A total of 147 fecal specimens were collected from pediatric patients aged <5 years, hospitalized with diarrhea in Chiang Mai, Thailand during January to December 2005. Group A rotavirus detection and genotype identification were performed using the protocols described previously (Khamrin et al., 2007b).

The nucleotide sequences of VP7 genes (1061 nt) of G9 rotaviruses detected in this study were compared with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search Tool) server. In addition, a total of 38 sequences of G9 reported previously from Thailand since 1989, were included in phylogenetic analysis together with the 2005 G9 strains and all sequences were analyzed by MEGA 4 software package (Tamura et al., 2007). The accession numbers for the VP7 sequences of the G9 strains isolated in Chiang Mai in 2005 are GQ149703 and GQ149704.

Of the 147 fecal specimens, 43 (29.3%) were tested positive for group A rotavirus by RT-PCR. Of these, five different G and P genotype combinations were indicated in this study, G1P[8] (27 strains), G2P[4] (12 strains), G9P[8] (2 strains), G3P[8] (1 strain), and G3P[10] (1 strain). The G9 rotavirus strains were detected in only 2 out of 43 (4.7%) specimens. The distribution of G9 rotavirus strains detected during the 1989–2005 surveillances in Chiang Mai, Thailand, is shown in Fig. 1. The G9 rotavirus in Thailand was first isolated in Chiang Mai in 1989 with a low prevalence of 1.98% (Urasawa et al., 1992). However, about a decade later in 1996–1997, the prevalence of G9 in Chiang Mai was shown to increase to 16.2% (Zhou et al., 2001). In 2000 and 2001 G9 became the most predominant genotype by as much as 75% and 92.2%, respectively (Khamrin et al., 2006). Then, the prevalence of G9 reached a peak of 100% in 2002 and decreased abruptly over the next 3 years, i.e. 16.7% in 2003, 32.1% in 2004 (Khamrin et al., 2007b), and 4.7% in 2005 (in this study) as shown in Fig. 1.

The nucleotide sequence of the VP7 gene of Thai G9 strains found in this study were compared with those of reference G9 strains as well as the G9 strains isolated previously in Thailand since 1989 in order to characterize their genetic evolutionary relationships. Phylogenetic analysis of the G9 rotavirus genotype, described by Hoshino et al. (2004), proposed temporal clustering of G9 into three major lineages (I, II, III) (Fig. 2). Most of the G9 rotavirus strains reported recently from several countries belong to lineage III (Hoshino et al., 2004). Two G9 strains (CMH017/05 and CMH020/05) of the present study as well as other G9 strains identified previously in Thailand clustered in lineage III (Fig. 2). However, the phylogenetic analysis of G9 demonstrated clearly that the G9 rotavirus strains isolated in Thailand since 1997 were less closely related to the G9 strains isolated previously in Chiang Mai in 1989 (Mc323 and Mc345). The two G9 rotavirus CMH017/05 and CMH020/05 strains detected in this study were clustered in a monophyletic branch with a 99.9% nucleotide sequence identity. From this phylogenetic analysis, it is interesting to point out that the G9 rotavirus strains Mc323 and Mc345, isolated from children with acute gastroenteritis in Chiang Mai in 1989, were most closely related to the porcine G9 rotavirus strain (CMP003) detected in the same geographical area in 2000 (Maneekarn et al., 2006). The finding provides evidence supporting the porcine origin of Mc323 and Mc345 human rotavirus strains. Moreover, partial VP4 sequences of the 2005 G9 rotavirus strains, CMH017/05 and CMH020/05, exhibited similar nucleotide identities of greater than 99.1% with the VP4 sequences of DH396 and SK430 G9P[8] strains isolated from Bangladesh in 2004–2005 (Paul et al., 2008), but lesser nucleotide sequence identity (98.6%) with VP4 sequences of other Thai G9 rotavirus strains (44vp4n, 51vp4n, and 52vp4n) which were isolated in Bangkok during the same study period (2004–2005) (Theamboonlers et al., 2008). Unfortunately, the VP4 sequences of the previous G9 strains isolated before 2005 in Chiang Mai are not available in databank. It was, therefore, not possible to analyze the relationship between these strains.

In order to determine whether the G9 rotavirus strains recently circulating in Chiang Mai were similar to those reported previously, the deduced amino acid sequences of VP7 genes of the G9 rotaviruses detected in Chiang Mai from 1989 to 2005 and the G9 strains detected in other regions of Thailand were aligned with each other (Fig. 3). The overall picture from this alignment revealed the distinction between the Thai G9 rotavirus strains isolated previously and those reported recently. It was interesting to note that one amino acid change from Thr to Ile was observed in antigenic region C at position 208 between the G9 strains isolated in 1989 and the strains of 1997–2005. In addition, two amino acid changes between these two groups were also found outside the antigenic regions, at position 44 from Val to Ala and position 77 from Ser to Leu. Furthermore, it was interesting to point out that the G9 strains detected from 2000 through 2005 had one amino acid changed from Pro to Ser at position 46 compared to the strains detected previously. However, the deletion or insertion of any amino acid residues in the VP7 gene was not observed.

The emergence and decline of G9 rotavirus genotype have been observed worldwide in several studies for more than a decade (Maneekarn and Ushijima, 2000; Rahman et al., 2005; Khamrin et al., 2006, 2007b; Samajdar et al., 2008; Wang et al., 2009). Our studies of rotavirus surveillances in Chiang Mai, Thailand, over five consecutive epidemic seasons revealed that the prevalence of rotavirus G9 have changed over time. Two G9 rotavirus strains Mc323 and Mc345 were first detected sporadically in Chiang Mai province in 1989. These strains exhibited VP4 genetic backgrounds closely related to porcine rotavirus strains which were detected for more than a decade afterward (Maneekarn et al., 2006). Both of these G9 strains (Mc323 and Mc345) were found in combination with P[19] genotype. These findings imply that G9P[19] strains first isolated in Chiang Mai are likely to be the ancestor of the current Thai G9 strains as well as the modern global G9 strains in lineage III (Martinez-Laso et al., 2009). However, the G9 strains detected later in 1996–1997 in the same geographical area were associated with either P[4] or P[6] genotypes (Zhou et al., 2001). Interestingly, since 2000, all the G9 strains currently detected in Chiang Mai were combined with P[8] genotype (Khamrin et al., 2006, 2007b). These observations suggest that after going through several reassortment events with the most common circulating P[8] genotype, those G9 strains may acquire the VP4 gene of P[8]

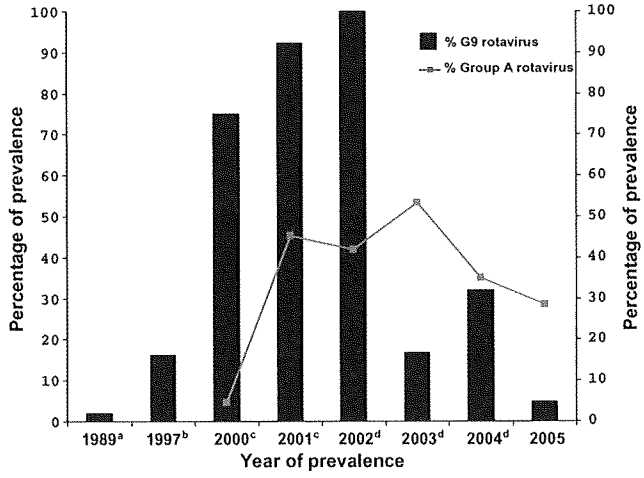

Fig. 1. Yearly distributions of rotavirus G9 genotype (indicated by solid bar) and annual rotavirus detection rate (indicated by gray line) among children hospitalized with diarrhea in Chiang Mai, Thailand, from 1989 to 2005. The data were published previously by: [a]Urasawa et al. (1992); [b]Zhou et al. (2001); [c]Khamrin et al. (2006); [d]Khamrin et al. (2007b).

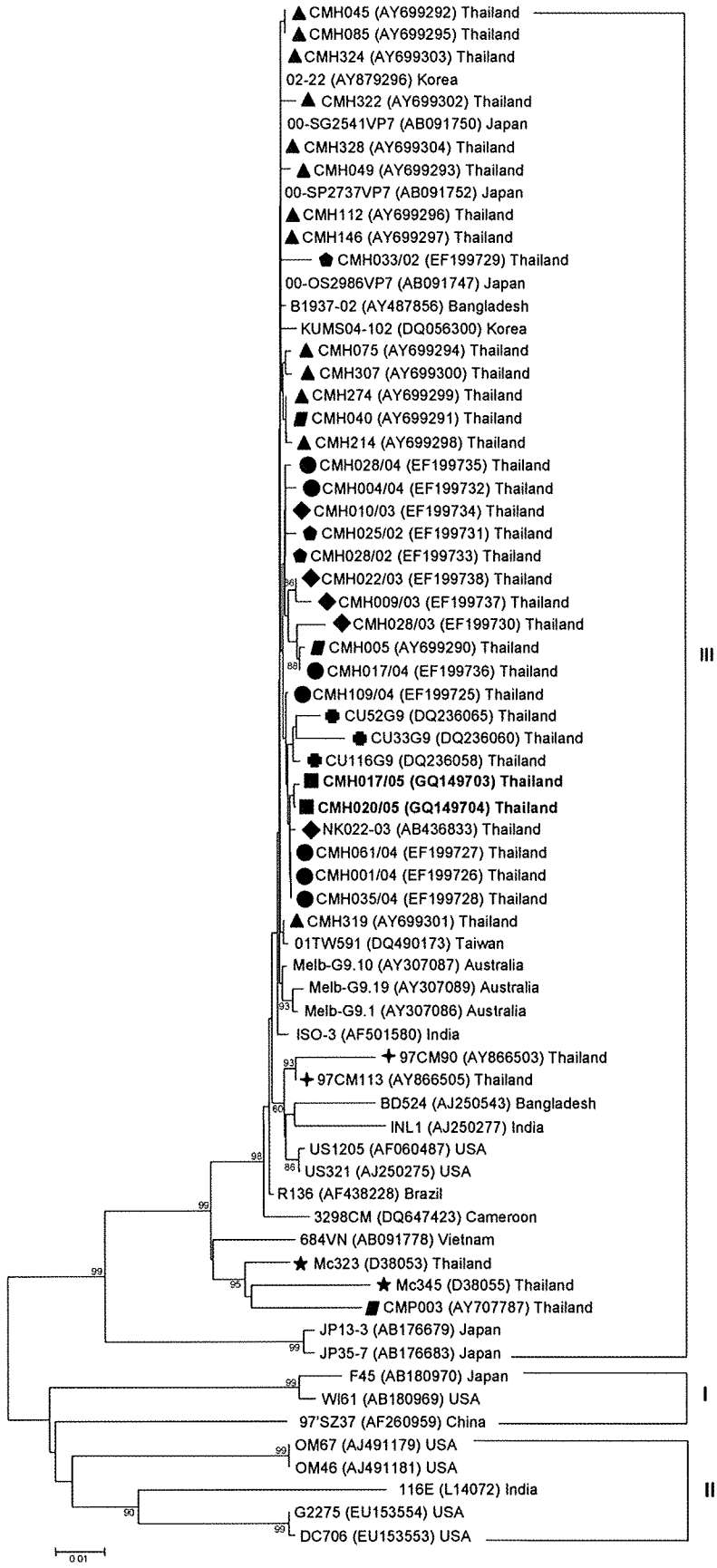

Fig. 2. Phylogenetic analysis of VP7 gene nucleotide sequences of rotavirus G9 strains. The tree was constructed by the neighbor-joining method using the MEGA 4 software package, and the numbers on each branch indicate the bootstrap values. The rotavirus G9 strains detected in the present study are presented in boldface and the strains detected previously in Thailand are marked by symbols according to the year of virus detection (★ 1989; ✚ 1997; ▰ 2000; ▲ 2001; ⬟ 2002; ◆ 2003; ● 2004; ■ 2005; ⯃ 2004–2005). The GenBank accession numbers of reference stains are given in parentheses followed by countries.

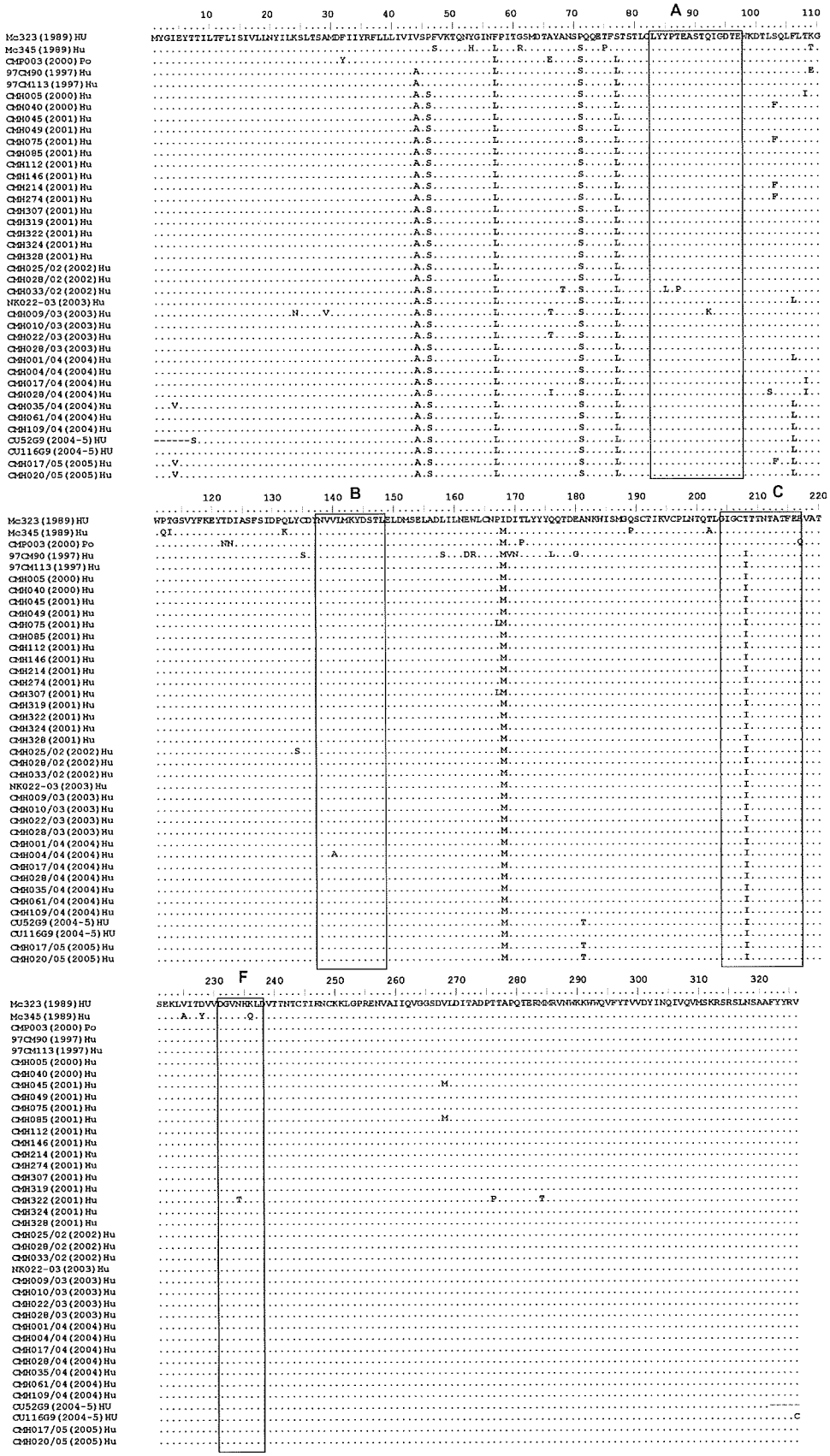

Fig. 3. Comparison of the deduced amino acid sequence of VP7 protein of G9 rotavirus strains detected in Thailand since 1989. Antigenic regions A, B, C, and F are shown in boxes and dots indicate amino acid residues identical with the sequence of Mc323 rotavirus strain.

specificity and became the predominant strain in this area. This finding indicates that the G9 rotavirus circulating in Chiang Mai, Thailand, has evolved over time. The reason for the decrease of G9 rotavirus in Chiang Mai, Thailand, in 2003–2005 is still unknown. One hypothesis is that most of the people in the area might have been exposed to G9 rotavirus infection during the years 2000–2002, when G9 prevalence reached its peak, and induced herd immunity in the area. Thereby, the majority of the people are immunized against G9 rotavirus infection.

Recently, sequence and phylogenetic analyses have demonstrated the existence of at least three distinct VP7 lineages among the G9 rotavirus strains (Hoshino et al., 2004; Cao et al., 2008). Most of the G9 rotaviruses, which are prevalent around the world, as well as Thai G9 strains, belong to lineage III. Our analysis revealed that the VP7 amino acid sequence of G9 rotavirus strains circulating in Chiang Mai since 1989–2005 were quite conserved. Within the antigenic regions (A, B, C, and F) of the VP7 gene, only one amino acid change was observed at position 208 in an antigenic region C from Thr to Ile in the Thai rotavirus strains detected in 1997 through 2005 as compared to the G9 strains in 1989. This change was also observed in other recent G9 strains isolated from Japan, Korea, Taiwan, Bangladesh, India, and Australia. It is not clear whether this change affects the nature of the viruses in terms of antigenicity, infectivity or pathogenicity. However, it has been reported that mutations of amino acid in VP7 gene at positions 94, 96, 97, 98, 99 in antigenic region A; 145–148 in region B; and 208, 211, 212, 213, 217, 221 in region C can alter the antigenicity of rotaviruses (Mackow et al., 1988; Taniguchi et al., 1988; Green and Kapikian, 1992; Hoshino et al., 1994). In fact, a similar phenomenon has also been observed in G3 rotavirus infection (Trinh et al., 2007). It has been postulated that amino acid substitutions at positions 96 and 213 might be involved in the emergence of G3 rotavirus strains in Japan, China, and Russia from 2001 to 2004. A comprehensive screening and genotypic characterization of circulating rotavirus strains could provide valuable insights into the biodiversity of the viruses currently circulating and the data could also be used to improve current vaccination strategies.

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Exceptionally high prevalence of porcine kobuviruses in piglets with diarrhea

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Keywords:	Porcine Kobuvirus, Piglets, Diarrhea, Thailand

May 20, 2009

Dear Dr. Drotman, D. Peter
Emerging Infectious Diseases

Title : Exceptionally high prevalence of porcine kobuviruses in piglets with diarrhea

Author(s) : Pattara Khamrin, Niwat Maneekarn, Aphisek Kongkaew, Sompreeya Kongkaew, Shoko Okitsu, and Hiroshi Ushijima
Corresponding Author : Prof. Hiroshi Ushijima

Attached herewith is a manuscript entitled "Exceptionally high prevalence of porcine kobuviruses in piglets with diarrhea" that I would like to submit for your consideration to publish as a "Letter" in the Emerging Infectious Diseases.

Since a new species of kobuvirus, named porcine kobuvirus, has been primarily recognized in 2008, a few epidemiological surveillance studies have been reported the detection of porcine kobuvirus thus far. This study reported the high prevalence of porcine kobuvirus in 97 out of 98 (99%) fecal specimens collected from piglets with diarrhea in Chiang Mai, Thailand. Phylogenetic evolutionary analysis confirmed that these porcine kobuvirus strains clustered in a monophyletic branch with all the porcine kobuviruses previously reported. At this time, it is not clear whether this agent associate with the diseases (including gastroenteritis) in piglets. In our study, the exceptional high prevalence of porcine kobuvirus (99%) was detected in piglets with diarrhea. This finding indicates the role of kobuviruses in diarrheal disease in piglets and provides additional information on their relationship to other porcine kobuviruses reported previously.

The final manuscript has been seen and approved by all the authors. The authors declare that they have no conflict of interest.

I would appreciate it very much if you could take our manuscript into your consideration. I am looking forward to hearing from you.

Best regards,

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LETTER

Exceptionally high prevalence of porcine kobuviruses in piglets with diarrhea

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To the Editor: To date, the kobuvirus genus consists of two officially recognized species, the Aichi virus and the bovine kobuvirus (1). The Aichi virus has been shown to associate with acute gastroenteritis in humans (2, 3, 4), while bovine kobuvirus has been detected only in cattle (5, 6). Most recently, a third candidate species of kobuvirus has been described in pigs from 2 different groups of investigators from Hungary and China (7, 8). This new candidate species of kobuvirus was serendipitously recognized in porcine stool specimens by unexpected amplification of PCR products (about 1,100 bp) using a primer pair for the detection of caliciviruses. The nucleotide sequences of these nonspecific PCR products were similar to those of the U-1 bovine kobuvirus and Aichi virus A846/88 reference strains with the sequence identities ranging from 73-79% at the nucleotide and 69-70% at the amino acid levels. The representative strain of a new candidate species of porcine kobuvirus, S-1-HUN, has been analyzed for its complete genome sequence and genetic organization (9). The RNA genome of the S-1-HUN strain comprises 8,210 nucleotides, with a genome organization analogous to picornaviruses. Therefore, this strain is tentatively classified as a new species of genus kobuvirus, and it is called porcine kobuvirus (7, 9).

Currently, 2 reports described the epidemiological feature of porcine kobuvirus in healthy piglets. Thirty-nine out of 60 (65%) stool samples collected from pigs in Hungary were positive for porcine kobuvirus by RT-PCR (9). Another report from China also demonstrated that the prevalence of porcine kobuvirus was 30% (97 out of 322) (8). These findings suggested that porcine kobuvirus infections are common in piglets. However, at this time it is not clear whether this agent associates with the diseases (including gastroenteritis) in piglets. Herewith, we have conducted an epidemiological survey of porcine kobuvirus and reported the detection of this

virus in stools of diarrheal piglets collected in Chiang Mai province, Thailand. Sequence and phylogenetic analyses of the porcine kobuvirus were examined to find out their evolutionary relationships with the kobuvirus strains previously reported.

A total of 98 porcine stool specimens were collected from diarrheic piglets during 2001 to 2003 in Chiang Mai province, Thailand. The presence of porcine kobuvirus in fecal specimens was detected by RT-PCR (9). The representative strains of porcine kobuvirus detected in our study were analyzed further by direct sequencing of their PCR amplicons (216 bp) using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequences of these portions were compared with those of reference strains available in the NCBI GenBank database using BLAST server. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 4 (10). The nucleotide sequences of porcine kobuvirus strains described in the present study were deposited in the GenBank under accession numbers GQ152093-GQ152122.

Surprisingly, the prevalence of porcine kobuvirus was extremely high in diarrheal piglets, reaching 99% (97 out of 98 specimens). Thirty representative strains of porcine kobuvirus detected in this study were randomly selected, sequenced, and analyzed for their evolutionary relationships with those other kobuvirus reference strains. The partial 3D region among all 30 porcine kobuvirus strains were highly conserved with the nucleotide sequences identities over 90%. In addition, our strains were most closely related to 2 porcine kobuvirus reference strains (S-1-HUN and Swine/2007/CHN) available in the GenBank database with the nucleotide sequence identity ranging from 91.5-96.3%. Phylogenetic analysis of partial 3D nucleotide sequences of our porcine kobuvirus strains together with those of published porcine kobuvirus reference strains as well as Aichi virus and bovine kobuvirus is shown in

the Figure. The phylogenetic tree confirmed that all of our strains belonged to porcine kobuvirus and formed a tight cluster in a monophyletic branch with other 2 porcine kobuvirus reference strains (S-1-HUN and Swine/2007/CHN), but are distantly related to standard strains of Aichi virus and bovine kobuvirus. Recently, 18 sequences of partial 3D region of the porcine kobuvirus strains detected in China have been deposited in the databank. Unfortunately, the target position of PCR amplification of the Chinese strains was different from our strains (8). It was, therefore, not possible to analyze the relationship between these strains.

So far, porcine kobuviruses have been reported only in healthy pigs (7, 8, 9). The association of this agent with the diseases in pigs remains unclear. In our study, the exceptionally high prevalence of porcine kobuviruses (99%) has been observed in piglets with acute gastroenteritis and those samples were negative for rotavirus infection as determined previously by RT-PCR. It is most likely that kobuviruses detected in the specimens might be the causative agent of severe diarrhea in these piglets. Extensive epidemiological surveillance and characterization of porcine kobuvirus strains from other geographical areas may help to understand the distribution, heterogeneity, and association of porcine kobuviruses with enteric diseases in pigs.

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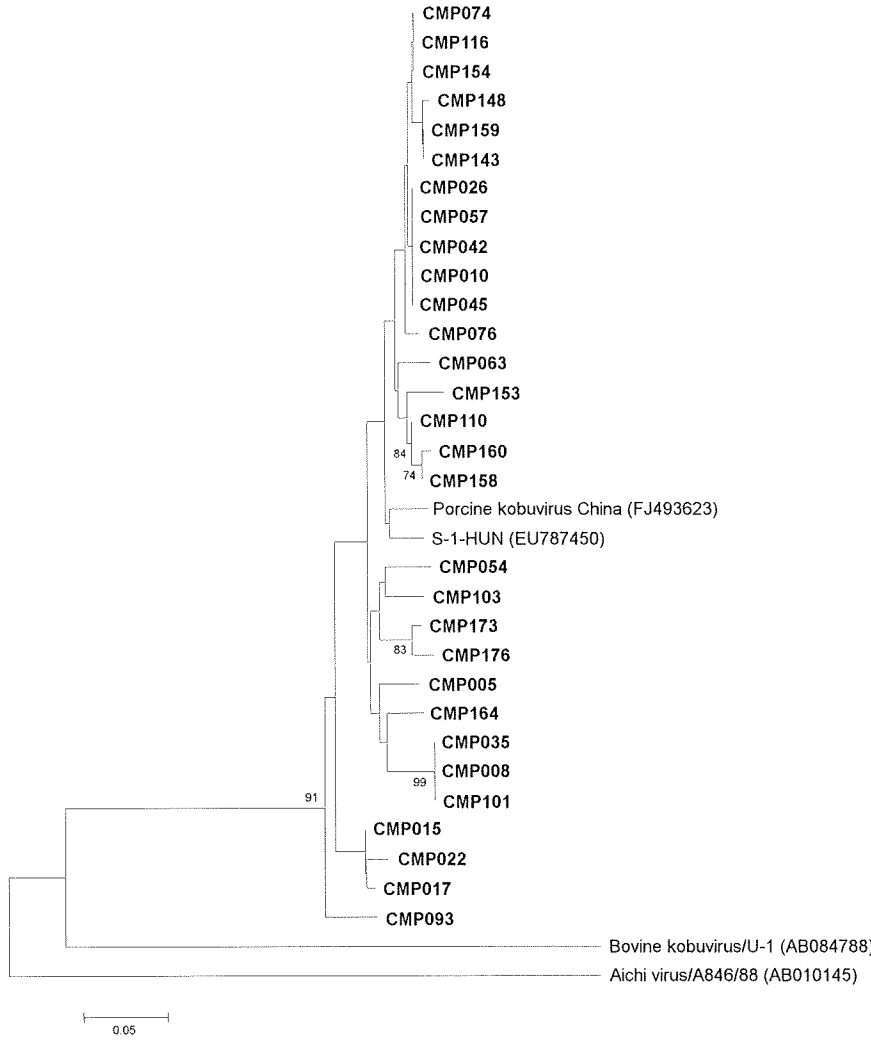
Figure legend

Phylogenetic analysis of the partial nucleotide sequence encoding the 3D region of porcine kobuviruses isolated in this study and other reference strains. The tree was generated on the basis of the neighbor-joining method using the MEGA 4 program.



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Diversity of Human Parechoviruses Isolated from Stool Samples Collected from Thai Children with Acute Gastroenteritis▽

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A total of 82 fecal specimens which were known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus and which were collected from infants and children with acute gastroenteritis in Chiang Mai, Thailand, from January to December 2005 were screened for human parechovirus (HPeV). HPeV was detected by reverse transcription-PCR with a primer pair that amplified the 5′ untranslated region of its genome and was genotyped by sequencing of the VP1 region. HPeV was detected in 12 of 82 specimens tested, and the detection rate was found to be 14.6%. The capsid VP1 gene was successfully sequenced from nine of the HPeV strains detected. The HPeV strains studied clustered into four different genotypes, HPeV genotype 1 (HPeV1) to HPeV4, and the majority of the strains studied (five strains) belonged to HPeV1. This is the first finding of HPeV from children with acute gastroenteritis in Thailand. In addition, the diversity of the Thai HPeV strains was also noted.

Parechoviruses are small, nonenveloped, positive-sense single-stranded RNA (ssRNA) viruses and belong to the large family of *Picornaviridae*, which is a highly diverse family of important pathogens of humans and animals. The *Parechovirus* genus was defined in the early 1990s (15, 18). The genus is composed of two species: Ljungan virus, isolated from bank voles (27), and human parechovirus (HPeV), a frequent human pathogen. On the basis of serological characteristics, two HPeVs were characterized in early 2004. However, they were first classified as echovirus types 22 and 23 within the *Enterovirus* genus but were later reclassified as HPeV genotype 1 (HPeV1) and HPeV2, respectively, on the basis of their distinctive biological and molecular properties. The genome of HPeV has an average length of 7,300 nucleotides and is packaged into an icosahedral capsid made up of multiple copies of each of the capsid proteins VP0, VP3, and VP1 (15, 33).

Previous findings revealed the genetic variability of HPeVs, and the number of newly identified HPeV genotypes has been on the increase. On the basis of VP1 sequence comparisons, nine HPeV types have been described to date (1–9, 16, 24, 34). In addition, HPeVs have been classified into 14 genotypes, as described elsewhere (http://www.picornaviridae.com/parechovirus/hpev/hpev.htm); however, the nucleotide sequences of new HPeV strains from genotypes 9 to 13 and corresponding studies have so far not been published.

Acute gastroenteritis is among the diseases that are the primary cause of morbidity in Thailand, as documented in a Thai annual report of epidemiological surveillance (http://epid.moph.go.th); and it is well established that rotaviruses, adenoviruses, astroviruses, and caliciviruses are the most important etiologic agents of acute gastroenteritis (14, 19, 20, 25). To date, however, there has been no report on HPeV infection in Thailand. The present study was aimed at screening stool samples collected from children with acute gastroenteritis in Chiang Mai, Thailand, for infection with HPeV, one of the less explored viral pathogens which has recently been reported to be associated with diarrhea, and characterizing the molecular properties of the HPeV strains detected.

MATERIALS AND METHODS

Clinical specimens. Eighty-two fecal specimens which had been shown to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus by reverse transcription (RT)-PCR and which were collected from infants and children with acute gastroenteritis in Chiang Mai, Thailand, from January to December 2005 were subjected to screening for HPeV. All stool samples were stored without additives at −30°C for up to 3 years before analysis. The fecal specimens were diluted with distilled water to 10% suspensions and were clarified by centrifugation at 10,000 × *g* for 10 min. The supernatants were collected and stored at −30°C until use for virus detection.

RNA extraction and RT. The RNA genome of HPeV was first extracted from 140 μl of a 10% fecal suspension by using a QIAamp viral RNA minikit (Qiagen, Inc., GmbH Hilden, Germany), according to the manufacturer's instructions. Then, for RT, 5 μl of the stored, extracted RNA was added to a reagent mixture consisting of 5× first-strand buffer (Invitrogen, Carlsbad, CA), 10 mM deoxynucleoside triphosphates (dNTPs; Roche, Mannheim, Germany), 0.1 M dithiothreitol (Invitrogen, Carlsbad, CA), SuperScript reverse transcriptase III (200 U/μl; Invitrogen, Carlsbad, CA), random primer (1 μg/μl; hexa-deoxyribonucleotide mixture; Takara, Shiga, Japan), RNase inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and distilled water. The total volume of the reaction mixture was 15 μl. The RT reaction was carried out at 50°C for 1 h, followed by 95°C for 5 min, and the reaction mixture was then held at 4°C. The cDNA was stored at −30°C until it was used for the PCRs (29, 35).

PCR for detection of HPeV. After the addition of 2 μl of cDNA to 23 μl of the reagent mixture containing 5× *Taq* DNA polymerase buffer (Promega, Madison, WI), dNTPs (10 mM), primers (20 μM), *Taq* DNA polymerase (5 U/μl; Promega), and distilled water, a screening PCR was conducted with primers ev22(+) and ev22(−) to amplify a 270-bp PCR product of the 5′ untranslated region

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TABLE 1. Oligonucleotide sequences of the primers used in this study and their positions

Primer	Gene	Sequence (5′ to 3′)[a]	Sense	Position[b]	Amplicon	Reference or source
ev22(+)	5′ UTR	CYCACACAGCCATCCTC	+	312–328	270	Joki-Korpela and Hyypia (17)
ev22(−)	5′ UTR	TRCGGGTACCTTCTGGG	−	581–565		
VP1-parEchoF1	VP1	CCAAAATTCRTGGGGTTC	+	2332–2349	760	Benschop et al. (6)
VP1-parEchoR1	VP1	AAACCYCTRTCTAAATAWGC	−	3090–3071		
Cap-parEcho-F	VP1	TCHACWTGGATGMGRAARAC	+	2162–2181	1076	This study
Cap-parEcho-R	VP1	TCYARYTCACAYTCYTCYTC	−	3237–3218		
3DparEcho-F	3D	GATTGGCACTTYATGATHAATG	+	6555–6576	383	This study
3DparEcho-R	3D	CAAATGAWTCTGCCATGAYAC	−	6937–6917		

[a] Y, C or T; R, G or A; W, A or T; H. A, C, or T; M, C or A.
[b] The sequence position is based on the full genome sequence of HPeV1 prototype strain Harris (GenBank accession number L02971).

(UTR) (17) (Table 1). The PCR protocol was 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min and with a final extension at 72°C for 7 min.

Genotyping by VP1 sequencing and primer designation. At first, to amplify the VP1 segment, the primers developed by Benschop and colleagues and described previously (6) were used. However, because of the failure to obtain PCR products from most of the HPeV-positive samples except one, two new primers were designed for the first PCR. Then, the nested PCR was performed with the inner primer pair described by Benschop and colleagues (6).

For primer designation, to obtain the full length of 702 bases of the VP1 capsid gene, alignment of the full genome sequences of reference strains of eight known HPeV genotypes available in the GenBank database was performed with Clustal X software to find the conserved regions, and the two new primers were designed to be specific for sequences outside the VP1 region. The oligonucleotide sequences of the newly developed primers and their positions are described in Table 1.

The first PCR was done with the newly designed primers; and the thermal cycle program was as follows: 5 min at 95°C, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. The nested PCR was conducted with the known primer pair VP1-parEchoF1 and VP1-parEchoR1 (6) at an annealing temperature of 48°C to generate a 760-bp product (Table 1). Analysis of the amplification products was performed by 1.5% agarose gel electrophoresis, and the bands were visualized by staining with SYBR Safe (Invitrogen, Tokyo, Japan) under UV light. The HPeV-positive samples were retested with another PCR by using a newly designed primer pair, 3DparEcho-F and 3DparEcho-R, derived from the 3D gene of the HPeV genome (Table 1).

Sequencing and phylogenetic analysis. The PCR amplicons of the VP1 gene were purified and sequenced in both directions by using a BigDye Terminator cycle sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). The primers used for amplification of the VP1 gene were used as sequencing primers. The sequence data were collected by an ABI Prism 310 genetic analyzer (Perkin Elmer-Applied Biosystems, Inc.).

The sequences of the VP1 segments from the HPeV strains obtained in the present study and other reference HPeV strains of nine known genotypes available in the GenBank database were compared. The sequence data and the data from the phylogenetic analysis were analyzed by using BioEdit (version 7.0.5) software. A parsimony analysis was also conducted by using the MEGA (molecular evolutionary genetics analysis; version 3.1) program to determine the evolutionary relationship among the sequences studied (22). The method was performed by using a close-neighbor interchange with a random option and with 500 bootstrap repetitions.

Nucleotide sequence accession numbers. The nucleotide sequences of the following reference HPeV strains described in this study have been deposited in the GenBank database under the accession numbers indicated in parentheses: HPeV1 strains Harris (L02971), 652281 (FJ373120), BNI-R09/03 (EU024632), BNI-R32/03 (EU024636), BNI-R15/03 (EU024633), BNI-788St (EF051629), 677033 (FJ373136), 69960AE (AM933170), A229-05 (AB300968), A234-05 (AB300969), A708-99 (AB300935), BNI-R04/03 (EU024631), A65-05 (AB300963), A222-05 (AB300967), BNI-R21/03 (EU024634), 652780 (FJ373127), 650648 (FJ373108), A191-05 (AB300966), A527-99 (AB300928), BNI-90/03 (EU024630), and BNI-R30/03 (EU024635); HPeV2 strain Williamson (AJ005695); HPeV3 strains Can82853-01 (AJ889918), 677146 (FJ373162), A415-01 (AB300945), A308/99 (AB084913), and 651689 (FJ373153); HPeV4 strains Fuk2001-282 (AB433630), NII370-93 (AB434673), T75-4077 (AM235750), 653046 (FJ373170), and K251176-02 (DQ315670); HPeV5 strains CT86-6760 (AF055846), T92-15 (AM235749), and 676618 (FJ373175); HPeV6 strains 2005-823 (EU077518), NII561-2000 (AB252582), BNI-67/03 (EU024629), and 650045 (FJ373178); HPeV7 strain PAK5045 (EU556224); HPeV8 strain BR/217/2006 (EU716175); and HPeV14 strain 451564 (FJ373179). For the Thai strains studied, the accession numbers are FJ648755 to FJ648762 and GQ149453.

RESULTS

Of the 82 samples tested, 12 were positive for HPeV, and the rate of detection of HPeV was 14.6%. All 12 patients whose stool samples were positive for HPeV were children ages 6 to 24 months. Of these, six patients (50%) were from 6 to 18 months of age.

For genotyping, the full-length VP1 capsid sequences from only nine strains were successfully amplified and sequenced. The full length of the VP1 sequences of the nine strains studied was 702 bases.

Figure 1 shows the phylogenetic tree constructed from the 624-nucleotide sequences of the partial VP1 segments of the reference HPeV strains and nine Thai strains found in this study. On the basis of the specific clustering of the isolates with known HPeV types obtained from the GenBank database, the strains studied could be identified as HPeV genotypes 1 to 4. The majority of the Thai strains (five strains) belonged to HPeV1, the largest cluster of HPeVs. One Thai strain clustered together with the Williamson strain into the HPeV2 cluster. The two other strains were HPeV3, the second largest cluster of HPeVs. The remaining strain was genotyped as HPeV4, and it clustered along with strain K251176, which was recently detected in The Netherlands.

For the HPeV1 strains studied, three strains were found to cluster closely together with prototype strain Harris, and the amino acid similarities between the three strains and strain Harris ranged from 92.7% to 93.7%, while the two remaining HPeV1 Thai strains were in the larger cluster consisting of recently detected HPeV1 strains, and the amino acid similarities between those two strains and strain Harris were less than 90% (86.4% and 89.6%, respectively).

In the case of the HPeV2 strains, the amino acid similarity between the Thai strains studied and strain Williamson was 95.2%, while the mean amino acid similarities between the

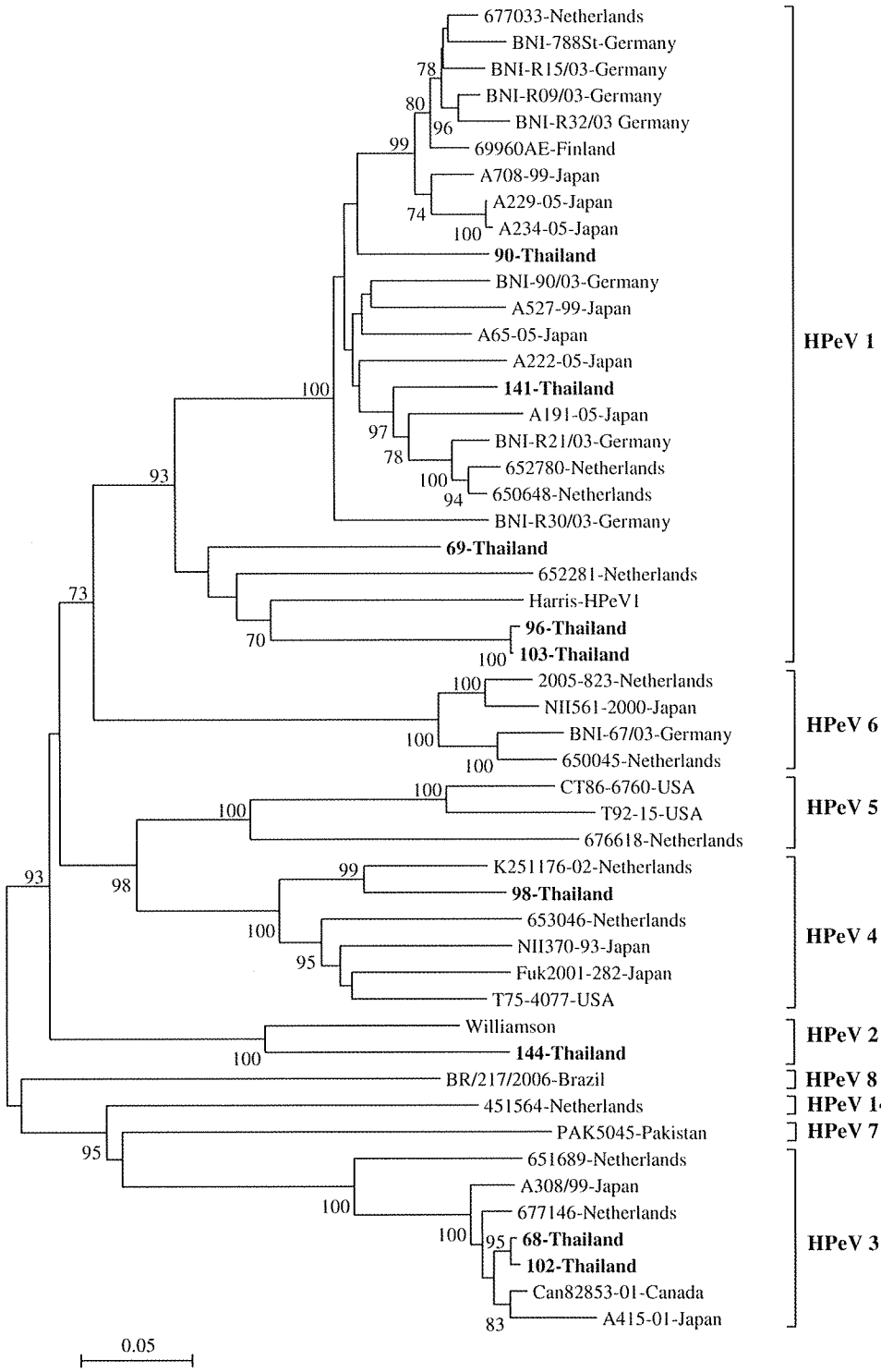

FIG. 1. Phylogenetic tree constructed from the 624-nucleotide sequences of the VP1 gene of the strains studied and reference HPeV strains with 500 bootstrap repetitions. Bootstrap values above 70% are shown at the branch nodes. The HPeV strains studied are in boldface type.

Thai strains and strains of the other genotypes ranged from 63.8% (with HPeV3) and 74.2% (with HPeV1).

The two Thai HPeV3 strains studied clustered closely together with Japanese strain A308/99, the prototype strain of HPeV3, and had 96.6% amino acid similarity to that strain.

Within the HPeV4 cluster, the amino acid similarity between the Thai strains studied and other strains ranged from 97.8% to 99.1%.

Alignment of the deduced amino acid sequences of the strains studied and global HPeV reference strains of HPeV