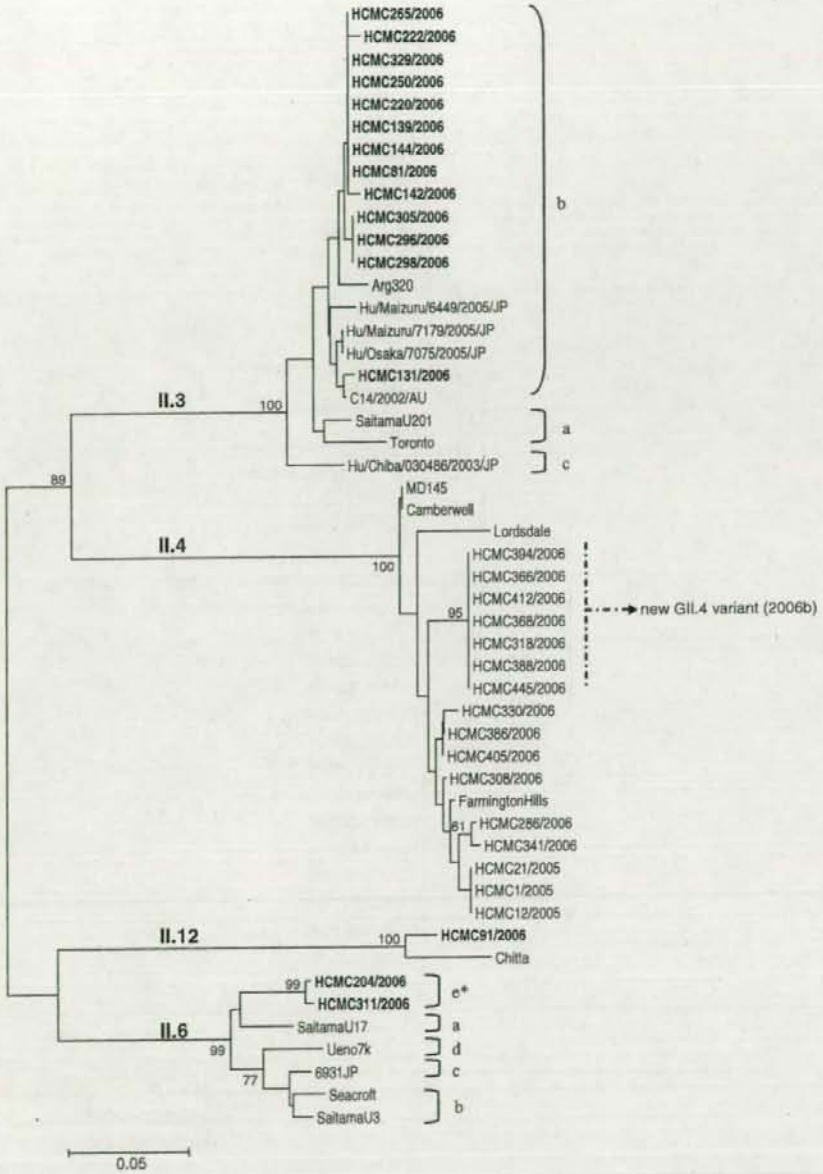

FIG. 1. Phylogenetic tree of the capsid region of 32 Vietnamese NoVs and other reference NoV strains available from GenBank. Vietnamese recombinant strains are in bold face. Genotypes and subgenotypes are indicated. Bootstrap values >75% are shown at the brach nodes.

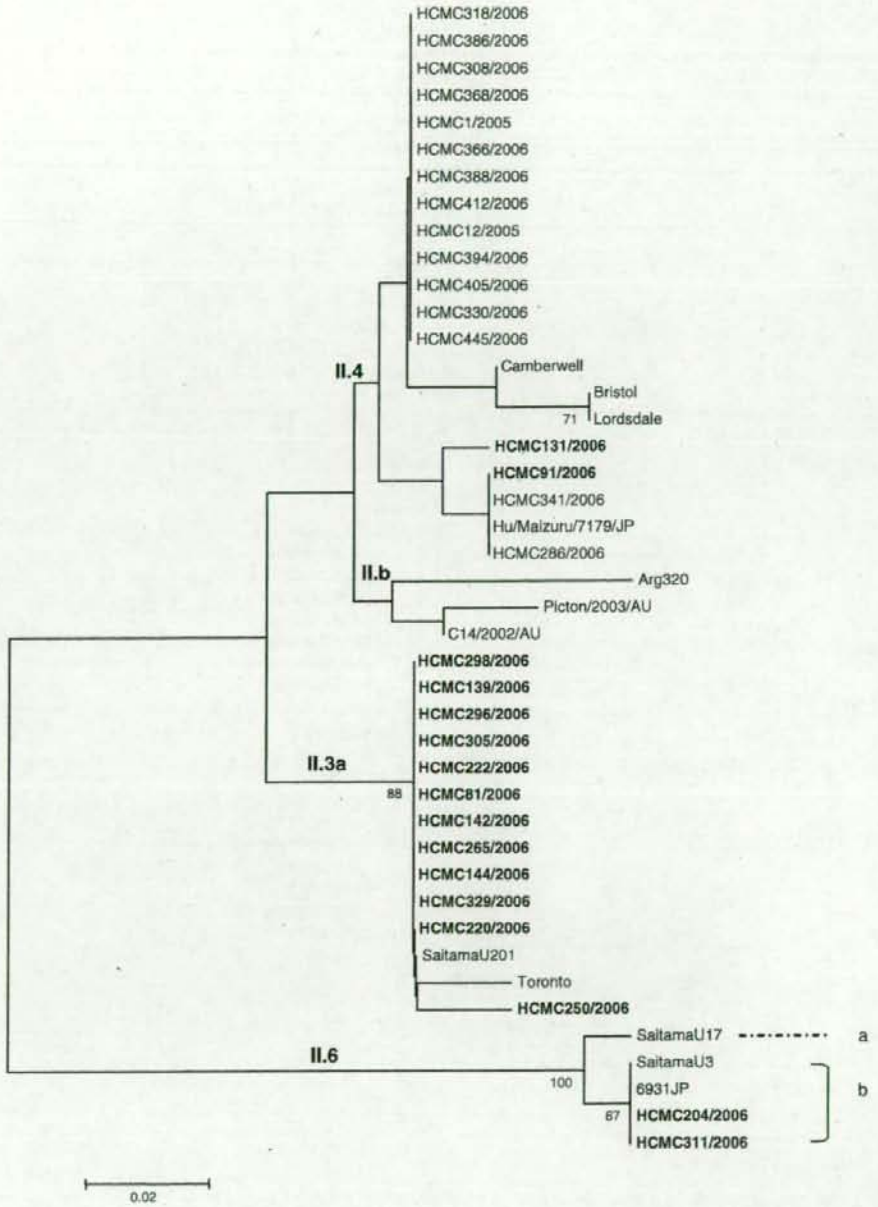

FIG. 2. Phylogenetic tree of the polymerase region of 32 Vietnamese NoVs and other reference NoV strains available from GenBank. Vietnamese recombinant strains are in bold face. Genotypes and subgenotypes are indicated.

TABLE 5

Molecular characteristics of NoVs strains detected among patients with acute gastroenteritis in the Children's Hospital 1, Ho Chi Minh City, during 2005–2006

Strain	Collection date[a]	Polymerase		Capsid		Type of recombination
		Genogroup/genotype	Representative	Genogroup/genotype	Representative	
HCMC1	Dec	II.4		II.4		
HCMC12	Dec	II.4		II.4		
HCMC21	Jan	II.4		II.4		
HCMC81	Feb	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC91	Feb	II.4	Lordsdale	II.12	Chitta	Intergenotype
HCMC131	Mar	II.4	Lordsdale	II.3b	Arg320	Intergenotype
HCMC139	Mar	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC142	Mar	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC144	Mar	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC204	May	II.6b	SaitamaU3	II.6e	N/A	Intersubgenotype
HCMC220	May	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC222	May	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC250	Jun	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC265	Jun	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC286	Aug	II.4		II.4		
HCMC296	Aug	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC298	Aug	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC305	Aug	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC308	Aug	II.4		II.4		
HCMC311	Aug	II.6b	SaitamaU3	II.6e	N/A	Intersubgenotype
HCMC318	Sep	II.4		II.4		
HCMC329	Sep	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC330	Sep	II.4		II.4		
HCMC341	Sep	II.4		II.4		
HCMC366	Oct	II.4		II.4		
HCMC368	Oct	II.4		II.4		
HCMC386	Oct	II.4		II.4		
HCMC388	Oct	II.4		II.4		
HCMC394	Oct	II.4		II.4		
HCMC405	Nov	II.4		II.4		
HCMC412	Nov	II.4		II.4		
HCMC445	Nov	II.4		II.4		

N/A, not applicable.

whereas the other strain, HCMC131, grouped with other GII.4 strains when polymerase-based grouping was performed. This type of recombination, GII.3b/GII.4, was similar to that of the NoV recombinant strain 5017/04/JP, which was reported formerly [36]. Similarly, the capsid-based GII.12 strain, HCMC91, bored a different genotype, GII.4 when a BLAST search was performed in the polymerase region. This strain also shared best identity, 96%, with the well-known GII.4/GII.12 recombinant strain SaitamaU1 [22] in both the polymerase and capsid region, demonstrating that HCMC91 was also a recombinant virus. Regarding two capsid-based GII.6e strains, HCMC204 and HCMC311, the polymerase-based phylogenetic tree clearly showed that they clustered together with other NoV strains into the GII.6b sublineage, therefore, these two Vietnamese strains were GII.6b/GII.6e recombinant strains. Altogether, half of the NoV (16/32) strains identified in this study were determined as recombinant viruses (Table 5).

Phylogenetic analysis of SaV strains and the identification of a novel recombination

Results of nucleotide sequencing of the 434 bp PCR product allowed us to analyze the molecular characteristics of both polymerase and the capsid region of SaV strains detected. Among six Vietnamese SaV strains, genotype GI.1, GI.2 and GII.1 were identified in two, one and one strain, respectively, and all of these four SaV strains maintained the same genogroup/genotype across polymerase and the capsid region (Fig. 3). However, the remaining two strains, HCMC86 and HCMC180, showed different genotypes when the polymerase-based and capsid-based phylogenetic analyses were conducted. These two SaV strains shared 100% nucleotide identity,

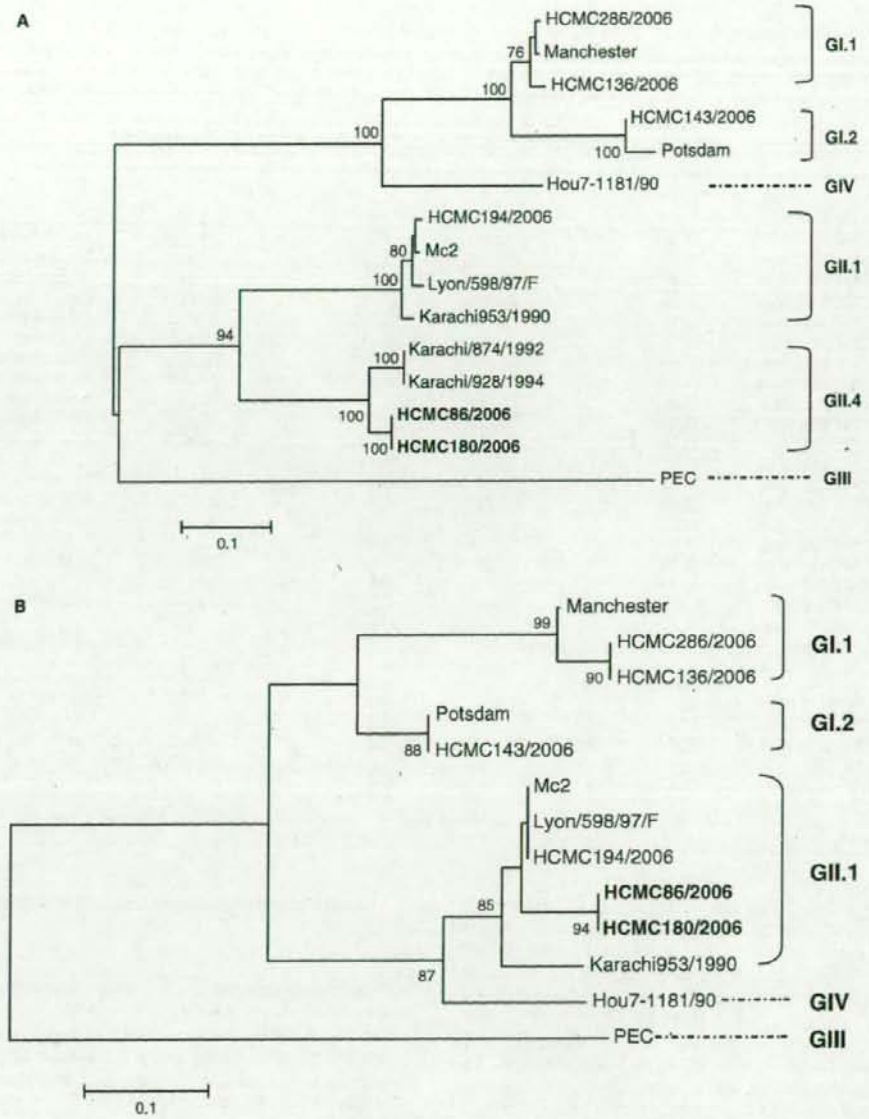

FIG. 3. Phylogenetic tree of the (**A**) capsid and (**B**) polymerase region of 32 Vietnamese SaVs and other reference SaVs. Two Vietnamese SaVs strain, HCMC86 and HCMC180, showed different genotypes when polymerase-based and capsid-based phylogenetic trees were constructed.

which indicates that they are the same strain. Nucleotide comparison showed that HCMC86 and HCMC180 had best identities (89.8–94.2%) with GII.1 strains in the polymerase region; however, they had higher homology with two Pakistani GII.4 SaV strains (Karachi/874 and Karachi/928) than GII.1 strains (94.2% vs. 67.7–68.1%) when a capsid-based comparative analysis was performed (Table 6).