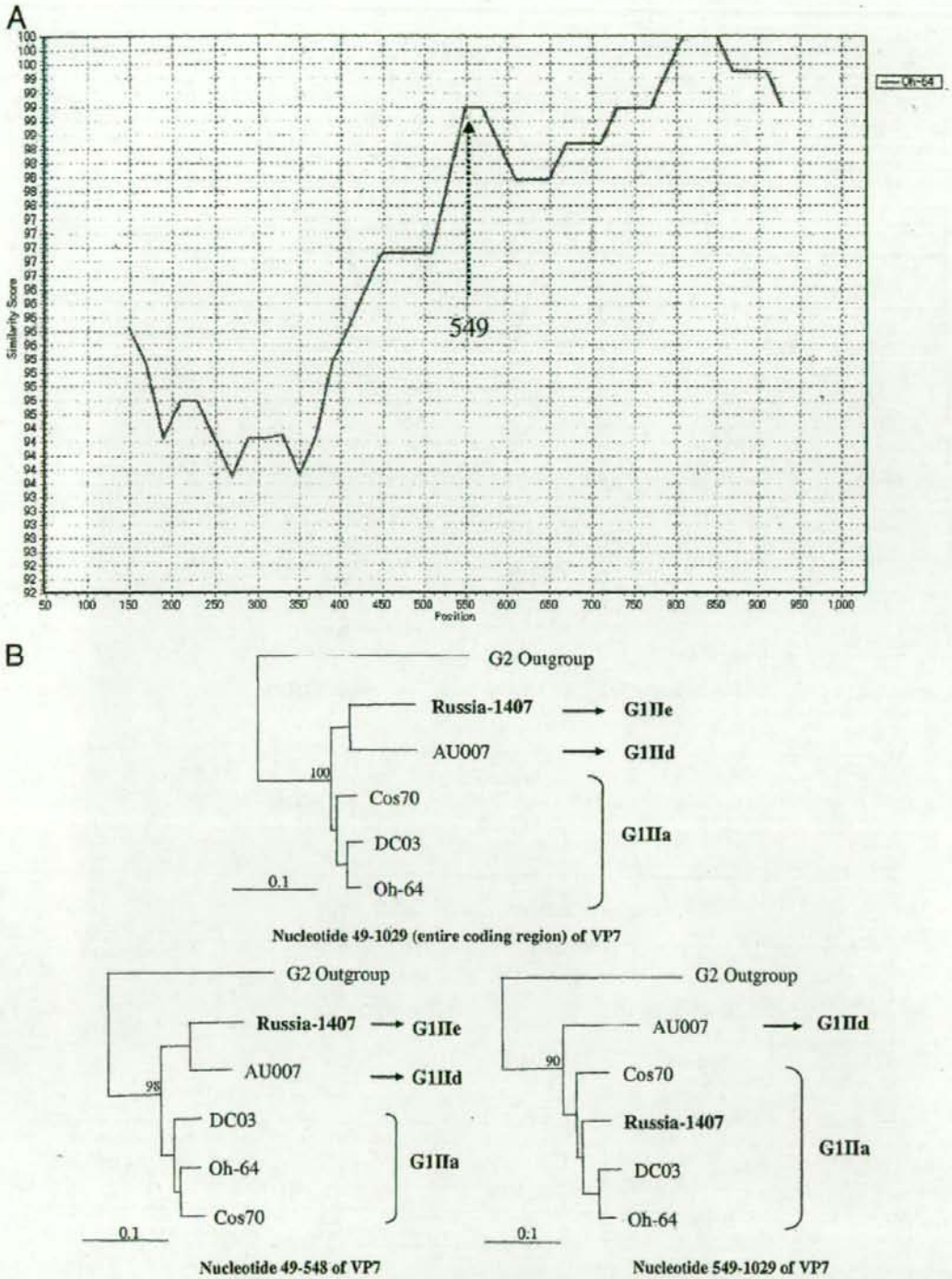

FIG. 4. Identification of novel recombinant G1 rotavirus strain Russia-1407. (A) Nucleotide identity comparison of the complete coding regions of VP7 genes of Russia-1407 (belonging to lineage IIe as a query strain) with that of OH-64 (belonging to lineage IIa as a reference strain). (B) Observation of changes of genotypes of Russia-1407 on the basis of phylogenetic trees of nucleotide sequences. The scale indicates nucleotide substitutions per position. Numbers in the branches indicate bootstrap values. Bootstrap values of 70% or higher are considered significant for the grouping.

and nucleotide substitution in producing antigenic variants for rotaviruses (3, 4) have been recognized, the intragenic recombination events in a few rotavirus strains were reported. Several studies showed the mixed infections with two different serotypes or with different variants belonging to the same serotype in one individual, therefore increasing the probability of reassortment or intragenic recombination in the evolutionary pathway of rotaviruses (3–5). RNA intragenic recombination is a mechanism for virus evolution (25). The primary mechanism involved in intragenic recombination in RNA viruses is the copy-choice model in which intragenic recombination is known to depend on various immunological and intracellular constraints, such as (i) successful coinfection of the host and in a single cell by two parental strains, (ii) efficient replication of parental viral genomes with template switching, and (iii) adaptation to different environments to be transmitted (25). The observation of the rotavirus intragenic recombinant strains detected in the study probably underscored that these strains theoretically might fulfill all prerequisites for their intragenic recombination. However, it is unclear that a copy-choice mechanism could work in rotaviruses where all replication occurs in particles and the replication complexes are anchored and separated from each other. The predicted amino acid sequences, including regions adjacent to the suggested breakpoints, were further analyzed. No same amino acid breakpoints were identified since the nucleotide breakpoints at positions 469 and 649 in Ban-59 are involved in coding for the amino acids leucine (position 141) and glutamine (position 201), respectively, and the nucleotide breakpoints at positions 449 and 549 in D and Russia-1407 are involved in coding for the amino acids tyrosine (position 134) and proline (position 167), respectively. Interestingly, the alignment of complete VP7 protein using CLUSTAL X showed the highly conserved region from amino acid 124 to amino acid 210 in G1 rotaviruses (data not shown). More interestingly, all of four amino acid breakpoints of three recombinant G1 rotaviruses were located in this region. Altogether, this highly conserved region in the VP7 gene was a favorable condition for recombination in G1 rotaviruses. However, further research should be conducted to elucidate the molecular mechanism of intragenic recombination in rotaviruses.

In conclusion, our results have described the genetic characterization of novel intragenic recombinant rotaviruses and have increased the evidence of worldwide distribution of intragenic recombinant rotaviruses. Intragenic recombination events in rotaviruses are not completely understood; further research should be conducted to investigate whether intragenic recombination can be potentially dangerous for host species, and it likely limits the rotavirus control programs and has major implications for rotavirus vaccine design.

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Short communication

Genetic heterogeneity, evolution and recombination in emerging G9 rotaviruses

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Abstract

G9 rotavirus is recognized as the emerging genotype spreading around the world. The rapidly increasing detection of this virus in association with the genetic heterogeneity raises questions regarding its origin and epidemiological importance. A total of 380 sequences of rotavirus G9 strains including our sequence data from Vietnam and Japan, which were detected from 1983 to 2006 in five different continents, were collected from GenBank to investigate their heterogeneity and evolution. A novel nomenclature for G9 rotaviruses is proposed, in which these viruses are clustered into 6 lineages with 11 sublineages. Multiple amino acid substitutions of VP7 specific for lineages and sublineages were found. Interestingly, six short amino acid motifs correctly defined phylogenetic lineages and sublineages. Another interesting finding was the identification of recombinant G9 rotavirus, bearing different genotype sequence. In view of rotavirus evolution, this report is an additional evidence to support the notion that there might exist a genomic relatedness between human and porcine rotaviruses.

Keywords: G9 rotavirus; Recombination; Lineage; Sublineage

1. The study

Rotavirus diarrhea is a significant disease of morbidity and mortality among children worldwide (Mulholland, 2004). G9 rotavirus was initially reported as the causative agent of acute gastroenteritis in the Philadelphia, the United States in 1983 (Clark et al., 1987). Soon after that, G9 was not detected for about a decade and then reemerged in the same city in 1995 (Clark et al., 2004). In Japan, the first G9 was sporadically identified in 1983 (Nakagomi et al., 1990). However, G9 was reported to be the most predominant genotype in Tokyo and Sapporo with a high detection rate of 52% and 71% during 1998–1999 (Zhou et al., 2003). In Thailand, during two consecutive years of epidemiological study, 1987–1989, G9 was first reported in the prevalence of about 2% (Urasawa et al., 1992). In 2000–2001, which was over a decade later, G9 infection had increased to an exceptionally high frequency with 91% in hospitalized children with acute gastroenteritis (Khamrin et al., 2006). Recently, the emerging G9 is recognized as one of the most widespread genotypes in causing acute gastroenteritis in children from many countries (Santos and Hoshino, 2005). The rapidly increasing G9 detection in association with the genetic heterogeneity raises intriguing questions regarding its origin and epidemiological importance.

A total of 380 sequences of G9, including our sequence data from Vietnam and Japan in 1996–2000 (Doan et al., 2003; Zhou et al., 2003), which did not include any gaps in alignment for the entire coding region of VP7 genes, were collected from GenBank. Sequence alignment was performed using CLUSTAL X. Phylogenetic trees with 100 bootstrap replicates of the nucleotide alignment datasets were generated using the neighbor-joining method. Genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot and bootscanning were used to compare recombinant rotavirus sequences (Salminen et al., 1995; Lole et al., 1999).

Detection period of 380 G9 strains expanded over two decades from 1983 to 2006. Fig. 1B revealed that G9 infection associated with acute gastroenteritis covered five different continents, including Asia (nine countries), Europe (eight countries), America (four countries), Oceania (one country)

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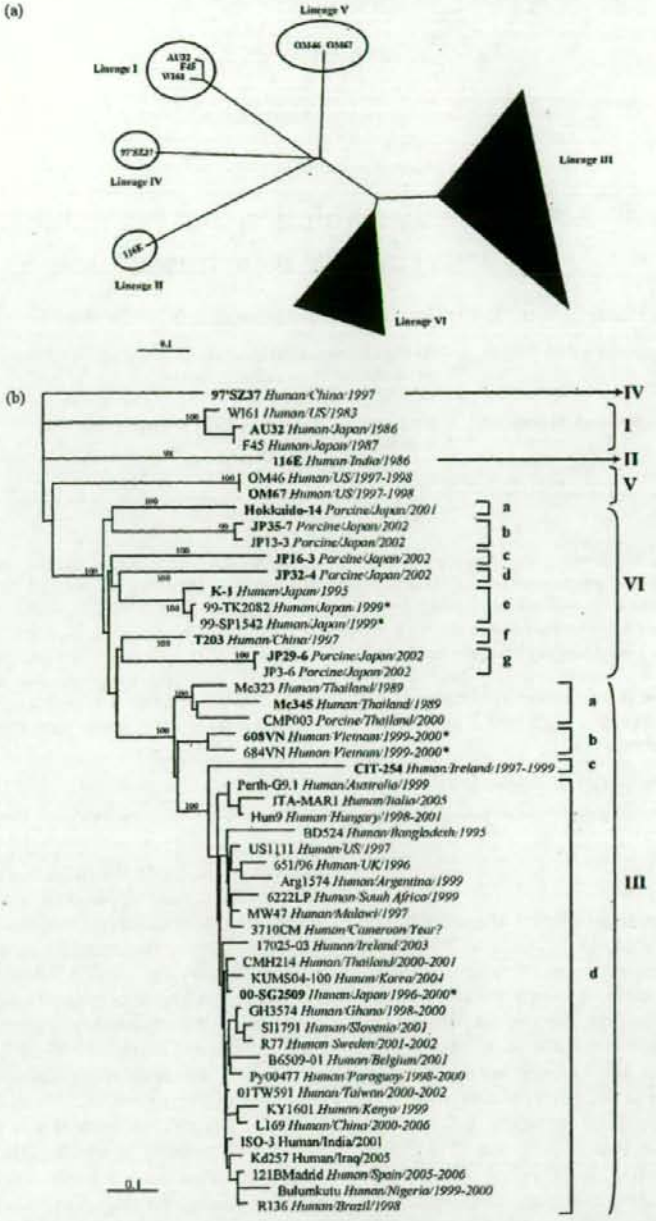

Fig. 1. Novel nomenclature of G9 rotaviruses. The phylogenetic trees were constructed from nucleotide sequences of VP7 genes of G9 rotavirus strains. The scale indicates nucleotide substitutions per position. Numbers in the branches indicate the bootstrap values. (A) Schematic presentation of six distinct G9 rotavirus lineages from I to VI. Black triangles contain rotavirus G9 strains belonging to lineages III and VI. (B) Novel classification of G9 rotavirus strains. Rotavirus strains were classified into multiple sublineages. *Our sequence data of G9 rotavirus strains. Rotavirus strains and their accession numbers used in this study were as follows: 97'SZ37 (AF260959), WI61 (AB180969), AU32 (AB045372), F45 (AB180970), 116E (L14072), OM46 (AJ491181), OM67 (AJ491179), Hokkaido-14 (AB176677), JP35-7 (AB176683), JP13-3 (AB176679), JP16-3 (AB176680), JP32-4 (AB176682), K-1 (AB045374), 99-TK2082 (AB091755), 99-SP1542 (AB091753), T203 (AY003871), JP29-6 (AB176681), JP3-6 (AB176678), Mc323 (D38053), Mc345 (D38055), CMP003 (AY707787), 608VN (AB091777), 684VN (AB091778), 6222LP (AF529871), 3710CM (AY816184), MW47 (AJ250544), BD524 (AJ250543), US1111 (AJ250269), Arg157 (AF323710), R136 (AF438228), CIT-254 (AF281044), Bulumkutu (AF359358), Hun9 (AJ605311), ITA-MAR1 (EF150338), B6509-01 (AY487895), GH3574 (AY211068), SI1791 (AY630924), R77 (AY196129), Py00477 (DQ015693), KY1601 (DQ822600), Perth-G9.1 (AY307094), 17025-03 (DQ207390), KUMSO4-100 (DQ056299), CMH214 (AY699298), 00-SG2509 (AB091748), ISO-3 (AF501580), 01TW591 (DQ490173), Kd257 (AB247943), and 651/96 (AJ401263).

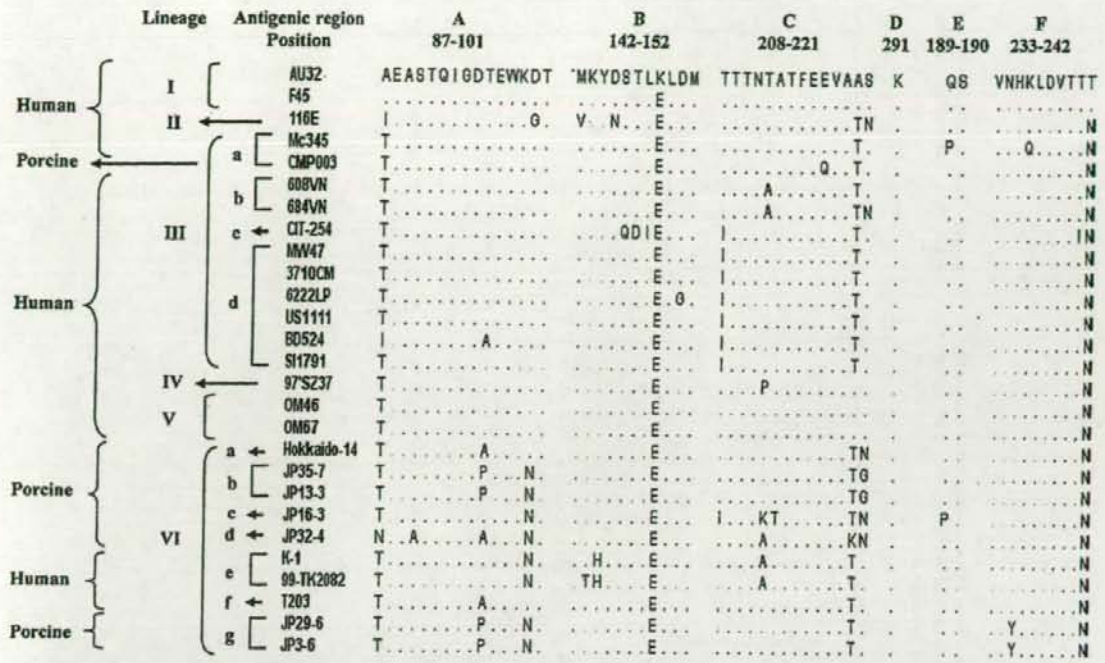

Fig. 2. The deduced amino acid sequences of antigenic regions of the VP7 gene of G9 rotavirus strains worldwide. Residues that match sequences of antigenic regions are denoted by dots. AU32 has been included as the G9 rotavirus standard.

and Africa (six countries). To investigate heterogeneity and evolution of rotavirus within G9 genotype, VP7 genes of worldwide G9 strains were used for genetic analysis. Fig. 1A demonstrated that phylogenetic analysis identified six distinct lineages. Of note, lineages I, II, IV and V were found only in humans; lineages III and VI were in both humans and porcine. Lineage III consisted of four sublineages. Of these, sublineages IIIa, IIIb and IIIc were exclusively limited to Thailand, Vietnam and Ireland, respectively. In contrast, sublineage IIIb was detected in many regions around the world. Moreover, lineage VI was further classified into seven sublineages. Of these, sublineages a–d and g were found only in humans and sublineages e and f were detected in porcine. The nucleotide identity of strains within each sublineage ranged from 96% to 100%, indicating only less than 5% of genetic difference among them. In contrast, sequence variation among strains between lineages was considerably higher, ranging from 5% to 14%.

Direct inspection of sequence alignment showed that there were three kinds of amino acid substitutions specific for lineages and sublineages (Table 1). For differentiation within lineages, 24 amino acid substitutions located at variable regions and conserved regions of VP7 genes were found, e.g., V at position 26 for lineage II; V at position 41 for lineage V. For differentiation within sublineages, seven amino acid substitutions located at variable regions and conserved regions of VP7 were identified, e.g., I at position 26 for sublineage VIb; H at position 144 for sublineage VIe. For specificity of lineages and sublineages, four amino acid substitutions located at variable regions were found, e.g., I at position 9 for lineage III and sublineages VIf and VIg. Of interest was the identification of only short signature sequences of VP7 gene, which correctly defined the phylogenetic lineages and sublineages. Seven amino acids at positions 9, 11, 17, 26, 29, 41, and 44 of variable regions formed identification codes for lineages, e.g. lineage I had a code of VTILAIV; lineage III had a code of ITVLAIA. This motif also made an identification code for sublineages, e.g. sublineages VIa and VIb had identification codes VTVLAIV and VTILVIV, respectively. Other five short motifs of different codes only for sublineage IIIc were also found. Fig. 2 showed the diversity of the amino acid substitutions in VP7 antigenic regions A–F of worldwide G9 strains. All lineages except for lineage I showed an amino acid substitution N at position 242 of the antigenic region F. Moreover, multiple amino acid variations that were specific for lineages and sublineages were also identified, e.g., G at position 100 for lineage II and H at position 144 for sublineage VIe. Of note, lineages III and VI shared two amino acid substitutions at positions 208 and 220 of the antigenic region C.

Fig. 3A showed evidence of the novel recombinant G9 bearing different genotype sequence when the nucleotide sequence of the G9 strain CIT-254 was compared with those of the G9 strain R136 and the G1 strain 87H140 using simplot and bootscanning. Strain CIT-254 was detected during the molecular surveillance of rotavirus infection in children with

Table 1

The amino acid substitutions from sequence data of the VP7 gene specific for lineages and sublineages. The amino acid substitutions highlighted in italics are specific for lineages. The amino acid substitutions highlighted in boldface are specific for sublineages, respectively. The amino acid substitutions highlighted in both italics and boldface are specific for both lineages and sublineages. Shaded boxes indicate variable regions located at rotavirus VP7. The phylogenetic lineage- and sublineage-defining sequences are indicated by a horizontal bar

Position			9	11	17	26	29	41	44	49	65	87	90	96	100	102/4/6	122/3/5/6	138/9	141	142	144	145	146/7/8	181	155/8	160	242	267
Amino acid			V	T	I	L	A	I	V	K	T	A	S	T	D	LQF	TDAS	NV	L	M	Y	D	STL	A	LL	L	T	E
I		AU32	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		F45	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
II		116E	*	*	*	*V*	*	*	*	*	*	*T*	*	*	*G*	*	*	*	*	*V*	*	*N*	*	*	*	*	*N*	*
III	A	Mc345	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
		CMP003	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
	B	608VN	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
		684VN	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
	C	CIT-254	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*RPI*	*SNVH*	*KL*	*R*	*	*	*	*QDI*	*	*IF*	*F*	*N*	*D*
	D	MW47	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
		3710CM	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
		6222LP	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
		US1111	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
		BD524	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
		SI1791	*I*	*	*V*	*	*	*	*A*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*D*
IV		97SZ37	*	*	*V*	*	*	*	*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*T*	*	*T*	*N*	*
V		OM46	*	*I*	*V*	*	*	*V*	*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*
		OM67	*	*I*	*V*	*	*	*V*	*	*	*	*T*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*N*	*

Table 1 (*Continued*)

VI	A	Hokkaido -14	•	•	V	•	•	•	•	•	•	T	•	A	•	•	•	•	•	•	•	•	•	•	•	•	N	•
	B	JP35-7	•	•	•	•	V	•	•	•	A	T	•	P	N	•	•	•	•	•	•	•	•	•	•	•	N	•
		JP13-3	•	•	•	•	V	•	•	•	A	T	•	P	N	•	•	•	•	•	•	•	•	•	•	•	N	•
	C	JP16-3	•	•	•	•	•	•	•	•	•	T	•	•	N	•	•	•	•	•	•	•	•	•	•	•	N	•
	D	JP32-4	•	I	V	•	•	•	I	R	•	N	A	A	N	•	•	•	•	•	•	•	•	•	•	•	N	•
	E	K-1	•	I	V	I	•	•	•	•	•	T	•	•	N	•	•	•	•	•	II	•	•	•	•	•	N	•
		99-TK2082	•	I	V	I	•	•	•	•	•	T	•	•	N	•	•	•	•	•	II	•	•	•	•	•	N	•
	F	T203	I	•	•	•	•	•	•	•	•	T	•	A	•	•	•	•	•	•	•	•	•	•	•	•	N	•
	G	JP29-6	I	•	V	•	•	•	I	•	•	T	•	P	N	•	•	•	•	•	•	•	•	•	•	•	N	•
		JP3-6	I		V	•	•	•	I	•	•	T	•	P	N	•	•	•	•	•	•	•	•	•	•	•	N	•

age of 2 years or younger over a 3-year period from 1997 to 1999 in Ireland (O'Halloran et al., 2002). The recombination break points were estimated at positions 380 and 520. Before position 380 and after position 520, the identity of CIT-254 and R136 was high (97–99%). From positions 380–520, their identity suddenly dropped and was distinctly different (78%); however, CIT-254 shared a high level of nucleotide identity (92%) with 87H140.

There are now numerous molecular epidemiological studies on G9 infection worldwide; its divergence of VP7 gene identified three lineages (Santos et al., 2002; Rahman et al., 2005; Steyer et al., 2005; Khamrin et al., 2006). With limited sequence variations, however, some G9 strains could not be classified into any clusters of above-mentioned classification scheme. The alignment of VP7 sequences we compiled from a number of G9 strains in different parts of the world available at the time of the study demonstrated six distinct lineages: lineage I (strains detected in the 1980s in the United States and Japan), lineage II (strain detected in 1986 and exclusively in India), lineage III (strains that emerged/reemerged in the mid-1990s), lineage IV (strain detected in 1997 and exclusively in China), lineage V (strains detected in 1997–1998 and exclusively in the United States) and lineage VI (strains detected in 1997–2002). This novel nomenclature should aid molecular characterization and description of sporadic cases as well as outbreak of G9 strains and also provide genetic and antigenic insights for future studies of this virus. Even though current vaccines have been proven to be highly efficacious against G9, some studies showed that symptomatic reinfection of vaccinated children with G9 was possible (Vesikari et al., 2004, 2006). Thus the novel nomenclature in the study should help to classify accurately G9 into proper clusters and to identify introduction and emergence of uncommon or new strains in many countries, especially which currently run the rotavirus vaccine program. Therefore, it could be a useful tool to demonstrate genetic diversity of G9 circulating in a community and also to explain situation of vaccine failure.

G9 rotavirus belonging to lineage III is the emerging genotype (Clark et al., 1987; Clark et al., 2004; Rahman et al., 2005; Santos and Hoshino, 2005). The origin of the emerging G9 in lineage III remains an enigma. In the previous report, it was unlikely that lineage III VP7 evolved from lineage I VP7 through antigenic drift and lineages I and III were more likely to have independent evolutionary origins (Hoshino et al., 2004). Interestingly, phylogenetic analysis showed that porcine G9 was genetically more closely related to recent human emerging G9 (90–94%) than classical human G9 (86–89%). Moreover, the VP7 gene of porcine G9 strain CMP003 detected in Thailand in 2000 was classified into the recent human G9 lineage. Some human G9 strains T203 from China and K-1, 99-TK2082 and 99-SP1542 from Japan were clustered into porcine lineage. More interestingly, the same amino acid substitutions in the VP7 antigenic region C were found at positions 208 and 220 among porcine strains and recent human emerging G9 strains. Taken together, VP7 genes of porcine G9 strains and recent human emerging G9 strains might have a common progenitor. In spite of their high identity in VP7 genes, majority

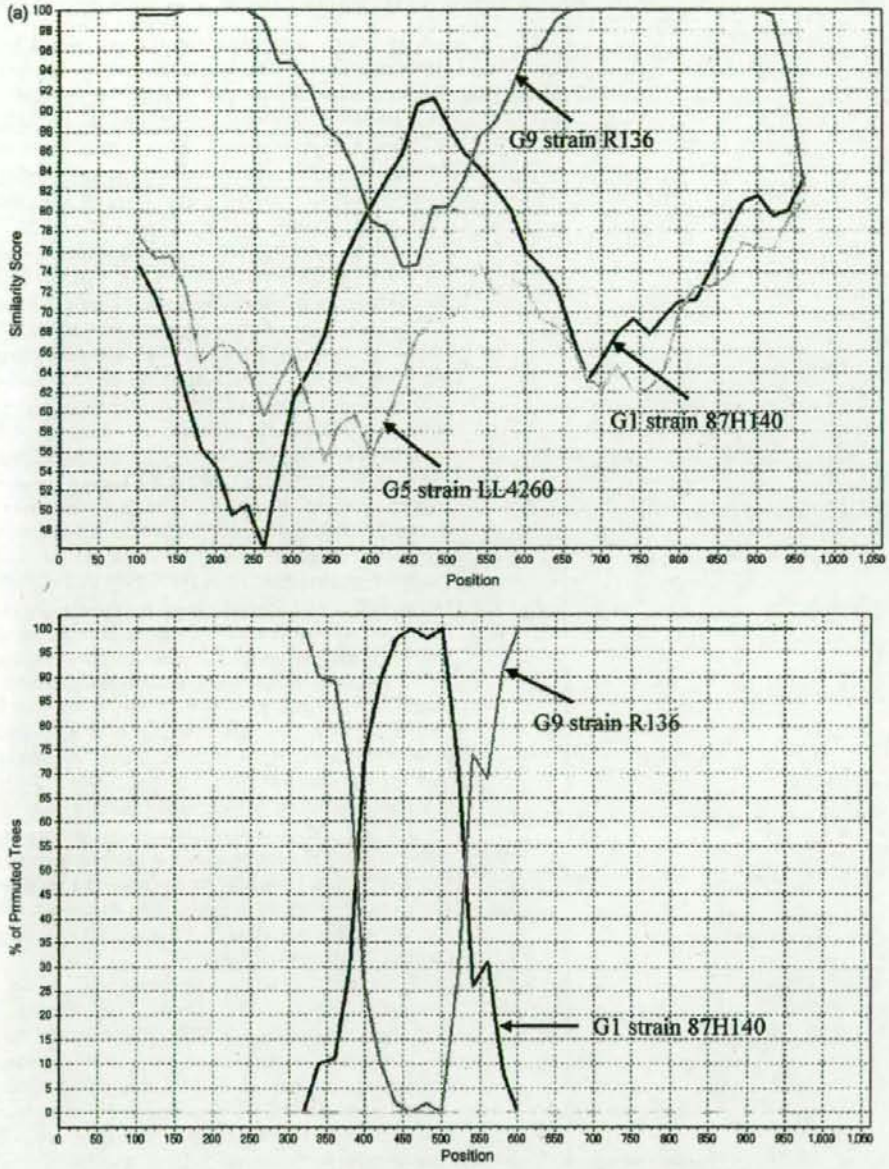

Fig. 3. Identification of recombinant G9 rotavirus. (A) Nucleotide identity comparison and bootscanning plots of the complete sequence of VP7 gene of CIT-254 as a query strain with those of R136 (known as G9) as a reference strain, 87H140 (known as G1) as a reference strain, and LL4260 (known as G5) as an outgroup strain. (B) Observation of changes of genotypes of CIT-254 on the basis of phylogenetic trees of nucleotide sequences. The scale indicates nucleotide substitutions per position. Numbers in the branches indicate the bootstrap values.

of porcine G9 rotaviruses possessed porcine-specific P genotypes that were unusual in human G9 rotaviruses except for Mc323 and Mc345 with P[19] (Okada et al., 2000; Maneekarn et al., 2006). Therefore the hypothesis of human G9 rotaviruses from porcine G9 rotaviruses as whole virion was unlikely. Possibly, the recently emerging G9 was introduced to humans by reassortant events. These findings highlight a possible route of zoonoses in humans as well as a possible explanation for the emergence of G9 rotaviruses. In view of G9 rotavirus evolution, this report is an additional evidence to support the notion that there might exist a genomic relatedness between human and porcine G9 rotaviruses.

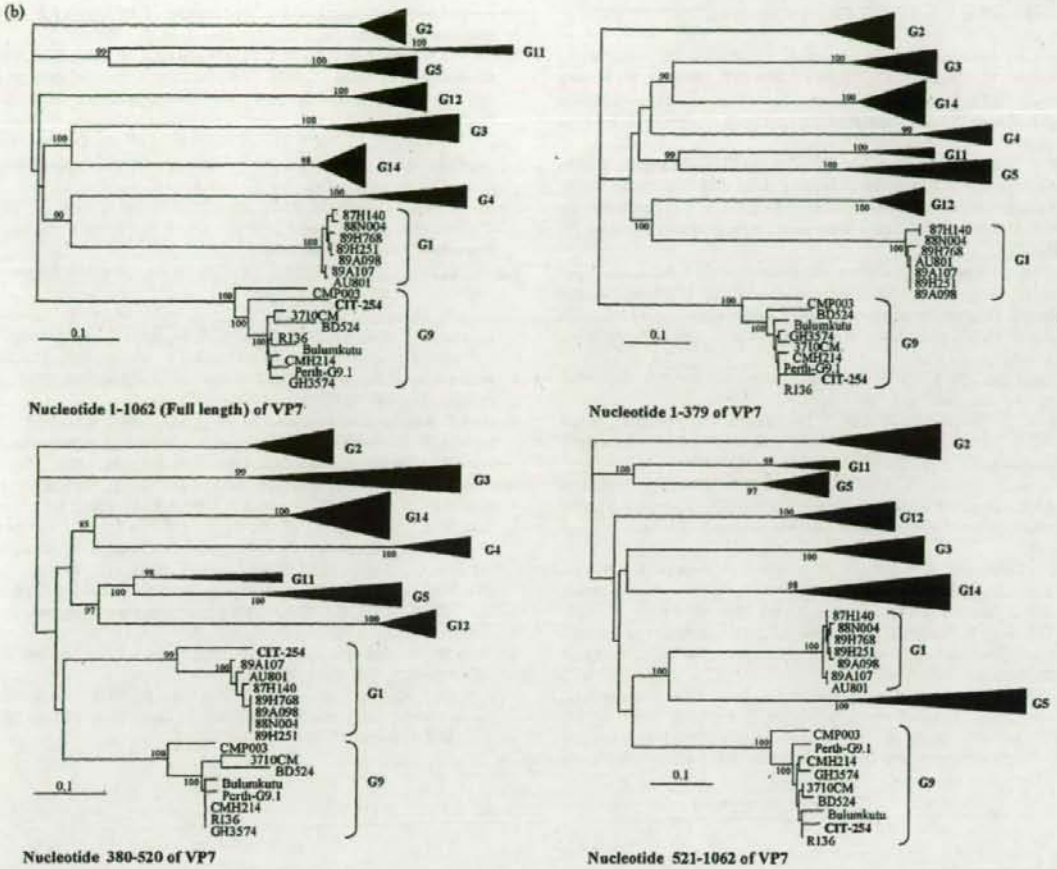

Fig. 3. (*Continued*).

RNA recombination plays a key role in virus evolution and it shapes a good deal of the virus diversity (Worobey and Holmes, 1999). Even though many studies conducted the surveillance on rotavirus infection around the world, reports documenting recombination in rotavirus are limited. To date, only two recombinant rotaviruses, CHW17 from China (recombination between G1 and G3) and ArgRes1723 from Argentina (recombination between sublineages Ib and Ic within G4), were detected (Suzuki et al., 1998; Parra et al., 2004). Remarkably, we found one novel recombinant rotavirus. Fig. 3B showed that CIT-254 clustered into different genotypes G1 and G9 when the different region of VP7 gene-based grouping was performed. This kind of phenomenon was recognized as an intergenotype recombination. These results are noteworthy because this provides evidence of recombination in G9 rotaviruses. This observation improves our current knowledge on rotavirus evolution as well as the origin of genetic heterogeneity. The further research should be conducted to investigate if this recombinant strain could be dominant in near future.

Acknowledgements

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Evaluation of Immunochromatography Tests for Detection of Rotavirus and Norovirus among Vietnamese Children with Acute Gastroenteritis and the Emergence of a Novel Norovirus GII.4 Variant

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Summary

A prospective study was conducted to evaluate two immunochromatography (ICG) tests for detection of group A rotavirus and norovirus GII, the commercial Dipstick 'Eiken' Rota kit (SA Scientific, USA) and the NV IC-1 stick (Immuno-Probe, Japan). Polymerase chain reaction (PCR) with specific primer pairs (Beg9 and VP7-1', for group A rotavirus; COG2F and G2SKR, for norovirus GII) was used as the reference method. The results of ICG tests were compared with those of reference method. The sensitivity, specificity and agreement between ICG tests and PCR were 87.8%, 93.3% and 89.4%, respectively, for rotavirus ICG test; and 73.7%, 100% and 95.2%, respectively, for norovirus ICG test. The immunochromatography assay for norovirus used in this study could detect not only common noroviruses, but also a novel norovirus GII.4 variant, which emerged in Ho Chi Minh City in 2006. Immunochromatography tests are easy, rapid and useful assays for detection of rotavirus and norovirus among pediatric patients with acute gastroenteritis in Vietnam.

Introduction

Rotavirus and norovirus are the two most common viral agents which cause acute gastroenteritis in infants and young children. In estimation, rotavirus causes ~22% of childhood diarrhea hospitalizations, and is responsible for about 611 000 childhood deaths per year worldwide, in which, most death cases belonged to the developing countries [1]. A review of food-related illness and death showed that norovirus accounted for ~23 million infections in all age groups in the United States [2]. Many epidemiological studies on rotavirus and recent reports about norovirus outbreaks in different countries confirm the disease burden of rotavirus and norovirus infections [3–9].

Rotavirus is a member of the *Reoviridae* family, which includes seven groups (A through G). Most diseases are due to group A rotavirus. Norovirus is one of the four members of the family *Caliciviridae*. Five genogroups of norovirus are thought to exist, though only norovirus GI, GII and GIV, are known to infect humans [10]. Epidemiological studies clearly indicated that norovirus GII is the main causative agent among noroviruses causing acute gastroenteritis [11–13].

After norovirus and rotavirus were discovered in stool sample by electron microscopic examination in 1972 and 1973, respectively [14, 15], several detection methods were developed to determine those viruses

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in fecal specimens. Enzyme-linked immunosorbent assays (ELISA) was the method of choice in many laboratories because it high sensitivity, and a built-in control for non-specific reaction. Recently, polymerase chain reaction (PCR) has been used broadly in epidemiological surveillances because of its ability to genotype samples that could not be typed by ELISA [16–20]. Those methods, however, need special equipment and are time consuming to perform. The development of rapid immunochromatography (ICG) based detection kits facilitates work in third-world countries, where there is lack of human and material resources for conducting laboratory tests, in hospital as well as in epidemiological researches. In this study, we evaluate two rapid ICG assays for detection of group A rotavirus and norovirus GII among pediatric patients with acute gastroenteritis in Ho Chi Minh City, Vietnam; and perform molecular analysis of norovirus strains detected.

Materials and Methods

Patients

A total of 30 out-patients and 74 in-patients who came to the Department of Gastroenterology, Children's Hospital 1, Ho Chi Minh City, with clinical diagnose of viral acute gastroenteritis in November 2006, were enrolled to this study. The patients were examined and followed-up by pediatricians. Severity of disease was assessed, based on the 20-point numerical score [21]. Another 10 healthy children were also recruited as controls.

Samples

One fecal specimen was collected from each child after coming to the out-clinic ward, for out-patients and controls, or after admitted to the department, for in-patients. Specimens were kept at +4°C, and checked for group A rotavirus and norovirus GII by ICG tests within less than 24 h after collecting. All stool samples were then stored at −20°C until checked further by monoplex PCR in the Department of Developmental Medical Sciences, The University of Tokyo, Japan.

Immunochromatography tests

Group A rotavirus was detected by using the Dipstick 'Eiken' Rota stick (SA Scientific, Texas, USA). This commercial kit has a capacity to detect a purified rotavirus antigen in a concentration of 100 ng/ml. Rotavirus G serotypes, including the five most common G-types: G1–G4 and G9, are detectable by this kit. The procedure was performed according to the manufacturer's instruction. Briefly, 12.5 mg of each fecal specimen was added to the reaction tube which contained 0.5 ml of reaction buffer, and was mixed with a mixer for 2 min. The mixture was then centrifuged at 2000 × g for 20 s before use.

Norovirus was determined by the NV IC-1 sticks lot 0609 (Immuno-Probe, Saitama, Japan), which was developed to determine norovirus GII/3 and GII/4, which are the most predominant norovirus genotypes globally. The 10% suspension was prepared with dilution buffer, then 80 µl of suspension and 20 µl of reaction buffer was mixed and left at room temperature for 3 min, before dropping onto the stick.

The reference test

Monoplex PCR was chosen as the reference test in this study. The fecal specimen was 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 procedure of reverse-transcription and PCR was carried out as previously described [22]. Briefly, 7.5 µl of the nucleic acid extract were mixed with a 7.5 µl reaction mixture, and then, the reaction tubes were incubated at 42°C for 1 h, followed by heating to 99°C for 5 min to inactivate the enzyme, and immediately cooling to 4°C.

Regarding PCR assay, 1 µl of cDNA was added to a reaction tube with 1.1 µl of 5X colorless GoTaq reaction buffer (Promega, WI, USA), 0.2 µl of each of the specific primers (33 µM each), 1.0 µl (2.5 mM/µl) of dNTPs (Roche, Mannheim, Germany) and 0.06 µl (5 U/µl) of GoTaq DNA polymerase (Promega), to achieve a total volume of 11.0 µl with the additional of MilliQ water. Each PCR cycle contained steps of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, for 35 cycles [23, 24]. All PCR products were electrophoresed in 1.5% agarose gel, followed by staining with ethidium bromide (0.5 µg/ml) and then visualized under ultraviolet light. Two specific primer pairs, Beg9–VP7-1′ for detection of group A rotavirus, and COG2F–G2SKR for norovirus GII [25–27], were used in this study (Table 1).

Sequence and phylogenetic analysis

PCR products of noroviruses detected were purified with the QIAquick PCR Purification Kit (Qiagen, Germany), and were sequenced by using 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. Sequence alignments were carried out by using the CLUSTAL W program, and the neighbor-joining method was used for the construction of the phylogenetic tree with MEGA2 version software [28], based on the classification of noroviruses by Okada *et al.* [29]. Nucleotide sequences of norovirus strains described in this study have been deposited in GenBank under accession numbers: EF198119–EF198137.

TABLE 1
Specific primers used in monoplex PCR

Primer	Target virus	Target region	Polarity	Sequence (5′ to 3′)[a]	Position	Amplicon
Beg9	Rotavirus group A	VP7 gene	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1–28	395 bp
VP7-1′	Rotavirus group A	VP7 gene	–	ACTGATCCTGTTGGCCATCCTTT	395–373	
COG2F	Norovirus genogroup II	Capsid	+	CARGARBCNATGTTYAGRTGGATGAG	5003–5028	387 bp
G2SKR	Norovirus genogroup II	Capsid	–	TACATRTTRCCHRTACGNCCRCC	5389–5367	

[a]Nucleotide, B: C, G, or T; H: A, C, or T; N: any base; R: A or G; Y: C or T.

TABLE 2
Characteristics of norovirus positive patients[a]

Isolate	Result of IC	Mixed infection[b]	GII.4 variant	Hospitalization	Age group[c]	Dehydration	Vesikari's score
VN1/06	+	–	4d	Yes	2	None	10
VN9/06	+	–	4new	Yes	3	None	6
VN26/06	+	–	4new	Yes	3	None	14
VN41/06	+	–	4new	Outpatient	2	None	N/A[d]
VN59/06	–	+	4new	Yes	3	None	13
VN73/06	+	–	4new	Yes	1	None	11
VN74/06	+	–	4d	Yes	2	None	11
VN75/06	+	–	4d	Yes	2	None	10
VN77/06	+	–	4new	Yes	2	1–5%	17
VN78/06	–	+	4new	Yes	3	None	14
VN79/06	–	+	4new	Yes	2	None	14
VN80/06	+	+	4d	Yes	2	None	15
VN82/06	–	+	4new	Yes	1	1–5%	18
VN84/06	+	–	4new	Yes	3	None	15
VN85/06	+	–	4d	Yes	3	None	15
VN87/06	+	–	4new	Yes	3	None	9
VN88/06	–	–	4new	Yes	2	None	16
VN89/06	+	–	4d	Yes	3	None	15
VN95/06	+	–	4d	Yes	3	None	10

[a]All 19 cases were identified as norovirus positive by monoplex PCR.
[b]Mixed infection between norovirus and rotavirus group A by reference tests.
[c]Group 1, <6 months; group 2, 6–11 months; group 3, 12–23 months; group 4, 24–35 months; group 5, 36 months and older.
[d]N/A, not applicable.

Results

Detection of target viruses by immunochromatography tests

Sixty-seven of 104 (64.4%) samples were found positive with group A rotavirus by Dipstick 'Eiken' Rota sticks. Norovirus GII was determined in 14 of 104 (13.5%) fecal specimens. There was no specimen which contained more than one target viral agent by ICG tests. All the sticks clearly showed the control lines and test line (if positive) at the appropriate position. Of 10 fecal specimens from healthy controls, all ICG assays showed negative results with target viruses.

Results of reference test

Monoplex PCR assays were performed separately for detection of group A rotavirus and norovirus GII. Seventy-four of 104 (71.2%) samples showed positive with group A rotavirus, and norovirus GII was found in 19 of 104 (18.3%) fecal specimens. Five samples were determined as coinfection between group A rotavirus and norovirus GII (Table 2).

Sensitivity and specificity of immunochromatography tests

In comparison with the reference test, there were two samples showed positive with rotavirus by ICG assay and negative by PCR. For norovirus, all cases

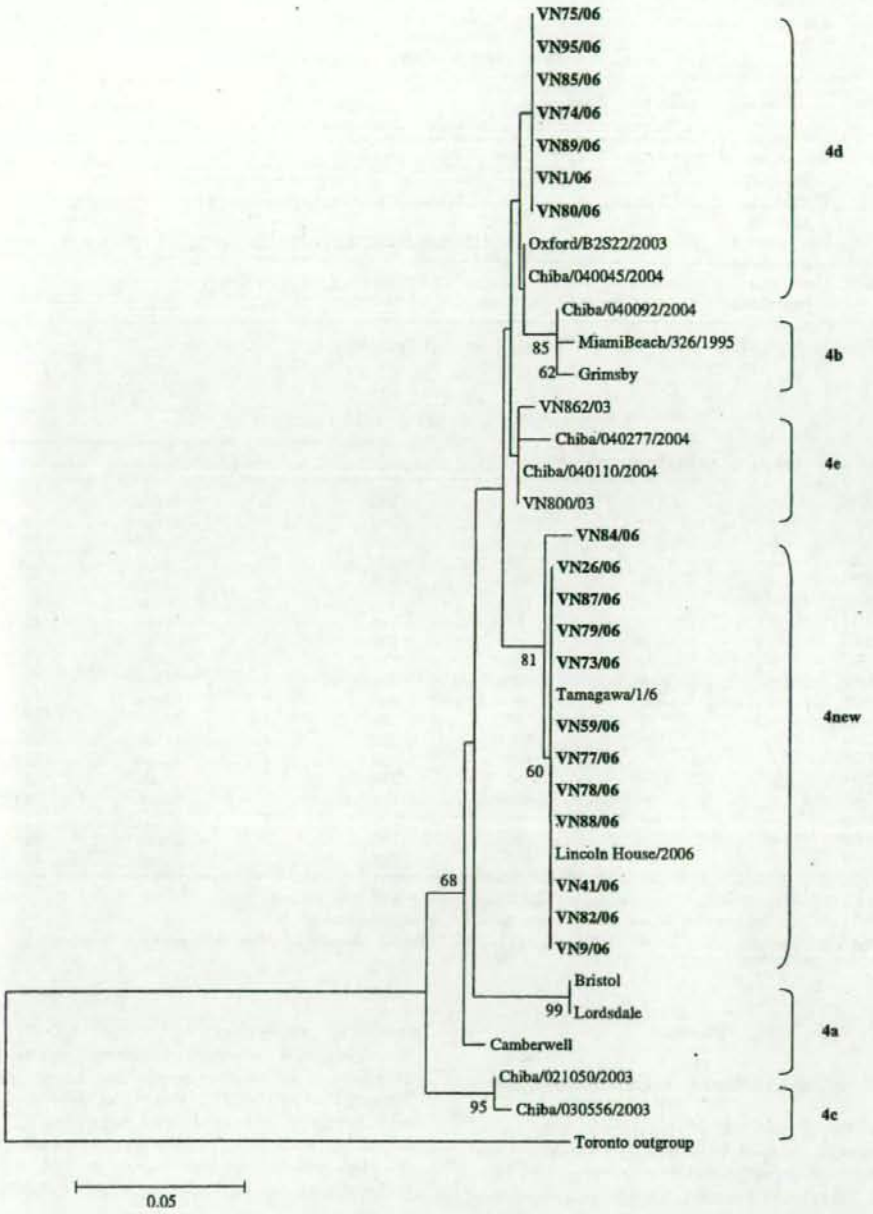

FIG. 1. Phylogenetic tree of a partial capsid gene (nt 5114–5321) of 19 Vietnamese noroviruses isolated in this study (in bold face) and other norovirus GII.4 strains. Bootstrap values >60% are indicated at the branch nodes. Vietnamese noroviruses detected in 2002–2003 surveillance, VN800/03 and VN862/03, are also shown. Toronto strain, GII.3 norovirus, is used as the out-group.

TABLE 3
Sensitivity, specificity and agreement of two IC tests for group A rotavirus and norovirus GII compared to reference test (monoplex PCR)

Parameter	Dipstick 'Eiken' Rota	NV IC-1 lot 0609
Sensitivity (%)	87.8	73.7
Specificity (%)	93.3	100
Agreement (%)	89.4	95.2

which were negative with norovirus by PCR also showed negative results with ICG test. The sensitivity, specificity and agreement of the two ICG tests, compared to PCR, are shown in Table 3.

Sequence analysis and phylogenetic tree of noroviruses detected

All 19 noroviruses detected by monoplex PCR were successfully sequenced at the capsid region. A BLAST search demonstrated that all studied isolates shared the greatest sequence identity with GII.4 norovirus strains. Interestingly, the phylogenetic tree of GII.4 noroviruses clearly showed that Vietnamese noroviruses isolated in this study belonged to two clusters, GII.4d and a novel variant which was distinct from previously described variants (Fig. 1).

Discussion

In this study, we conducted a prospective study to evaluate two immunochromatography tests for detection of group A rotavirus and norovirus GII, which were considered to be the two most common causes of acute gastroenteritis worldwide. To our knowledge, there has been no other report on evaluation of ICG test in viral enteropathogens among diarrheal children in Vietnam. The performance of ICG tests is easy (does not need special equipment), and rapid (15–20 min). This assay would be suitable for developing countries, where morbidity and mortality of diarrhea is high, and where laboratory assays to conduct epidemiological surveillances, as well as to monitor diarrhea outbreaks are limited.

The results of ICG test for rotavirus in this study was comparable with other evaluation studies. Bon *et al.* [30] evaluated seven ICG assays for the rapid detection of group A rotavirus, of them, four assays showed the sensitivity <90%. However, the reference method in that study was ELISA. PCR assay, the method of choice in detection of viral pathogens in many laboratories nowadays, was chosen as the reference test in this study, and made the results of comparison between test assay and reference assay more accurate.

Fourteen of 19 (73.7%) norovirus strains positive by PCR were detected by ICG test in this study. Of the five negative specimens, four were mixed infection between norovirus and rotavirus, which might cause the low concentration of target viruses in fecal specimens. Furthermore, five specimens which were negative by ICG test showed the faint bands when visualized under ultraviolet light, when amplified by PCR (data not shown). Although the ICG test using in this study has a capacity to detect norovirus in a concentration of 10^6 copies per 1 g of stool (Immuno-Probe, unpublished data), the low concentration of virus in fecal specimen might explain the fault negative results in rapid ICG tests. On the other hand, the results of ICG assay for norovirus detection in this study were comparable with that of other studies. Okame *et al.* [31] reported a comparison result between rapid ICG assay and PCR, with the sensitivity, specificity and concordance ratio 72.7%, 90.9% and 80.0%, respectively. It is noteworthy that ICG sticks using in this study could detect not only norovirus GII.4 which belonged to common variant (4d), but also novel norovirus GII.4 variant (Table 2).

The phylogenetic tree clearly showed that noroviruses isolated in Ho Chi Minh City were clustered into two different variants, and differed from noroviruses detected previously in this city, which belonged to variant GII.4e (Fig. 1). It is of interest that majority of the Vietnamese noroviruses in this study and the other two unique norovirus strains, Tamagawa/1/6 in Japan and Lincoln House/2006 in United Kingdom, made a novel cluster, tentatively called GII.4f. The mutation accumulation could cause a novel variant virus, and make it become the predominant strain in an outbreak [32]. There was a new norovirus GII.4 variant which caused Europe-wide outbreaks, and then became the major cause of norovirus outbreaks in many parts of the world [33, 34]. The mass detection of novel norovirus GII.4 variant in Vietnam, as well as in Japan and UK in the year 2006 highlights the ability of the spread of this norovirus variant worldwide.

Immunochromatography tests are easy, rapid and useful assays for detection of rotavirus and norovirus among pediatric patients with acute gastroenteritis in Vietnam.

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Changing Pattern of Rotavirus G Genotype Distribution in Chiang Mai, Thailand From 2002 to 2004: Decline of G9 and Reemergence of G1 and G2

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Group A rotaviruses are the most common cause of acute viral diarrhea in humans and animals throughout the world. Previous surveillance studies of group A rotaviruses in Thailand indicated that the dominant types of rotaviruses were changing from time to time. During 2000 and 2001, the G9 rotavirus emerged as the most prevalent genotype, with an exceptionally high frequency (91.6%) in Chiang Mai, Thailand. In the year 2002–2004, group A rotavirus was detected in 98 out of 263 (37.3%) fecal specimens collected from children hospitalized with diarrhea. Of these, 40 (40.8%) were G9P[8], 33 (33.7%) were G1P[8], 23 (23.5%) were G2P[4], and 2 (2.0%) were G3P[9]. The G9P[8] was found to be the most predominant strain in 2002, but the prevalence rate abruptly decreased during the period 2003–2004. In addition, G2P[4] reemerged in the epidemic season of 2003, whereas G1P[8] became the most predominant strain in the following year (2004). Phylogenetic analysis of the VP7 genes revealed that G1, G2, and G9 rotavirus strains clustered together with recently circulating strains, which were isolated from different regional settings in Thailand. In conclusion, the study demonstrated a decrease of incidence of G9P[8] and reemergence of G1P[8] and G2P[4] rotaviruses in Chiang Mai, Thailand during the period 2002–2004. These data imply that the distribution of group A rotavirus genotypes circulating in Chiang Mai, Thailand, changes over time. ***J. Med. Virol. 79:1775–1782, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: rotavirus; genotype; G1; G2; G9; Thailand

INTRODUCTION

Group A rotavirus is the most important etiologic agent of gastroenteritis and severe diarrhea in infants and young children, and in a large variety of young animal species [Estes, 2001]. The global mortality associated with rotavirus infection has been estimated at 454,000–705,000 deaths annually [Parashar et al., 2006]. Rotavirus is a member of the *Reoviridae* family. The virus contains two outer capsid proteins, VP4 and VP7, which allow classification of the rotavirus into P (protease-sensitive) and G (glycoprotein) genotypes, respectively. Based on both antigenic and genetic differences, thus far, 15 distinct G genotypes and 27 P genotypes have been identified [Rao et al., 2000; Estes, 2001; McNeal et al., 2005; Rahman et al., 2005; Martella et al., 2006; Khamrin et al., 2007a; Steyer et al., 2007]. Epidemiological studies around the world have demonstrated that rotavirus strains bearing the G and P combinations of G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G9P[6] are responsible for most rotavirus infections. However, other G and P types (i.e., G5, G6, G8, G10, G12, P[9], P[11], and P[14]) have also been detected in different geographical areas of the world [Gentsch et al., 2005; Santos and Hoshino, 2005]. Therefore, due to the diversity of strains in different parts of the world, knowledge of molecular epidemiology and antigenic diversity of rotaviruses that circulate currently is imperative for the development of a suitable and efficacious rotavirus vaccine.

In Thailand, rotavirus is the leading pathogen that causes diarrhea in children, and it is responsible for about 27–55% of diarrheal diseases in hospitalized

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cases [Maneekarn and Ushijima, 2000; Jiraphongsa et al., 2005; Khamrin et al., 2006]. Rotavirus surveillance studies in Thailand have been reviewed by Maneekarn and Ushijima [2000]. The study of rotavirus infection in Thailand was initially performed approximately two decades ago. The accumulated data from those surveillance studies indicate that the predominant serotype of rotaviruses in each epidemic season change over time. It was demonstrated that G4 was the most predominant genotype from 1982 to 1984, G1 from 1985 to 1987, both G1 and G2 were co-predominant from 1987 to 1988, and G1 from 1988 to 1989. However, from 1990 to 1993, G2 and G3 were the most predominant genotypes. Recently, the Bureau of Epidemiology, Thai National Institute of Health (NIH) reported on the sentinel surveillance of rotavirus infection from six hospitals located in different regions of Thailand. In this study, the G9 rotavirus was the most predominant genotype from 2001 to 2002, while both G2 and G9 were co-predominant genotypes in 2003 [Jiraphongsa et al., 2005]. In addition, a rotavirus surveillance study in Bangkok, Thailand from 2002 to 2004 revealed that G2 was the most predominant genotype, followed by much less prevalent of G9 and G1 genotypes [Theamboonlers et al., 2005].

In Chiang Mai, Thailand, Urasawa et al. [1992] reported the prevalence of rotavirus infection during two consecutive study periods from September 1987 to April 1988 and December 1988 to June 1989. In the first half of the study, G1 and G2 were co-predominant genotypes, while in the second half G1 became the most predominant genotype followed by G2 and G4. In addition, that study also described the first detection of human rotavirus G9 in Thailand at the same time as those initially reported elsewhere in the world. The prevalence of the G9 rotavirus increased between 1996 and 1997 to 16.2% and it was found to be the third most common genotype after G1 and G2 [Zhou et al., 2001]. Recently, from 2000 to 2001, G9 was reported in Chiang Mai as the most predominant genotype, with an exceptionally high frequency (91.6%) in children hospitalized with diarrhea [Khamrin et al., 2006].

In this study, the changing pattern of group A rotavirus genotype distribution in children hospitalized with diarrhea in Chiang Mai, Thailand from 2002 to 2004 is described.

MATERIALS AND METHODS

Specimen Collection

A total of 263 fecal specimens were collected from pediatric patients aged less than 5 years, who were hospitalized with diarrhea at the McCormick Hospital, Chiang Mai, Thailand. The study period was from January 2002 to December 2004.

Detection of Group A Rotavirus

The RNA genome of rotavirus was first extracted from 10% fecal suspension supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Germany). The presence of the group A rotavirus in fecal specimens was detected by RT-PCR using a protocol described previously [Yan et al., 2004]. All of the rotavirus positive samples were examined further for G and P genotypes by RT-PCR and multiplex PCR-genotyping methods.

RT-PCR and Multiplex-PCR for G and P Genotyping

The extracted dsRNA 5 μl was added to 0.5 μl of 50% dimethyl sulfoxide before being heated at 95°C for 5 min and then rapidly cooled on ice. The RT-PCR was carried out according to the methods described previously by Das et al. [1994], Gentsch et al. [1992], and Gouvea et al. [1990]. For PCR amplification of the VP7 gene, a 1,062 bp fragment was generated using Beg9 (forward) and End9 (reverse) primers. For PCR amplification of the VP4 gene, an 876 bp fragment was generated using Con3 as a forward primer and Con2 as a reverse primer. The G genotyping was performed using a pool of different primers specific for G1-G4, G8, and G9 [Gouvea et al., 1990; Das et al., 1994]. The VP4 gene was characterized by using a pool of different P genotype-specific primers for P[4], P[6], and P[8]-P[10] [Gentsch et al., 1992].

Sequence Analysis of the VP7 Gene

The representative strains of predominant rotavirus G1, G2, and G9 genotypes were randomly selected for examination of their evolutionary relationships with those other strains circulating in different regions of the world. The PCR products of the gene encoding the entire VP7 protein were gel purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). The primers used for amplification of the VP7 gene were also used as sequencing primers. The nucleotide sequences of VP7 genes from the representative strains of G1, G2, and G9 were compared with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using the basic local alignment search tool server (BLAST) [Altschul et al., 1990]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [Kumar et al., 2004].

Nucleotide Sequence Accession Numbers

The nucleotide sequences of rotavirus strains described in this study have been deposited in the GenBank database. The accession numbers for the VP7 sequences of the representative G1, G2, and G9 strains isolated from 2002 to 2004 in Chiang Mai city are as follows: G1 (EF199711-EF199717); G2 (EF199718-EF199724); G9 (EF199725-EF199738).

RESULTS

Detection of the Group A Rotavirus and Distribution of G and P Genotypes

From a total of 263 fecal specimens collected from pediatric patients hospitalized with diarrhea, 98 (37.3%) were found positive for group A rotavirus by using the PCR-screening method. All of the rotavirus positive samples were characterized further for identification of their G and P genotypes. Of these, all of the tested samples could be assigned to their G and P genotypes and mixed infections of G or P types were not found. The distribution of G and P genotype combinations is shown in Table I. Four different G and P genotype combinations, G1P[8], G2P[4], G3P[9], and G9P[8], were found in this study.

Changing Distribution of G1, G2, and G9 Genotypes

The distribution of circulating rotavirus G genotypes detected during the 2002–2004 surveillance study period in Chiang Mai, Thailand is shown in Figure 1. The G9 rotavirus in Thailand was first isolated in Chiang Mai in 1989 with a low prevalence [Urasawa et al., 1992] but by 2000 and 2001 it had become the most predominant genotype by as much as 75% and 92.2%, respectively [Khamrin et al., 2006]. Interestingly, this follow-up surveillance study revealed that G9 reached a peak of 100% prevalence rate in 2002 (Table I, Fig. 1). However, the prevalence of G9 decreased abruptly over the next 2 years, that is, 16.7% in 2003 and 32.1% in 2004. It was interesting to note that G2 was detected with a low prevalence of 1.9% in 2001 and it was undetectable in 2002. Unexpectedly, G2 reemerged and rose to 83.3% as a predominant genotype in 2003 and then declined rapidly to about 5.4% in 2004. In contrast, G1, which was not detected in the Chiang Mai area for four consecutive years (2000–2003), suddenly reemerged as the most prevalent genotype in 2004 at a prevalence rate of 58.9%, followed by G9 (32.1%), G2 (5.4%), and G3 (3.6%).

VP7 Sequence Analysis of G9 Rotaviruses

A total of 14 G9 rotavirus strains (CMH025/02, CMH028/02, CMH033/02, CMH009/03, CMH010/03, CMH022/03, CMH028/03, CMH001/04, CMH004/04, CMH017/04, CMH028/04, CMH035/04, CMH061/04, CMH109/04) were selected based on the difference in the year of detection from 2002 to 2004 and their VP7 genes were sequenced. When comparing the VP7 sequences of these G9 strains with those of reference strains, including the representative of G9 strains isolated from several other parts of the world, and also G9 rotaviruses isolated previously in Thailand since 1989 by phylogenetic analysis (Fig. 2), three major lineages of G9 were observed, which were similar to those described by Hoshino et al. [2004, 2005]. In addition, the phylogenetic tree revealed that all of the 14 G9 representative strains detected in this study clustered with the G9 reference strains in lineage 3. These G9 rotavirus strains exhibited similar nucleotide identities of greater than 98.4–100% with each other and were closely related to the G9 strain isolated in 2000 (strain CMH005) in the same area (Chiang Mai), with similar nucleotide identities ranging from 98.9% to 99.8% (data not shown). In addition, the sequences of these G9 strains were also similar to those of G9 rotaviruses isolated in Bangkok, Thailand from 2002 to 2004 (strain CU57G9, CU52G9, CU53G9, CU33G9, and CU116G9) [Theamboonlers et al., 2005], with a similar nucleotide identity ranging from 98.2% to 99.8% (data not shown). Furthermore, one G9 isolate, CMH033/02, detected in this study in 2002, was slightly different from other G9 strains. The CMH033/02 was related more closely to the Bangladesh B1937-02 strain (99.2% at the nucleotide level) than to the G9 strains isolated in Thailand between 2003 and 2004. However, the phylogenetic tree clearly demonstrated that the G9 strains isolated in this study (2002–2004) appeared to be not so closely related to G9 strains isolated in 1989 (Mc323 and Mc345) and 1997 (97CM90 and 97CM113), even though all of these G9 strains were classified into lineage 3 and isolated from the same geographical area of Chiang Mai, Thailand.

VP7 Sequence Analysis of G2 Rotaviruses

The complete nucleotide sequences of VP7 genes of seven representative G2 strains (CMH017/03, CMH019/03, CMH027/03, CMH030/03, CMH032/03, CMH033/03, CMH041/03) which predominated in 2003 were

TABLE I. Distribution of G and P Genotype Combinations of Human Group A Rotavirus Detected in Chiang Mai, Thailand From 2000 to 2004

	No. of strains detected in (%)					
Genotype	2000[a]	2001[a]	2002	2003	2004	Total
G1P[8]	—	—	—	—	33 (58.9)	33
G2P[4]	1 (25.0)	2 (1.9)	—	20 (83.3)	3 (5.4)	26
G3P[3]	—	1 (1.0)	—	—	—	1
G3P[8]	—	5 (4.9)	—	—	—	5
G3P[9]	—	—	—	—	2 (3.6)	2
G9P[8]	3 (75.0)	95 (92.2)	18 (100)	4 (16.7)	18 (32.1)	138
Total	4	103	18	24	56	205

[a]The data were published previously by Khamrin et al. [2006].

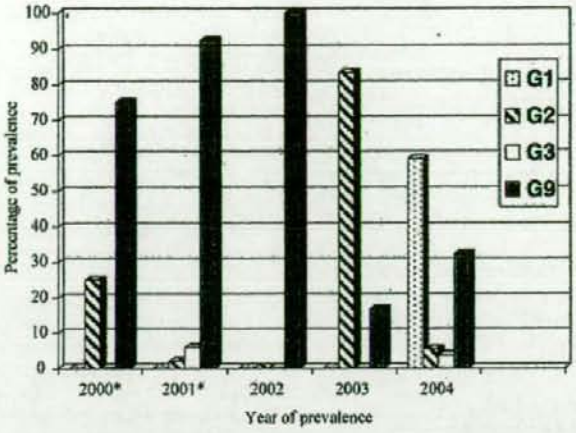

Fig. 1. Yearly distributions of rotavirus G genotypes among children hospitalized with diarrhea in Chiang Mai, Thailand from 2000 to 2004. *The data were published previously by Khamrin et al. [2006].

selected and sequenced. The phylogenetic analysis shown in Figure 3 revealed the existence of at least three major lineages of G2, similar to that suggested by Page and Steele [2004a]. It was of note, however, that in the phylogenetic tree, one additional separate branch was demonstrated and designated as lineage IV. The novel G2 lineage IV was comprised exclusively of two animal G2 strains, the porcine isolates; CMP034 detected in Thailand and 34461-4 detected in Spain (Fig. 3). All of the seven human G2 rotavirus representative strains isolated in Chiang Mai in 2003 as well as G2 strains isolated from other Asian countries were classified into lineage II. Comparing the nucleotide sequences of VP7 genes among these G2 strains revealed similarity more than 99%. Moreover, our G2 strains appeared to be more similar to other Thai G2 rotavirus strains (27G2, 30G2, and 59G2), which were isolated in Bangkok during the same period (2002–2004) [Theamboonlers et al., 2005]. They were all clustered in a monophyletic branch with a similarity of nucleotide sequence identity ranging from 99.3% to 100% (data not shown). However, the phylogenetic tree demonstrated clearly that all G2 strains described in this study were less closely related to the G2 strain isolated previously in Chiang Mai in 2001 (CMH277), even though they were all in the same lineage and isolated from the same geographical area.

VP7 Sequence Analysis of G1 Rotaviruses

The nucleotide sequence of the VP7 gene of seven representative G1 Thai strains (CMH022/04, CMH036/04, CMH040/04, CMH041/04, CMH042/04, CMH048/04, CMH056/04) were compared with those of reference strains and their phylogenetic relationships were analyzed. Phylogenetic evolutionary analysis of G1 by Berois et al. [2003] and Jin et al. [1996] proposed temporal clustering of G1 into four major lineages. Recently, however, Arista et al. [2006] reported three additional G1 lineages (lineages V, VI, VII) by comparing G1 strains that had circulated in Italy for 19 consecutive years with those circulating worldwide. As shown in Figure 4, all of our G1 strains were clustered in lineage I. In addition, within lineage I, which could be divided into three discrete sub-lineages (Ia, Ib, Ic). The G1 representative strains analyzed in this study exhibited 97.6–100% nucleotide identity to each other and fell into sub-lineage Ic. It is interesting to point out that four representative strains isolated in this study (CMH036/04, CMH042/04, CMH048/04, CMH056/04) formed the same cluster with G1 rotaviruses isolated previously in Thailand (Bangkok and Chiang Mai), Bangladesh, India, and Ireland with a nucleotide sequence identity ranging from 98.8% to 100%, while another three strains (CMH022/04, CMH040/04, CMH041/04) were closely related to three Vietnamese and one Thai G1 strains in a separate branch, with a nucleotide sequence identity of 99.5–100%.

DISCUSSION

The data obtained from this study and those of previous reports [Urasawa et al., 1992; Zhou et al., 2001; Khamrin et al., 2006] have provided several important features of the epidemiology of rotavirus in Chiang Mai, Thailand. These included (i) G9 as the most predominant genotype from 2000 to 2002, which declined abruptly from 2003 to 2004, (ii) G2, which reemerged in the epidemic season of 2003, and (iii) G1, which became the most prevalent in 2004 after being undetectable from 2000 to 2003. Similar studies conducted in other regions of Thailand also indicated a similar shift of rotavirus genotype distribution [Jiraphongsa et al., 2005; Theamboonlers et al., 2005].