CHAPTER V

RESULTS

1. Detection of diarrheal viruses by RT- multiplex PCR

A total of 332 stool specimens collected in one year round in 2008 were screened for 10 types of diarrheal viruses. Before conducting the molecular detection of 10 types of diarrheal viruses, the positive controls which are known to be positive for SaV, AiV, RCV, HPeV, NoV GI, NoV GII, EV, AdV, RAV, AstV from the previous studies were used as positive control in this novel RT-multiplex PCR method. The expected PCR product sizes of SaV, AiV, RCV, HPeV, NoV GI, NoV GII, EV, AdV, RAV, and AstV were 100 bp, 158 bp, 205 bp, 270 bp, 330 bp, 387 bp, 440 bp, 482 bp, 569 bp, and 719 bp, respectively. All the positive controls showed the correct PCR product sizes as shown in Figure 1.

By screening with multiplex-PCR, 4.2% were positive for 5 types of diarrheal viruses. Among these, AdV, EV, AiV, NoV GII, and HPeV were detected (Figures 2, 3, 4, 5, and 6, respectively), while SaV, RCV, NoV GI, RAV, and AstV were not found in this study. AdV and EV were detected as the most prevalent viruses in this study (1.2%, 4 out of 332), followed by AiV (0.9%, 3 out of 332) and NoV GII (0.6%, 2 out of 332), respectively. In addition, mixed infection of 2 viruses between NoV GII and HPeV was also detected in one fecal specimen (0.3%), as shown in Table 8.

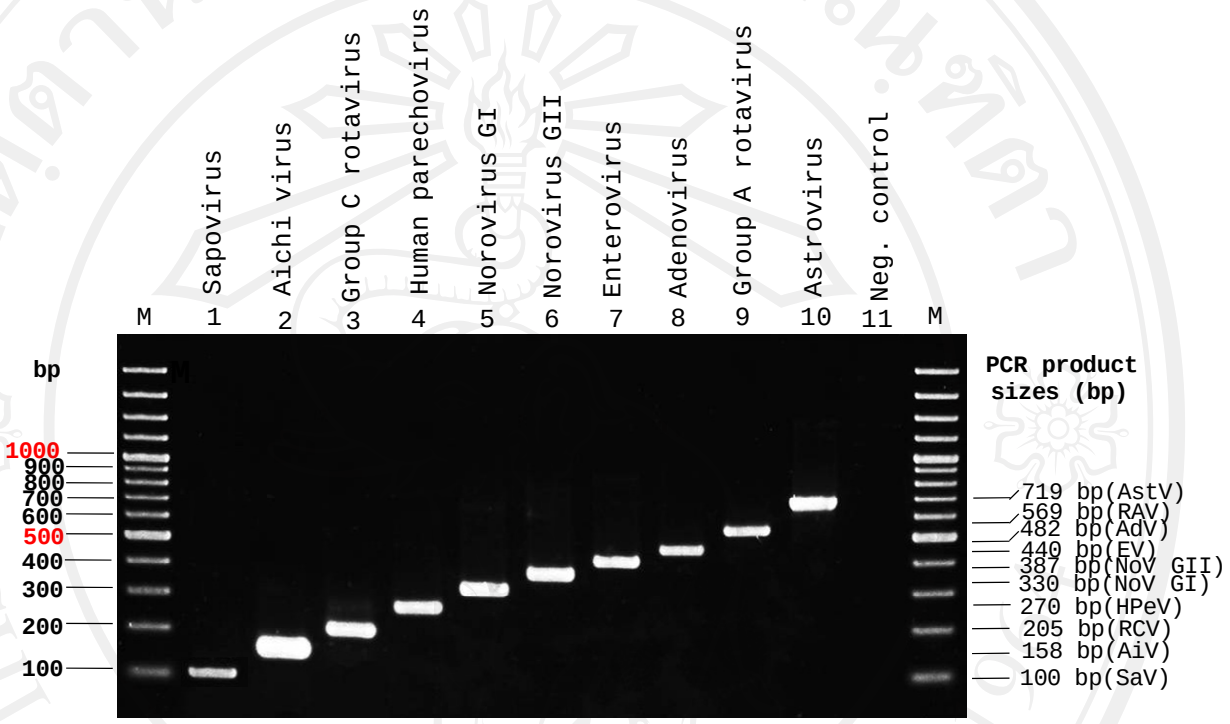


Figure 1 Agarose gel electrophoresis demonstrating the expected PCR product size of 10 viruses. Lane 1, SaV (100 bp); lane 2, AiV (158 bp); lane 3, RCV (205 bp); lane 4, HPeV (270 bp); lane 5, NoV GI (330 bp); lane 6, NoV GII (382 bp); lane 7, EV (440 bp); lane 8, AdV (487 bp); lane 9, RAV (569 bp); lane 10, AstV (719 bp); lane 11, a negative control. Lane M, 100 bp DNA ladder.

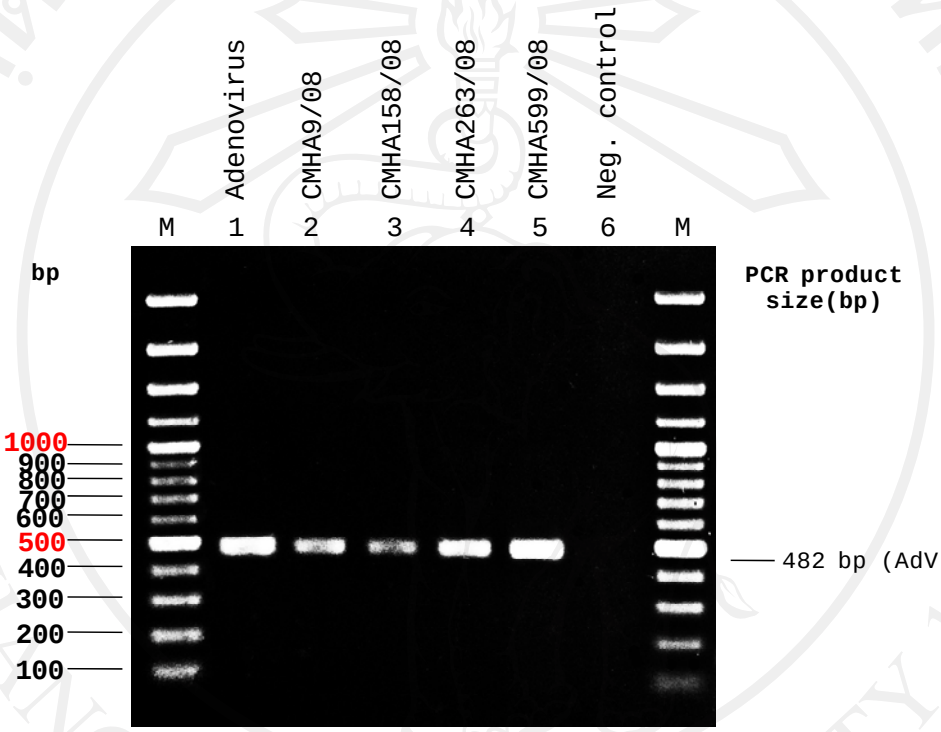


Figure 2 Agarose gel electrophoresis demonstrating the expected PCR product size of adenoviruses. Lane 1, reference strain of AdV; lanes 2-5, tested samples (CMHA9/08, CMHA158/08, CMHA263/08, and CMHA599/08) that were positive for AdV, and lane 6, a negative control. Lane M, 100 bp DNA ladder.

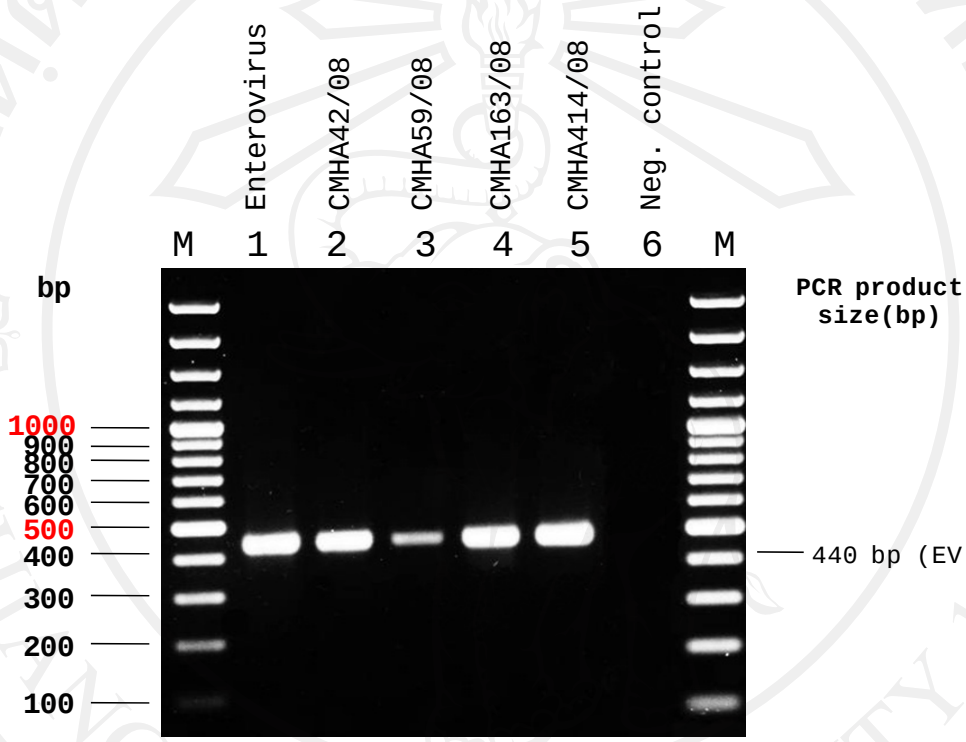


Figure 3 Agarose gel electrophoresis demonstrating the expected PCR product size of enteroviruses. Lane 1, reference strain of EV; lanes 2-5, tested samples (CMHA42/08, CMHA59/08, CMHA136/08, and CMHA414/08) that were positive for EV, and lane 6, a negative control. Lane M, 100 bp DNA ladder.

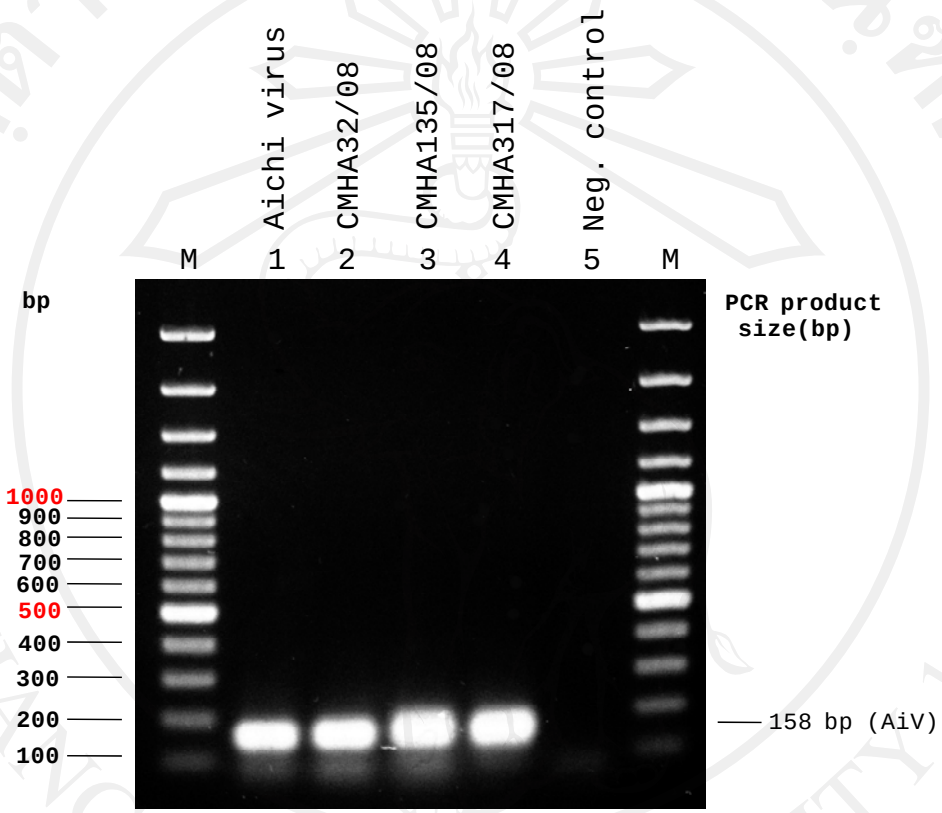


Figure 4 Agarose gel electrophoresis demonstrating the expected PCR product size of Aichi viruses. Lane 1, reference strain of AiV; lanes 2-4, tested samples (CMHA32/08, CMHA135/08, and CMHA317/08) that were positive for AiV, and lane 5, a negative control. Lane M, 100 bp DNA ladder.

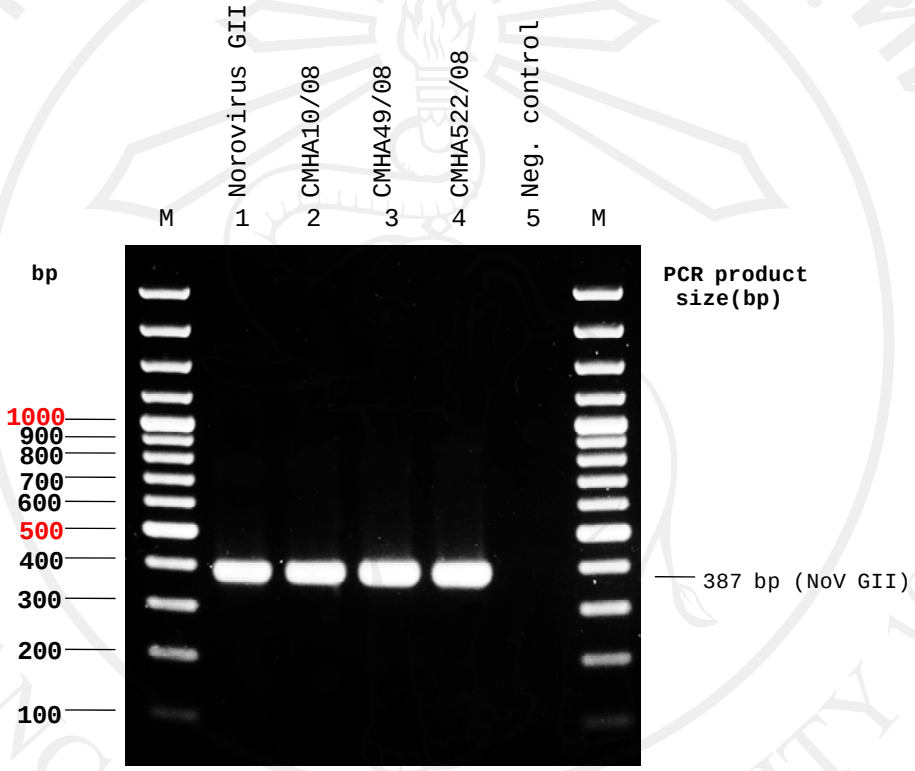


Figure 5 Agarose gel electrophoresis demonstrating the expected PCR product size of noroviruses GII. Lane 1, reference strain of NoV GII; lanes 2-4, tested samples (CMHA10/08, CMHA49/08, and CMHA522/08) that were positive for NoV GII, and lane 5, a negative control. Lane M, 100 bp DNA ladder.

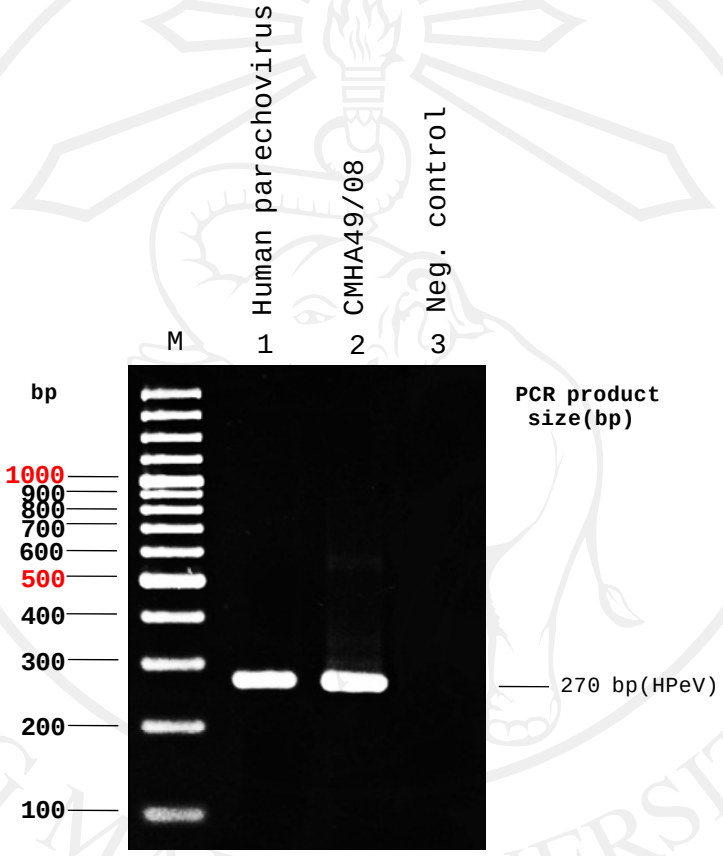


Figure 6 Agarose gel electrophoresis demonstrating the expected PCR product size of human parechoviruses. Lane 1, reference strain of HPeV; lane 2, tested samples (CMHA49/08) that were positive for HPeV, and lane 3, a negative control. Lane M, 100 bp DNA ladder.

Table 8 Prevalence of diarrheal viruses in adults with diarrhea in Chiang Mai, Thailand in 2008 determined by RT-multiplex PCR

Number of fecal specimens tested	Number of positive-diarrheal viruses (%)											Total (%)
	Sapovirus	Aichi virus	Group C rotavirus	Human parechovirus	Norovirus GI	Norovirus GII	Enterovirus	Adenovirus	Group A rotavirus	Astrovirus	Mixed infection	
332	0/332 (0)	3/332 (0.9)	0/332 (0)	0/332 (0)	0/332 (0)	2/332 (0.6)	4/332 (1.2)	4/332 (1.2)	0/332 (0)	0/332 (0)	1*/332 (0.3)	14 (4.2)

* Mixed-infection of 2 viruses between norovirus GII and human parechovirus.

2. Nucleotide sequencing and phylogenetic analyses of adenoviruses, enterovirus, Aichi viruses, noroviruses GII and human parechoviruses

The PCR products obtained from the specimens that positive for diarrheal viruses were purified, and sequenced by using Big-Dye Terminator Cycle Sequencing kit (ABI PRISM 3100, Carlsbad, USA). Among these 14 specimens, AdV and EV each was found in 4 samples. AiV and NoV GII each was found in 3 and 2 samples, respectively. HPeV was detected in 1 sample. The obtained sequences were compared with the reference sequences by searching for the close by related reference sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) server from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). Those reference sequences obtained from BLAST search, the viruses of each genotypes/ genogroups/ subgroups/ species, as well as our study sequences were aligned using ClustalX software, and further classified for their genotypes/ genogroups/ subgroups/ species by phylogenetic analysis using MEGA 4 software (Tamura et al., 2007).

1.1 Analysis of partial capsid gene of adenoviruses

The partial hexon gene (482 bp) of AdV capsid was sequenced by using Ad2 specific primer. From BLAST search and clustalX alignment, 4 AdVs found in this study (CMHA9/08, CMHA158/08, CMHA263/08, and CMHA599/08) were genetically variable. Two AdV sequences (CMHA263/08 and CMHA599/08) were most closely related to the prototype strain of AdV40 (X51782) at 99% nucleotide sequence identity. The CMHA263/08 strain was most closely related to HME562 strain (EF570136), while the CMHA 599/08 was most closely related to Dugan strain (AB330121), 6643 strain (FJ167565), HME562 strain (EF570136) at 99% nucleotide

sequence identity. In addition, the CMHA9/08 strain showed nucleotide sequence identity at 99% with AdV25 prototype strain (DQ149623) and BP-1 strain (AB330106) while CMHA158/08 was closely related (99% identity) with AV-3153 strain (AB330105) and prototype strain of AdV24 (DQ149622), of subgroup D.

The phylogenetic analysis of partial hexon gene sequence of AdV demonstrated clearly that 4 AdV strains detected in this study were classified into 3 genotypes of 2 distinct subgroups. CMHA9/08 and CMHA158/08 belonged to genotypes AdV25 and AdV24 of subgroup D. The CMHA263/08 and CMHA599/08 belonged to AdV40 and both strains were in subgroup F, as shown in Figure 7. It is interesting to note that, AdV strain detected in this study were quite difference from the AdVs isolated previously in children in Chiang Mai in 2007, which belonged to AdV1, AdV3, and AdV41 genotypes. Comparing 4 AdV strains identified in this study with those of the AdV strains isolated previously in children in Chiang Mai (CMHA9/07, CMHA25/07, and CMHA16/07) revealed the nucleotide sequence identity ranging only from 77-89%.

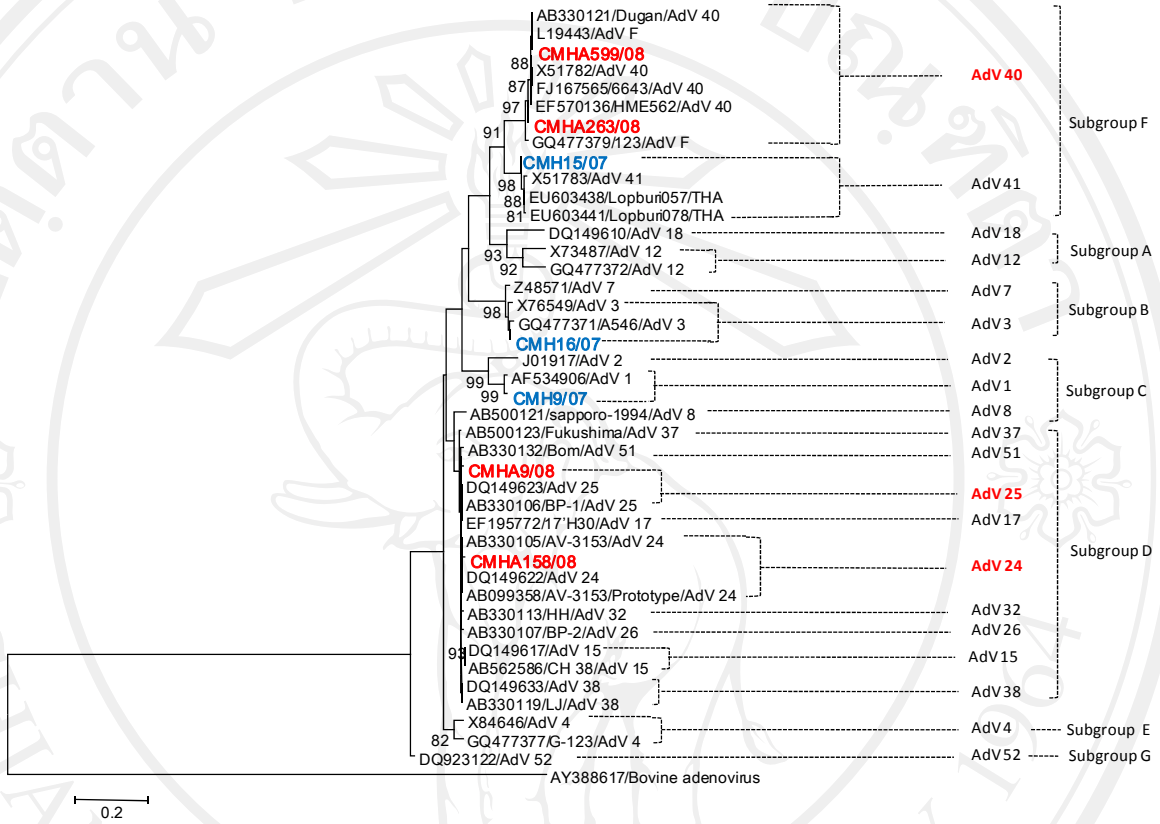


Figure 7 Phylogenetic analysis of the partial nucleotide sequences (482 bp) of the hexon gene of adenoviruses. The GenBank accession numbers of reference strains were indicated proximal to the strain name. The AdV strains detected in this study and previous study in children were indicated in red and blue, respectively. The tree was constructed according to neighbor-joining method. The number on each branch indicates the bootstrap value.

2.2 Analysis of partial 5' untranslated region (5'UTR) of enteroviruses

The 4 EV strains (CMHA42/08, CMHA59/08, CMHA136/08, and CMHA414/08) found in this study were genetically variable. The CMH59/08 strain showed highly nucleotide sequence identity (96%) with echovirus 30 (AM237034) reference strain and belonged to EV of species B. Comparing the nucleotide sequence of CMHA59/08 with those of EVs in species B isolated previously in children in Chiang Mai (CMH86/07, CMH134/07, and CMH135/07), it was observed that the sequence identities were ranging from 93-99%. The CMHA42/08 strain was identical (100%) to poliovirus genotype 3, MF1 strain (AJ783739), 31974 strain (FJ460227) and HeB strain (FJ859192). It is interesting to note that CMHA136/08 strain is most closely related to CMH116/07 strain which was isolated previously form a child in Chiang Mai at 95% nucleotide sequence identity, and also similar to MOR83 reference strain (EF015020). All of these strains were coxsackievirus A20. Moreover, the CMHA414/08 strain shared nucleotide sequence identity with BAN04-1067 reference strain (EF015010) of enterovirus 99 at 90% and with enterovirus 99 prototype strain (EF555644) at 83%.

Based on the phylogenetic analysis of EV 5'UTR sequence, 4 strains of EV found in this study belonged to 4 genotypes of 2 species (B and C) of EV (Figure 8). The CMHA42/08 was the poliovirus 3, CMHA136/08 was coxsackie virus A20, CMHA414/08 was enterovirus 99, and all 3 strains were clusted within EV species C. The CMHA59/08 was the only EV strain clusted in EV species B and was identified as Echovirus 30. Moreover, phylogenetic tree clearly demonstrated that CMHA136/08 belonged to coxsackie virus A20 and was the only strain that showed nucleotide sequence most closely related to EV (CMH116/07) which was isolated

previously in Chiang Mai in 2007. The data confirmed that coxsackievirus A20 was circulated in Chiang Mai and caused diarrhea both in children and adults.

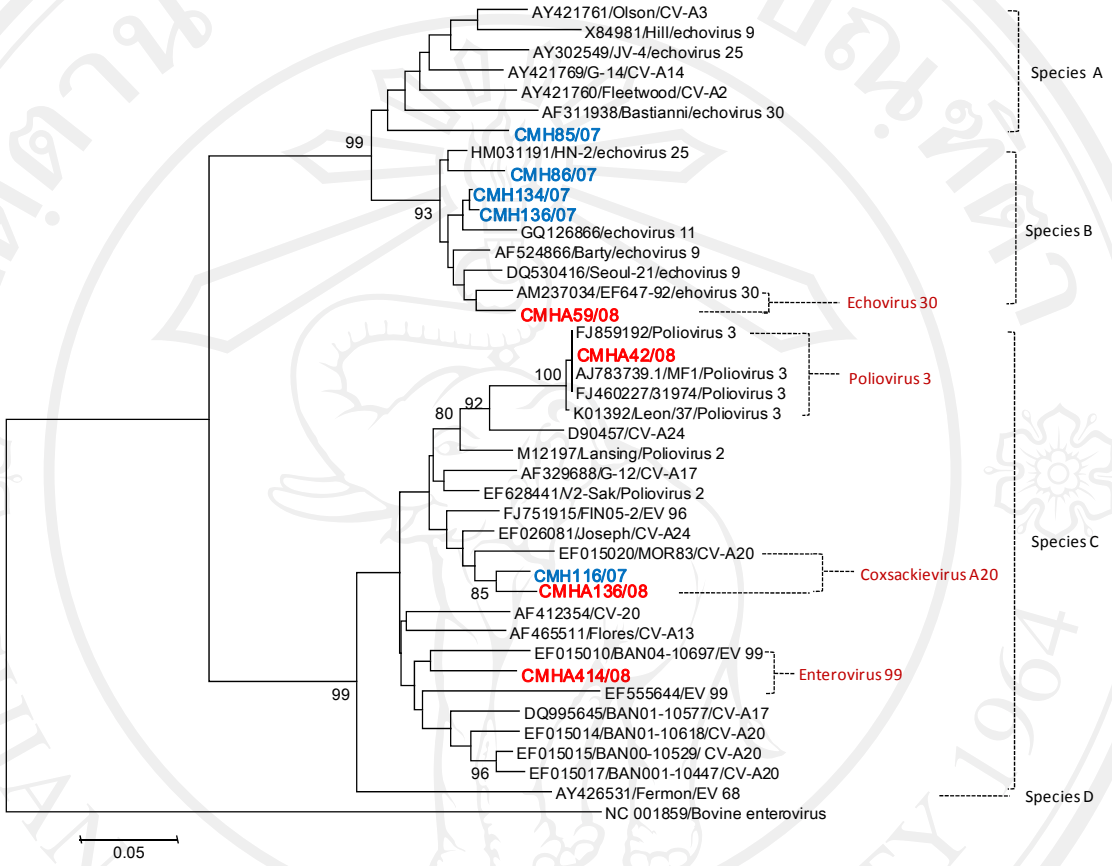


Figure 8 Phylogenetic analysis of the partial nucleotide sequence (440 bp) of the 5' untranslated region (5' UTR) of enteroviruses. The GenBank accession numbers of reference strains were indicated proximal to the strain name. EV detected in adults in this study and previous studies in children were indicated in red and blue, respectively. The tree was constructed according to neighbor-joining method. The number on each branch indicates the bootstrap value.

2.3 Analysis of the 3C and the N terminus of 3D regions (3CD) of Aichi viruses

In the present study, 3 AiVs strains (CMHA32/08, CMHA135/08, and CMHA317/08) were detected by RT-multiplex PCR screening method. For characterization of their genotypes, the partial 3CD region was amplified and the PCR product size was 519 bp as shown in Figure 9. The PCR amplicons of 519 bp were subjected to direct sequencing. The obtained sequences were compared to those of AiV strains available in the GenBank database by BLAST program and clustalX alignment. Sequence analysis revealed that 2 AiV strains (CMHA32/08 and CMHA317/08) showed highly nucleotide sequence similarity among themselves, and also similar to these of AiV genotype B, Chsh3 (FJ890521), Chsh4 (FJ890522), and Chsh6 (FJ890517) reference strains from China at 98% nucleotide sequence identity. In addition, the nucleotide sequence of one strain of AiV found in this study (CMHA135/08) was most closely related to AiV genotype A, 494/97 (AB092828), A1471/96 (AB034650), 364/96 (AB092826), and J-482 (EF079154) reference strains from Japan at the nucleotide sequence identity of 96%. It is interesting to point out that the CMHA135/08 AiV identified in this study is not so closely related with the AiV T-132/02 strain, the only one AiV previously found in children with diarrhea in Chiang Mai area in 2002. Nucleotide sequence identities between these 2 strains were only 94%.

The phylogenetic analysis shown in Figure 10 clearly demonstrated that all 3 AiV strains from adults with diarrhea were clustered into two major genotypes, genotypes A and B. The CMHA135/08 strain belonged to genotype A with the nucleotide sequence similar to the AiV strain (EF079160) which was isolated previously from children in Chiang Mai, in 2002. The other two AiV strains

(CMHA32/08 and CMHA317/08) were clustered closely together with other AiV genotype B reference strains. The data clearly demonstrated that AiV of both genotypes A and B were circulating in this area in adults with diarrhea in Chiang Mai, Thailand.

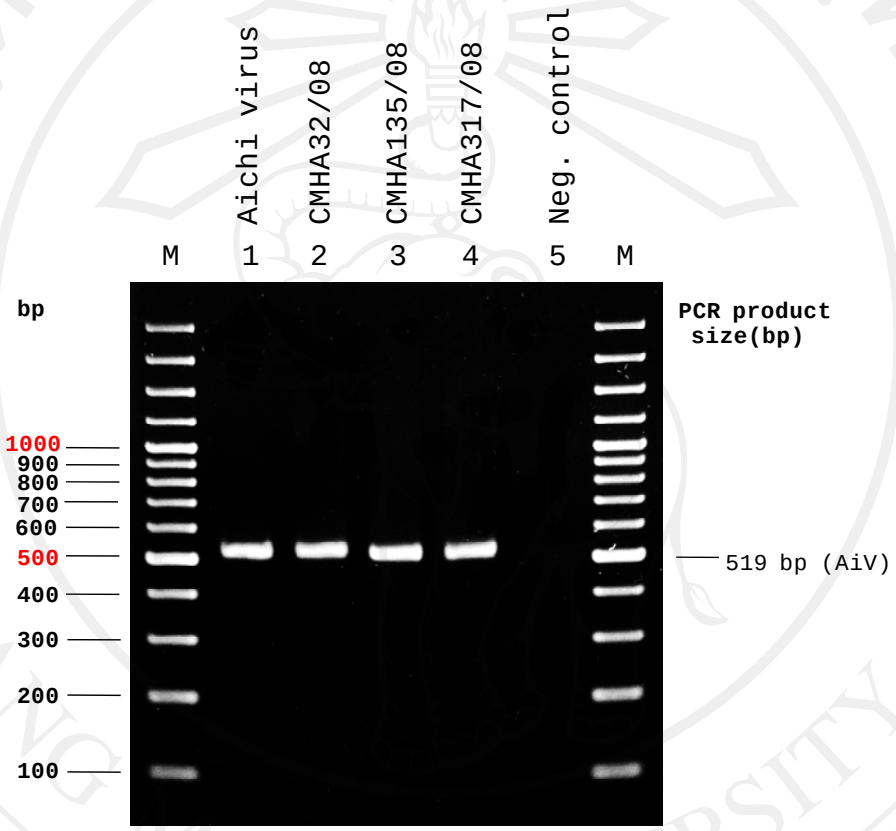


Figure 9 Agarose gel electrophoresis demonstrating the expected PCR product size of Aichi viruses at the 3CD regions. Lane 1, reference strain of AiV; lanes 2-4 test samples (CMHA32/08, CMHA135/08, and CMHA317/08) that were positive for AiV; lane 5, a negative control reaction. Lane M, 100 bp DNA ladder.

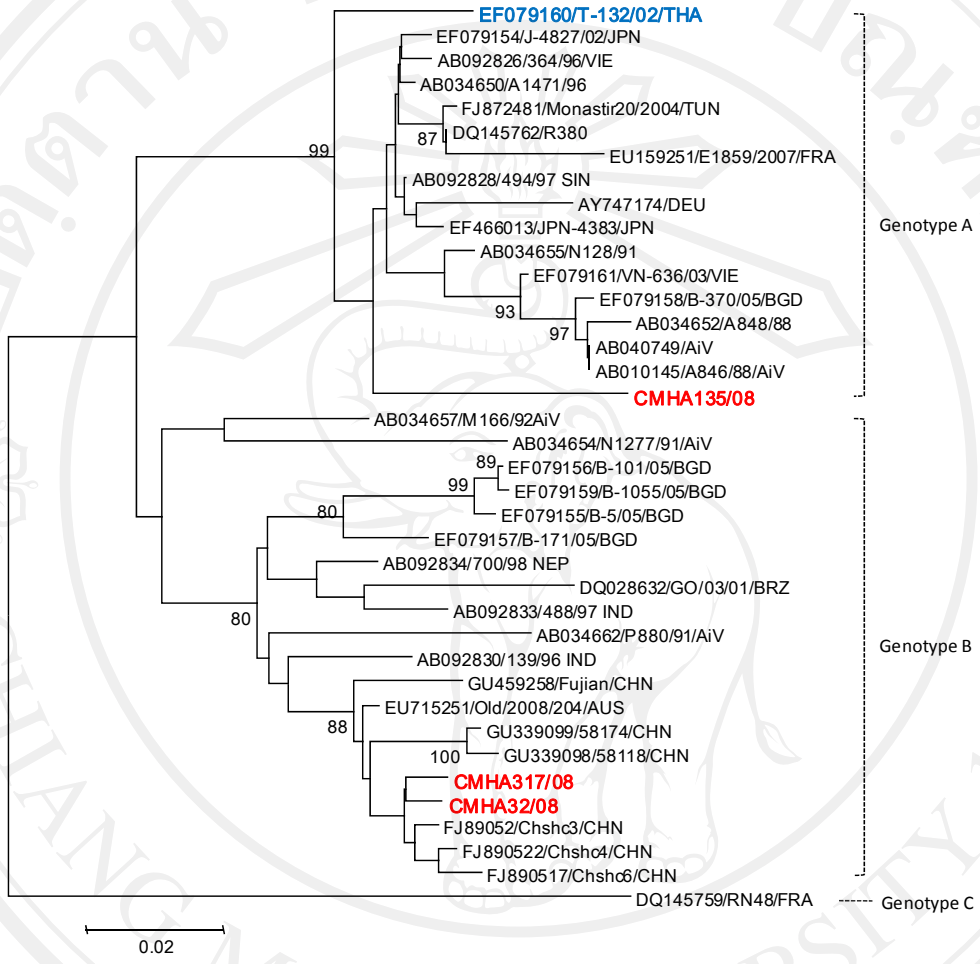


Figure 10 Phylogenetic analysis of the 3CD region nucleotide sequences (519 bp) of Aichi viruses. The GenBank accession numbers of reference strains were indicated proximal to the strain name. AiV detected in this study and previous studies in children were indicated in red and blue, respectively. The tree was constructed according to the neighbor-joining method. The number on each branch indicates the bootstrap value.

2.4 Analysis of partial capsid gene of noroviruses GII

The nucleotide sequences of partial capsid gene of NoV GII strains (CMHA10/08, CMHA49/08, and CMHA552/08) obtained from this study were compared to those of NoV strains available in the GenBank data base using the BLAST program and clustalX alignment. Two strains of NoV GII (CMHA552/08 and CMHA49/08) were identical (100% identity) to Nagano strain (AB541303) which was the GII/4 strain reported from Japan, while sharing lesser degree of identity (99%) to Beijing/74 (EU703746), Beijing/334 (EU703755), CHD-032304 (HM624049), Shanghai/SH2 (GU991353) which were reported from China, and also similar to Sakai4 (AB541344), Chiba5 (AB541234) which were reported from Japan, and Seoul/027 (HM636147) from Korea. The other NoV GII strain, CMHA10/08 shared a great homology at 98% with NZ327 (EF187497) which was reported from New Zealand. Moreover, this CMHA10/08 also showed high degree of sequence identity (97%) with other NoV GII/4 strain isolated from Japan, United Kingdom, and Thailand. Those included Hokkaido5 (AB541267), Amori2 (AB447343), B4S6 (AY587958), CMH38/02 (EF600760), CMH041/02 (EF600762), CMH43/02 (EF600764), and Sakaeo-14 (AY646868). In addition, CMHA10/08 showed less sequence identity (80%) with other 3 NoV GII/4 strains (CMH150/07, CMH153/07, and CMH155/07) isolated previously from children with diarrhea in Chiang Mai in 2007.

Based on the phylogenetic relationships and the classification scheme in Figure 11, all NoVs detected in the present study (CMHA10/08, CMHA49/08, and CMHA552/08) belonged to NoV GII/4 which carried nucleotide sequence somewhat

differed from the NoV GII/4 strains isolated previously from children in Chiang Mai area.

The NoV GII/4 were further characterized for their variant genotypes by nucleotide sequencing and phylogenetic analysis as shown in Figure 12. From sequence alignment of NoV GII/4 strains in the present study with NoV GII/4 varaint strain available in the GenBank database using ClustalX software revealed that CMHA49/08 strain was identical (100% identity) to Nagano2 strain (AB541303) which were the NoV GII/4 variant strains reported from Japan. In addition, CMHA49/08 showed high sequence identity (97-98%) with other NoV GII/4 variant strains detected in Japan including Hokkaido4 (AB541266), FUMI (AB543808), Osaka3 (AB541324), Saga1 (AB447456), and Hokkaido3 (AB447440). Moreover, the CMHA552/08 strain was most similar to Nagano2 (AB541303) at the nucleotide sequence identity of 98%, while sharing lesser degree of identity (97%) to NoV GII/4 variant strains from Japan [Osaka3 (AB541324), Saga1 (AB447456), and Hokkaido3 (AB447440)]. Comparing of two NoV GII/4 variant strains (CMHA49/08 and CMHA552/08) with the NoV GII/4 variant strains detected previously in children in Chiang Mai showed 81-84% nucleotide identity. Another NoV GII/4 variant strain (CMHA10/08) showed high degree of sequence identity (97%) with the NoV GII/4 variant strains isolated from Germany [Mannheim131 (GQ303445)], Australia [NSW892U (HM748973)], and Japan [Aichi1 (AB541202), Osaka1 (AB541320)], and showed 95-96% nucleotide identity with Niigata1 strain (AB541310), Iwate3 (AB541272) which were those reported from Japan, and NSW001P (GQ845367) from Germany. In contrast, the CMHA10/08 was compared with the NoV GII/4

variant strain from previously detected in children in Chiang Mai in 2007 revealed only 84% nucleotide sequence identity.

Based on the phylogenetic relationships and the classification of NoV GII/4 variant scheme, NoV GII/4 vairants detected in the present study could be divided into two variants, NoV GII/4 2006a variant and NoV GII/4 2006b variant (Figure 13). The CMHA10/08 belonged to NoV GII/4-2006a variant while CMHA49/08 and CMHA552/08 belonged to NoV GII/4-2006b variant. Two NoV GII/4 variant strains, CMHA49/08 and CMHA552/08 were most closely related to Osaka3 (AB541324) and Nagano2 (AB541303) which were NoV GII/4 variants reported from Japan, and shared a great sequence identity (83%) with CMH158/07 and CMH160/07 strains which were isolated previously from children in 2007 in Chiang Mai. Moreover, One strain of NoV GII/4 variant (CMHA10/08) was most similar (88%) to NoV GII/4 variant strains (CMH150/07, CMH153/07, and CMH155/07) which were isolated from children in Chiang Mai area in 2007.

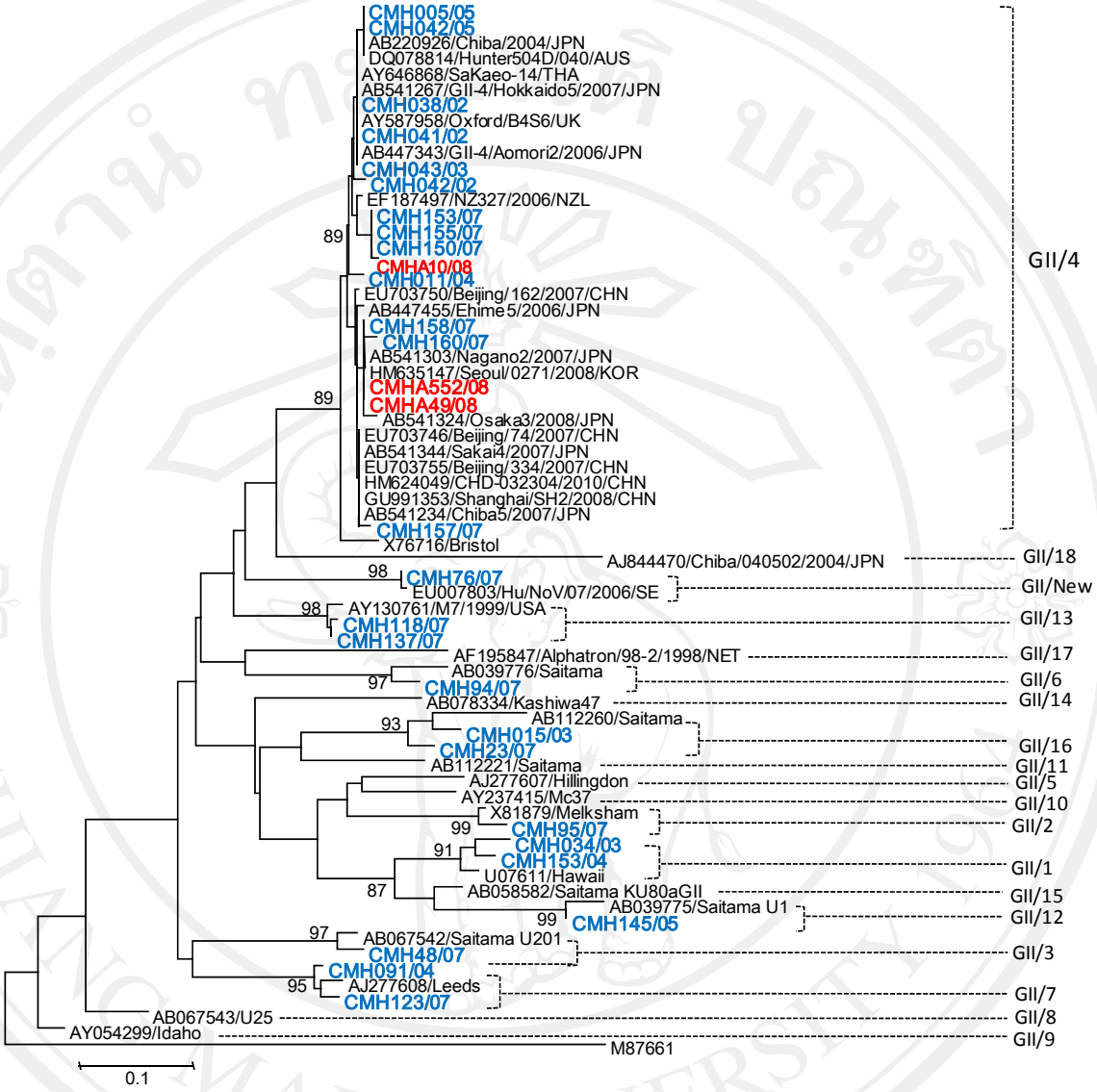


Figure 11 Phylogenetic analysis of the partial nucleotide sequences (387 bp) of capsid gene of noroviruses genogroup II. The GenBank accession numbers of reference strains were indicated proximal to the strain name. NoV GII detected in this study and in previous study in children were indicated in red and blue, respectively. The tree was constructed according to the neighbor-joining method. The number on each branch indicates the bootstrap value.

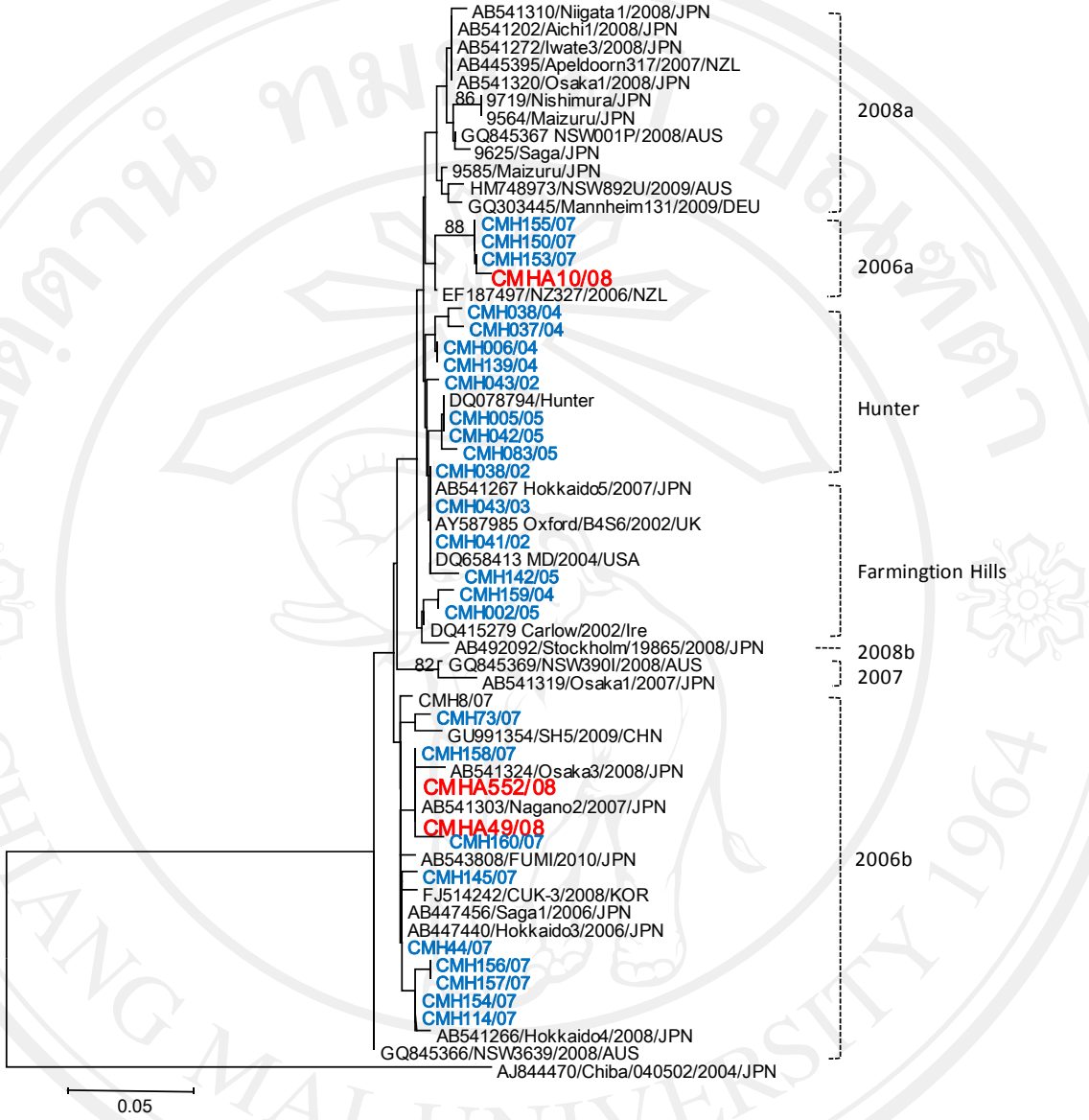


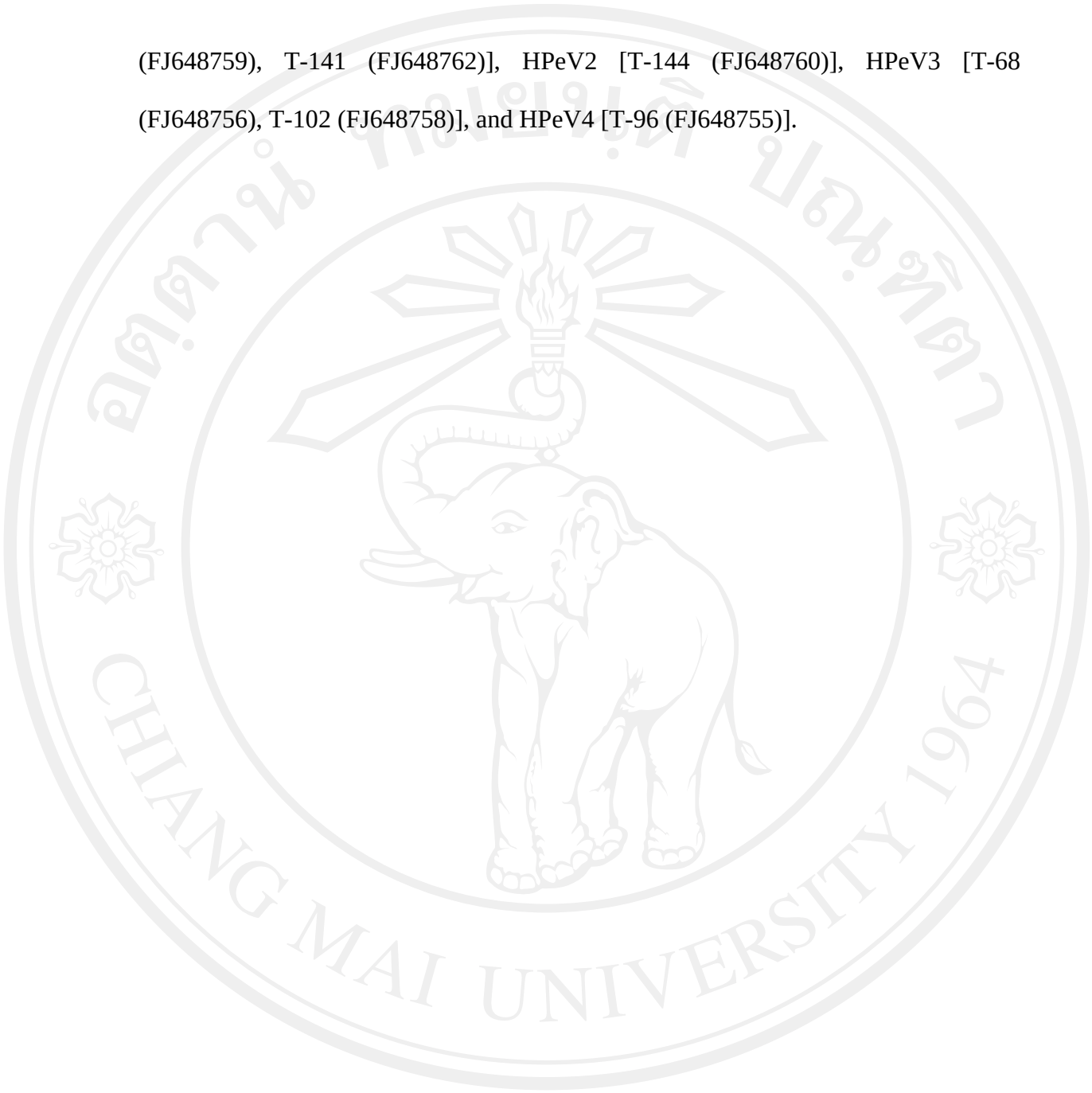
Figure 12 Phylogenetic analysis of the partial nucleotide sequences (387 bp) of capsid gene of noroviruses genogroup II/4 variants. The GenBank accession numbers of reference strains were indicated proximal to the strain name. NoV GII/4 variant detected in this study and previous study in children were indicated in red and blue, respectively. The tree was constructed according to neighbor-joining method. The number on each branch indicates the bootstrap value.

2.5 Analysis of partial capsid gene of human parechovirus

Classification of HPeV into genotype is based on the VP1 capsid gene. Partial VP1 nucleotide sequence of capsid gene sequence was amplified by specific primers which targeted to amplify the 760 bp PCR product of partial VP1 gene. If the PCR product of 760 bp was not amplified, nested-PCR was performed the PCR product of 477 bp. The gel electrophoresis demonstrating the PCR product of 477 bp is shown in Figure 13. The partial VP1 gene of 477 bp was purified and sequenced by using HPeV-VP1-R as a sequencing primer. From BLAST search and ClustalX alignment, 1 strain of HPeV (CMHA49/08) found in the present study was most closely related to T-141 strain isolated previously from a child in Chiang Mai, Thailand in 2005 (FJ648762) at 96% nucleotide sequence identity. When comparing the sequence with other Thai HPeV strains previously isolated in Chiang Mai area in 2005 [T-69 (FJ648761), T-96 (FJ648757), and T-103 (FJ648759)], the nucleotide sequence identity was only 75% although all these strains were HPeV1. Moreover, The CMHA49/08 strain was also similar to HPeV strains reported from Netherland [03-0812 (AB443809), 677008 (FJ373135), K63-94 (GQ183025), 7555312 (FM178558)], Germany [BNI-R21 (EU024634)], and Japan [A10987 (AB112487), JP-8275 (HQ163882)] with the nucleotide sequence identities ranging from 88-90%.

Phylogenetic analysis of partial VP1 gene (Figure 14) confirmed that CMHA49/08 strain was most closely related to T-141 strain (FJ648762) but quite different from the other HPeV strains isolated previously in children in Chiang Mai in 2005. The HPeV strains previously isolated in Chiang Mai were classified into 4 distinct genotypes including HPeV1 [T-69(FJ648761), T-96 (FJ648757), T-103

(FJ648759), T-141 (FJ648762)], HPeV2 [T-144 (FJ648760)], HPeV3 [T-68 (FJ648756), T-102 (FJ648758)], and HPeV4 [T-96 (FJ648755)].



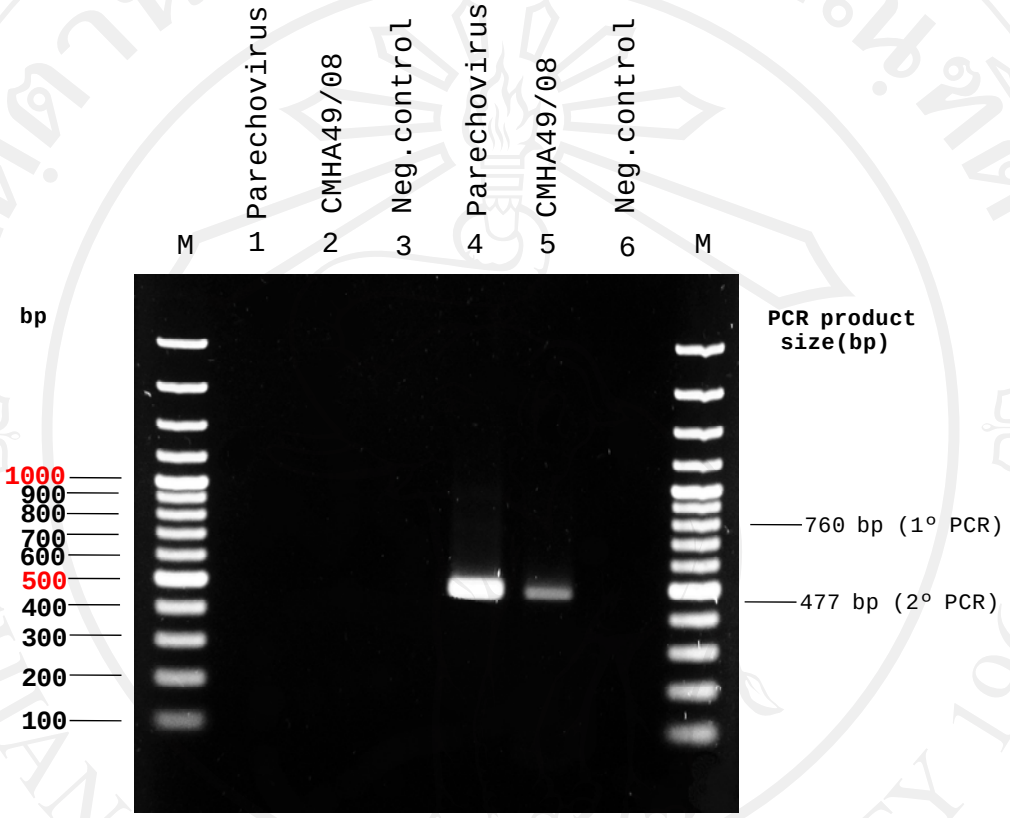


Figure 13 Agarose gel electrophoresis demonstrating the expected PCR product size of human parechoviruses using specific primer for VP1 capsid gene. Lanes 1 and 4, reference strain of HPeV; lanes 2 and 5, HPeV positive (CMHA49/08); lanes 3 and 6, negative controls. Lane M, 100 bp DNA ladder.

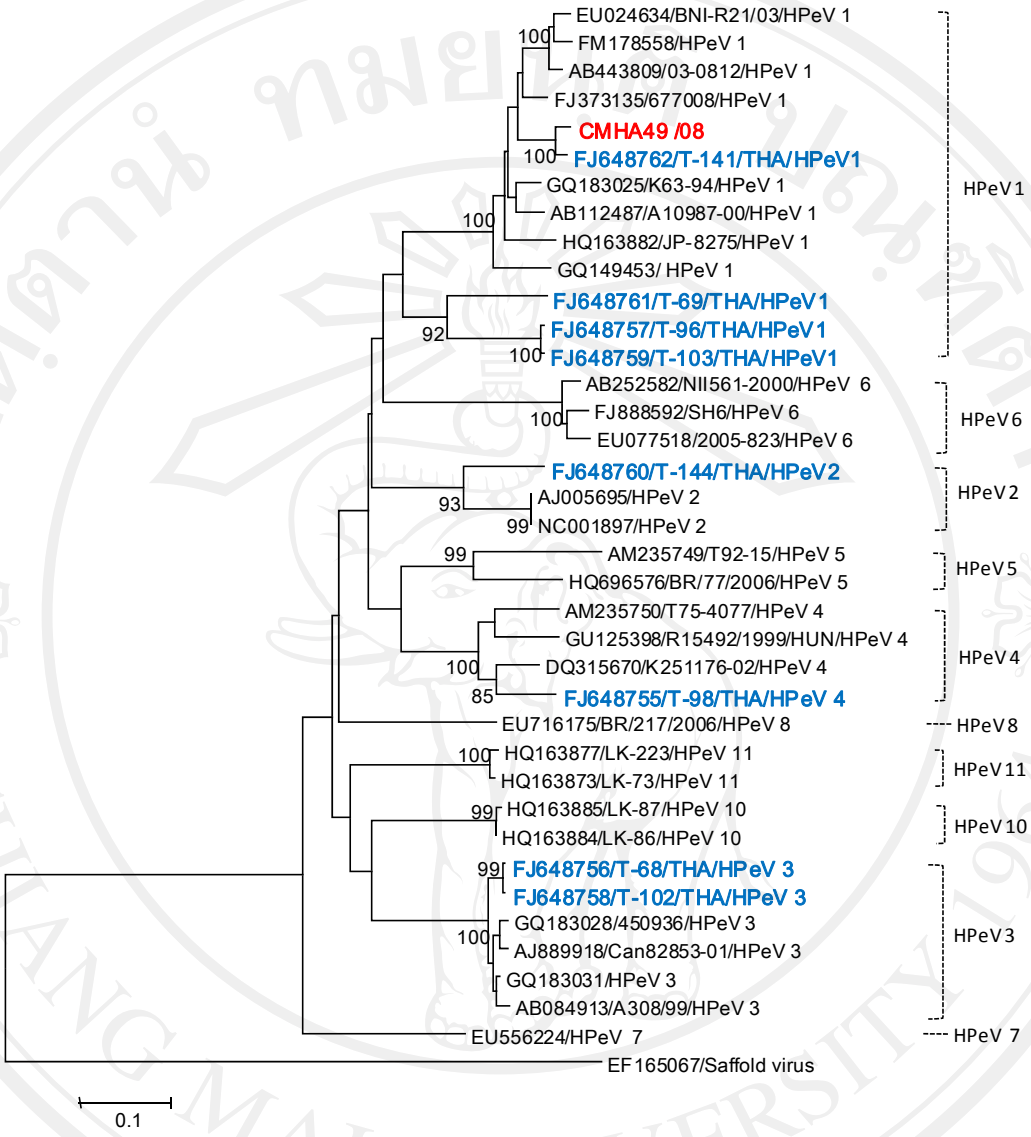


Figure 14 Phylogenetic analysis of the partial nucleotide sequences (477 bp) of capsid gene of human parechoviruses. The GenBank accession numbers of reference strains were indicated proximal to the strain name. HPeV detected in this study and previous studies in children in Chiang Mai were indicated in red and blue, respectively. The tree was constructed according to neighbor-joining method and the number on each branch indicates the bootstrap value.