Prevalence of human enterovirus infection and complete coding sequence analysis of human enterovirus 71 among patients with hand, foot and mouth disease and herpangina in Thailand, 2008-2014

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ความชุกของฮิวแมนเอนเทอโรไวรัสและการวิเคราะห์ลักษณะทางพันธุกรรมของฮิวแมนเอนเทอโรไวรัส 71 ในผู้ป่วยเด็กไทยที่เป็นโรคมือเท้าปากและ herpangina ระหว่างปี 2551- 2557

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จอน เมาฬีกุลไพโรจน์ : ความชุกของฮิวแมนเอนเทอโรไวรัสและการวิเคราะห์ลักษณะทางพันธุกรรมของฮิวแมนเอนเทอโรไวรัส 71 ในผู้ป่วยเด็กไทยที่เป็นโรคมือเท้าปากและ herpangina ระหว่างปี 2551- 2557 (Prevalence of human enterovirus infection and complete coding sequence analysis of human enterovirus 71 among patients with hand, foot and mouth disease and herpangina in Thailand, 2008-2014) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ. ยง ภู่วรวรรณ, 85 หน้า.

โรคมือเท้าปาก (HFMD) เกิดจากการติดเชื้อ Human enterovirus (HEV) ส่งผลกระทบต่อหลายประเทศทั่วโลกโดยเฉพาะประเทศในภูมิภาคเอเชียแปซิฟิก จากการศึกษาที่ผ่านมาแสดงให้เห็นว่าการระบาดของ HEV มักจะติดต่อจากคนสู่คนได้อย่างรวดเร็ว นอกจากนี้ การติดเชื้อของ HEV บางสายพันธุ์เช่น enterovirus 71 (EV71) สามารถทำให้เกิดภาวะแทรกซ้อนทางระบบประสาทได้ งานวิจัยนี้ได้ทำการศึกษาทางระบาดวิทยาเชิงชีวโมเลกุลของเชื้อ HEV ในตัวอย่างผู้ป่วยโรคมือเท้าปากและ herpangina ในประเทศไทยในปี 2008-2013 และวิเคราะห์ข้อมูลวิวัฒนาการของ EV71จากตัวอย่างที่พบในช่วงปี 2012-2014 การตรวจและระบุสายพันธุ์ HEV ในประเทศไทยได้ทำในตัวอย่างทั้งหมด 584 ตัวอย่าง โดยได้นำตัวอย่างเหล่านี้มาตรวจหา EV สายพันธุ์อื่นๆ โดยการตรวจยีน VP1 ด้วยไพรเมอร์ CODEHOP ผลการศึกษาพบตัวอย่างที่มีไวรัสเพิ่มอีก 115 ตัวอย่าง ซึ่งทำให้อัตราการตรวจจับเพิ่มขึ้นเป็น 61% โดยตรวจพบ HEV ในตัวอย่างเหล่านี้ทั้งหมด 4 สายพันธุ์ ได้แก่กลุ่ม A, B, C, และ D จากข้อมูลในช่วงที่ทำการศึกษาพบว่ามีความชุกของ HEV-A มากที่สุด โดยพบมากถึง 93.5% (n = 603) นอกจากนี้พบ HEV-B 5.74% (n = 37), HEV-C 0.930% (n = 6) และ HEV-D 0.155% (n = 1) ช่วงเวลาที่พบการระบาดของ HEV มากที่สุดคือช่วงต้นปี (เดือนมกราคมถึงกุมภาพันธ์) และช่วงหน้าฝน (เดือนมิถุนายนถึงสิงหาคม) จากการวิเคราะห์ลำดับนิวคลีโอไทด์จีโนมของเชื้อ EV71 จำนวน 14 ตัวอย่าง ผลการศึกษาพบว่าตัวอย่างจำนวน 13 ตัวอย่างนั้นถูกจัดอยู่ใน subgenotype B5 และหนึ่งตัวใน C4 โดยมีการเปลี่ยนแปลงทางพันธุกรรมของเชื้อไวรัส HEV 71 ในกลุ่ม B5 อย่างชัดเจนหลังการวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการเชื้อที่ตรวจพบในช่วงการระบาดของโรคมือเท้าปากในปี 2012 นั้นแยกออกจากเชื้อที่พบหลังการระบาด ในการวิเคราะห์ recombination งานวิจัยได้นำลำดับนิวคลีโอไทด์ของจีโนมของเชื้อ EV71 ในประเทศมาเปรียบเทียบกับนิวคลีโอไทด์ของ enterovirus สายพันธุ์ต้นแบบที่อยู่ในตระกูล A โดยผลจาก similarity plot และ bootscan ชี้ให้เห็นว่านิวคลีโอไทด์ของไวรัสจากผู้ป่วย ที่มีอาการหนักและเสียชีวิต (THA_1219) ที่จัดอยู่ใน subgroup C4 มีการเกิด recombination ในบริเวณ P2 และ P3 ของจีโนม โดยมีลักษณะทางพันธุกรรมใกล้เคียงสายพันธุ์ต้นแบบของ coxsackievirus A4, 14 และ 16 มากกว่าสายพันธุ์ต้นแบบของตน ความรู้จากการศึกษานี้เป็นประโยชน์ในการเฝ้าระวังและป้องกันการแพร่ระบาดของโรคมือเท้าปากและเป็นข้อมูลสำคัญในการศึกษาวิวัฒนาการของ EV71 ต่อไป

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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JOHN MAULEEKOONPHAIROJ: Prevalence of human enterovirus infection and complete coding sequence analysis of human enterovirus 71 among patients with hand, foot and mouth disease and herpangina in Thailand, 2008-2014. ADVISOR: PROF. YONG POOVORAWAN, M.D., 85 pp.

Hand, foot, and mouth disease (HFMD) due to human enterovirus (HEV) infections affects multiple countries worldwide especially in the Asia-Pacific region. Previous studies showed an outbreak of HEV are often fast spread and easily transmitted with some enterovirus infection, for example enterovirus 71(EV71), could potentially develop into neurological complication. The study examined prevalence of enterovirus among patients with HFMD and herpangina in Thailand and evolution of EV71 through conducting a complete genome characterization on circulating strains. To detect and identify all circulating HEV in Thailand a subset of 584 from1221 clinical specimens, from 2008 to 2013, which was negative form specific primers amplification were further detected for other enterovirus detection through amplification of partial VP1 gene with CODEHOP primers. One hundred and fifteen clinical specimens were identified resulted in an increase in detection rate to 61%. All 4 species of HEV, A, B, C, and D, were detected and result show highest prevalence of HEV in HEV-A of 93.5% (n=603) follow by 5.74% (n=37) in HEV-B, 0.930% (n=6) in HEV-C and 0.155% (n=1) in HEV-D. More HEV were detected at the begining of the year, January and Febuary, and during rainy season, June - August. The complete genomic sequences of 14 EV71 strains isolated from children with hand, foot and mouth disease in Thailand from 2012 to 2014 were determined and compared to enterovirus group A prototypes. Phylogenetic analysis revealed that 13 strains resembled B5 subgroup, while one strain from a fatal case designated THA_1219 belonged to C4 subgroup. The phygenetic analysis demonstrates distinct separation of B5 subgenotype between strains detected during and post-HFMD outbreak. Similarity plot and bootscan analyses suggested that THA_1219 underwent recombination in the P2 and P3 regions where such region are closer related to coxsackievirus A4, 14 and 16 than EV71 prototype strain. The study reports first complete enterovirus prevalence in Thailand and demonstrate evolution dynamics of the EV71 strain from fatal patient.

Field of Study: Medical Science Student's Signature .....

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ABBREVIATION

BLAST	Basic Local Alignment Search Tool
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
CODEHOP	Concensus Degenerate Oligonucleotide Primer
CV-A	Coxsackievirus A
CV-B	Coxsackievirus B
EV71	Enterovirus 71
GAPDH	Glyceraldehyde 3-phosphate dehydrogease
HA	Herpangina
HEV (A-D)	Human Enterovirus Species (A-D)
HFMD	Hand, Foot, and Mouth Disease
NJ	Neighbor Joining Method
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
rpm	round per minute
Tm	Melting temperature
VP (1-4)	Viral capsid protein (1-4)
3'UTR	3'untranslated region
5'UTR	5'untranslated region

CHAPTER 1

Introduction

Throughout Asia-Pacific region hand, foot, and mouth disease (HFMD) is one of the major public health issues affecting children with multiple outbreaks recorded and enteroviruses showed to be a common causative agent (2). Recent outbreak in Thailand occurred in 2012 with 3-fold increase in incidence rate in comparison to an average incidence from 2007 to 2011 (3). With no effective antiviral treatment and vaccine, surveillance system demonstrated to be the most promising method in reduced the spread of the virus, by early intervention, and is enforced in multiple countries (4, 5). Though enterovirus 71 (EV71) and coxsackievirus A16 (CV-A16) was reported to be a major cause of an outbreak in Asia pacific region, many other enteroviruses can cause HFMD as well with increasing numbers of coxsackievirus A6 (CV-A6) and A 10 (CV-A10) pandemic in recent years (3, 6), therefore an effective surveillance system must have the ability to detect and identify all enterovirus serotypes. With current surveillance system in Thailand only detect limited serotypes (3, 7, 8), it is crucial in clinical management and disease control to establish a virological surveillance which are able to detect all enteroviruses.

One of the major drives of enteroviruses evolution showed to be it ability to extensively mutate and recombinant creating virus with new pathogenic allowing potential for highly virulent strain to emerge (12). Recombination between EV-71 and

other human enterovirus A species has been widely reported in different countries, especially where co-circulation of multiple strain of viruses were found.

The most common cases of HFMD worldwide showed association with EV71 and CV-A16 infection, however, HFMD cases due to infection of CV-A16 and other enteroviruses reported in a lower incidence of severe complication when compare to EV71 (9, 10). Most EV71 pandemics often associated with fatalities as virus has potential to infect the central nervous system (CNS) resulted in severe neurologic disease therefore attention is required for an extensive research (11). The complete genome sequencing would give valuable information in the study of genetic basis of EV71 strains circulating in Thailand. The data from this study would play an important role in evolutional study of the virus to further use in prevention, control and development of EV71 vaccine.

CHAPTER 2

Literature review

HFMD or Herpangina (HA) are widely associated and is an important diagnostic indicator of enterovirus infection (11). In Thailand, HFMD was commonly found in children who were 5 years old or younger but were generally self-limited (7). HFMD is described in children present with brief febrile illness with papulovesicular rashes on the plams and soles and multiple oral ulcers while HA is characterized in children with febrile illness and multiple oral ulcers that predominantly located in the anterior pharyngeal folds, uvula, tonsils and soft palate (13). The infection of enteroviruses in the central nervous system (CNS) can cause severe complication resulted in acute flaccid paralysis, aseptic meningitis or encephalitis etc. (14).

Enterovirus is a single-stranded, positive-sense RNA virus with the genome approximately 7.4 kb from Picornaviridae family (15). The viral RNA is capsulated in a small, non-enveloped, icosahedral particle as show in figure 1a. Viral capsid contains 60 identical subunits with each subunit assembled from four structural proteins (VP1-4); The VP1, VP2, and VP3 proteins are exposed to the external environment including host antibody while VP4 are completely internalized (16). All structural proteins are located in the P1 region of the genome with non-structural proteins located in P2 and P3 region with containing 2A-C genes and 3A-D genes, respectively as show in figure 1b.

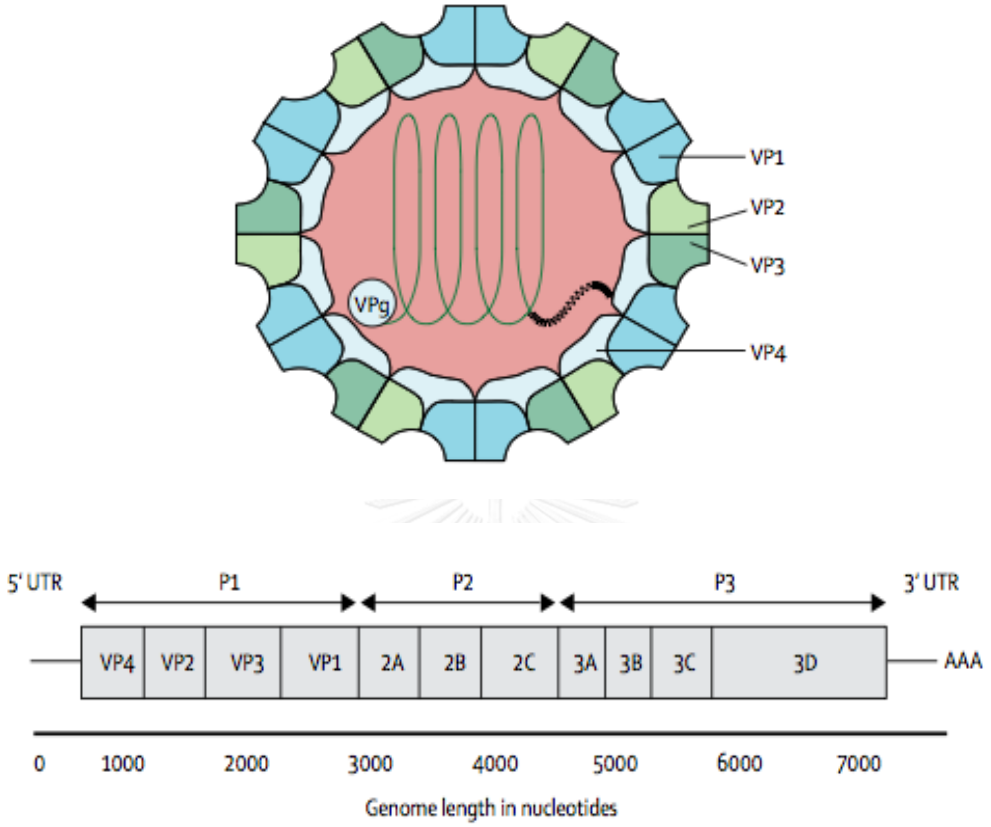


Figure 1. *(a) Structure and (b) Genome structure of Enterovirus 71 (2)*

The summary of piconaviruses' life cycle is represented in figure 2. The viral entry into host cells occur upon binding of the virus to a specific receptors on cell membrane, which resulted in cell membrane pore formation in the cell membrane, allowing entry of the virus into host cell. After virus entry, it undergoes uncoating result in a release of the viral genome into the cytoplasm. The positive-sense viral RNA then act as an mRNA and uses host cellular protein synthesis machinery. The viral proteases cleave polypeptides translated from viral RNA into 11 structural and non-structural proteins. Non-structural proteins interact with the viral RNA, other nonstructural proteins, and host cell components; viral protease 2A shutdown host proteins synthesis, viral

polypeptide 3C interacts with the clover-leaf structure in the 5' UTR and play roles in viral replication initiation while the replications are driven by viral 3D RNA polymerase. The polymerase misincorporates nucleotides during the copying event making virus undergoes rapid mutation in the host cell. RNA and viral capsid then assembled and packaged into virus particle in the cytoplasm and release once infected cell is lysed (1)

Due to the absence of lipid bilayer envelop, human enteroviruses are highly stable in host environment and able to survive when exposed to human gastric acid, organic solvent, alcohol, freezing and can also survive at room temperature for several days (2), Virus can spread through contact with contaminated vesicular fluid, oral secretions, surfaces and respiratory droplets with viral transmission occurs predominantly via the faeco-oral route (17). Without effective antiviral treatment or vaccine, disrupting person-to-person viral transmission and contact with contaminated area, including closing school, childcare or public function involving children, are the most efficient way to control outbreak, in order to do so, effective surveillance must be established.

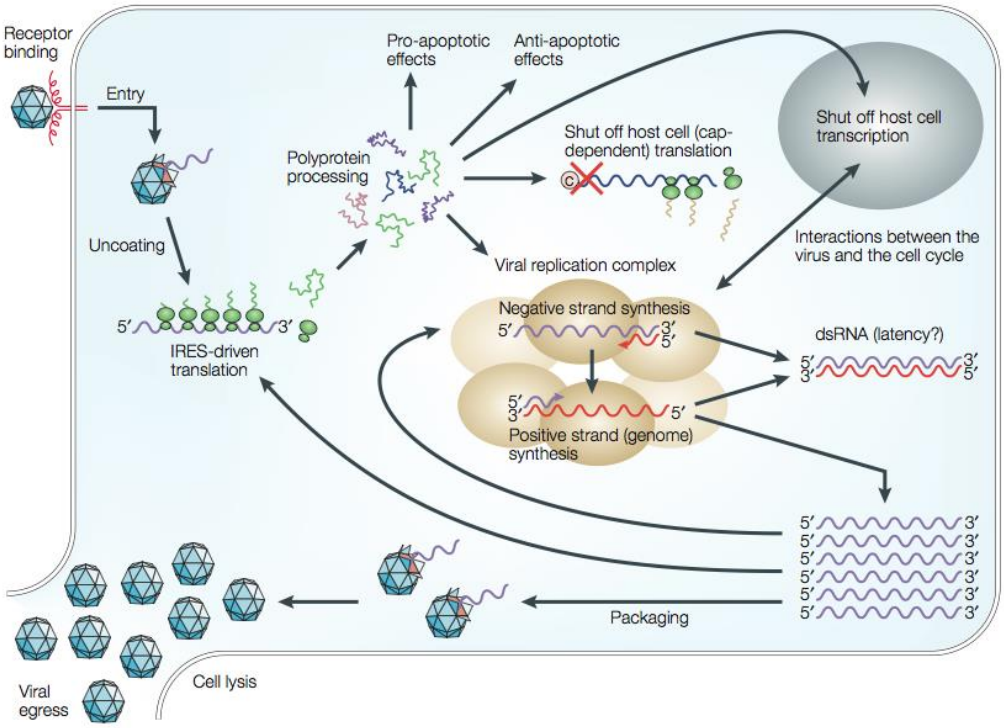


Figure 2: Summary of piconavirus life cycle (1).

There are over 100 serotypes of Human enterovirus, enteroviruses mutate and evolve rapidly therefore surveillance system must be able to detect all of these serotypes in order to monitor transmission, spread and evolution of these viruses. The traditionally enterovirus infections are detected and serotyped by virus isolation in cell culture and neutralization using reference antisera, however this is time consuming, as many enteroviruses do not grow readily in cell culture, labour intensive, as multiple cell lines are required in order to detect all enteroviruses, and unable to detect new serotypes before antisera are available (18), therefore the method is unsuitable in clinical environment where detection has to be rapid for both clinical management and disease control. The application of molecular diagnostic has the advantage of speed over virus isolation and serotyping. Due to it high sensitive, it can be performed directly

on original clinical specimen and able to detect even when small amount of specimen presence, i.e. in the spinal fluid (19). The molecular diagnostic test target to amplify 5'-untranslated region, which is highly conserve site among enteroviruses (20), however serotyping using 5'-untranslated region is more likely to give false serotyping as it is highly conserve and can only be characterized into two cluster (21). Furthermore, recombination has been reported in 5' UTR (22). Serotyping of enteroviruses is defined by their viral capsid proteins property, VP1 is the major external surface accessible protein of the picornaviruses' virion, which, contribute to more neutralization sites when compare with VP2 and VP3 (23). VP1 gene sequences contain serotype-specific information and showed through phylogenetic analysis to be highly correlated to serotyping with neutralization tests (24); therefore it would be a suitable target to be amplified and sequenced for detection and characterization of enteroviruses.

Multiple methods use VP1 region to detect and serotype enterovirus has been published but mostly only target a subset of enterovirus serotype as increase specificity by highly degenerate primer would reduce sensitivity and excess nonspecific amplification will outcompete virus making it hard to identify. Consensus degenerate hybrid oligonucleotide primer (CODEHOP) is a reverse transcription-seminested PCR technique that was claimed to detect and identify all enterovirus serotypes from original clinical specimens (25). Amplification using CODEHOP were designed to facilitate sequencing of a fragment of VP1 gene, which contain enough information for virus characterization. To encounter the nonspecific amplification problem, the CODEHOP consists of two regions Degenerate Core and Consensus clamp. The degenerate core region uses inosine to make the region highly degenerative, designed from conserved motif when amino acid sequences are aligned, allowing board target specificity while consensus clamp are non-degenerate driven the hybridization of primer to template,

therefore increases the stability allowing higher annealing temperature to be use and in turn diminish nonspecific amplification (26). CODEHOP had been reported to performed better in comparison to other methods and able to effectively detects and serotype enteroviruses in clinical setting (22).

The surveillance of EV71-associated with HFMD in Thailand was first reported internationally in 2011; the study detected stored clinical specimens from 2008-2011(8), and followed by an epidemiology and seroepidemiology reported in 2014 (27). Based on phylogenetic analysis of partial VP1 sequence, multiple subgenotypes of EV71 were reported in 2008 however there were a shifted resulted in predominantly only B5 subgenotype by 2013(27). Various genotypes detected in 2008 of 14 cases includes C4, C1, C2 and B5 deduced to only C4 and B5 in 2009 of 10 EV71 cases and only B5 in 2010, 2011, and 2013 with 2, 11, and 15 cases were identify, respectively. The outbreak of HFMD resulted in an increased in cases in 2012 , especially from June to August of that year, and even though coxsackievirus A6 was reported as a causative agent of this outbreak, 58 cases of EV71 were identified. Of all 58 cases detected in 2012, only 2 were phylogenetically categorized as subgenotype C4 while the rest belong to subgenotype B5. These data show a continuous circulation of B5 in Thailand. EV71 B5 had been a cause for large outbreak in multiple countries since 1997 including Malaysia, Brunei, Singapore, and Taiwan. C4 that was found co-circulating with B5 in 2008, 2009 and 2012 was reported to be the cause of a large-scale outbreak in China since 1998 with a continuing outbreak with higher mobility and mortality year by year (28, 29). The C4a EV71 is also often associated with neurovirulence where large outbreak resulted in fatal neurological complication creating big concern in public health especially in Mainland China (30, 31).

Through rapid evolution, viruses are able to create genetic diversity to benefit it spread in human population and recombination were found to be method convenient to virus in facilitating the viral fitness including improved virulence of the virus. The recombination of enteroviruses is presumably occur through copy choice mechanism where viral RNA-dependent RNA polymerase initiated replication on one viral RNA

before switch template and continues to replicate on the other viral RNA molecule (32). The recombination can be expected between EV71 and other virus include CV-A16 and -A8 as previously reported by Chan and AbuBaker (33). Since, the recombination of EV71 in a structural region may create a less fit variant as virus capsid gene contain crucial genetic information required viral recognition to host cell receptor, recombination in the non-structural genes were found to be a hotspot in generating gene diversity.

Numerous studies on enteroviruses focused on the structural region where it can be use in determination of the virus serotype while the rest of the genome remain uncertain. To investigate molecular characteristic of currently circulating EV71 in Thailand, this study performed 14 complete genome sequencing from 2012 HFMD outbreak till 2014. The complete genome sequences include EV71 derived from clinical specimen during and after the HFMD outbreak and clinical specimen of patient with mild and severe EV71 infection and sequence were further subjected for recombination analysis, which was suggested to be a possible driving force of a virus to increase fitness.

CHAPTER 3

Experiment

3.1 Prevalence of human enterovirus infection

Research methodology

Population Study

Clinical specimens of pediatric patients who were suspected with HFMD and HA which were collected from 2008 till 2013 were use in this study. Patients who were suspected for HFMD were presented with painful blisters in the oropharynx and blister on the plams, soles, knees, elbow, and/or buttocks while patients who were suspected for HA were present with painful blisters in the mouth only, predominantly on the soft plate.

Sample size

The calculation of sample size is based on information from the database where infectious rate of enterovirus in Asia is on average around 58.3%.

Calculation: $n = \frac{Z^2 pq}{d^2}$

Where: Z is the z-score using confidence interval of 95% = 1.96

p is infectious rate of enterovirus

q = 1-p

d is the desired margin of error = 5%

$$n = \frac{(1.96)2 * (0.583) * (1-0.583)}{((5/100) * 0.583)^2} = 1099.1$$

Therefore, the study required at least 1100 samples of Thai population.

Sample collection

The specimens included stool, rectal swab, throat swab and vesicle fluid spinal fluid. These specimens were collected through suspension with 3 ml of 0.1% PBS, divided into aliquots.

RNA extraction

The Viral Nucleic acid extraction kit (RBC Bioscience, Taipei, Taiwan) were used for extraction of RNA. 400 µl VB lysis buffer were added to 200 µl of clinical specimen and incubated at room temperature for 10 minutes. Additions of 500 µl of 95% ethanol were added to the lysate. The mixtures were vortex. VB filter Columns were placed in 2 ml collection tube and 1100 µl of the lysate mixtures were transferred into the columns. The columns were centrifuged at maximum speed for 1 minute and the filtrates were discarded. Two washes were performed by centrifugation at maximum speed for 30 seconds each with 600 µl of W1 wash buffer. The column matrixs were centrifuged to dry for 3 minutes and nucleic acids were eluted into 1.5 ml microcentrifuge tube with 50 µl of RNase-free water.

Determination of serotype

Synthesis of cDNA by Reverse Transcription

The reverse transcription used specific primers, AN32, AN33, AN34, and AN35, from Nix et al. (25) and reversed used ImProm-II™ Reverse Transcription System (Promega, Madison, WI). The primers designed downstream from all reverse primers that were used for the amplification of VP1 sequence in PCR,

The reaction mixture were prepared accorded to following:

- 1X of Improm™ II Reaction buffer
- 1µl of Improm™ II Reverse Transcriptase
- 0.5 mM of dNTP mix
- 3 mM of $MgCl_2$
- 1U/µl of RNase inhibitor

Mixtures were made up to the final concentration of 15 µl per reaction

5.0 µl of RNA template and cDNA primers (20 pmol of gene-specific primers; AN32, AN33, AN34, and AN35) were added to a 1.5 ml microcentrifuge tube, denatured at 70°C for 5 minutes and chilled on ice. The reaction mixture, contained nuclease-free water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor, was added to the combination of RNA template and cDNA primers on ice made up the final volume of 20 µl. The mixture was incubated at room temperature for 5 minutes, at 42°C for 2 hours and inactivation of reverse transcriptase at 70°C for 15 mins

Confirmation of RNA extraction

the success of RNA extraction was determined with detection of universal housekeeping gene, GAPDH. The detail of GAPDH primers are represented in Table 2. Gel electrophoresis was performed for visual analysis. The PCR products were run on 2% agarose gel with 100 bp DNA Ladder as a marker to separate the products by sizes before staining with Ethidium bromide. The band should appear at ~500 bp marker under UV transilluminator.

Gene amplification through PCR

The study followed method described by Nix et al. (25), where detail of primers is show in table 2. The cDNA synthesized were used as template for amplification of VP1 gene with reaction mixture followed table 1. The semi-nested PCR used primers 222 and 224 for PCR 1 and primers AN88 and AN89 for PCR 2 followed conditions describe in table 3.

Table 1: *Reaction Composition for conducting PCR*

Reagent	Concentration
2 x Perfect*Taq* Plus MasterMix	1.25 units DNA Polymerase
	1x PCR buffer
	200 μM of each dNTP
10x PerfectLoad Dye Concentrate	1x PerfectLoad Dye Concentrate
Forward primer	0.24 μM
Reverse primer	0.24 μM
cDNA Template	1.5
Total volume	12.5 μl

Table 2: *Primers use for detection of GAPDH gene, cDNA synthesis and PCR amplification for detection of enterovirues, Primer AN_32, AN_33, AN_34, AN_35, 222, 224, AN_89 and AN_88 are primer from Nix et al. (25).*

Primer name	Sequence (5' to 3')	Genes
GAPDH_F	GTGAAGGTCGGAGTCAACGG	GAPDH
GAPDH_R	GTTGTCATGGATGACCTTGGC	GAPDH
AN_32	GTYTGCCA	VP1
AN_33	GAYTGCCA	VP1
AN_34	CCRTCRTA	VP1
AN_35	RCTYTGCCA	VP1
224	GCIATGYTIGGIACICAYRT	VP3
222	CICCIGGIGGIAYRWACAT	VP1
AN_89	CCAGCACTGACAGCAGYNGARAYNGG	VP1
AN_88	TACTGGACCACCTGGNGGNAYRWACAT	VP1

Table 3: *Condition for performing PCR for detection of GAPDH gene and semi-nested PCR use in detection of enteroviruses*

PCR reaction	Duration	Temperature			Cycle(s)
		GAPDH (min.)	CODEHOP (min.)		
			1st PCR	2nd PCR	
Initial denaturation	3.00	94ºC	94ºC	94ºC	1
Denaturation	0.50	94ºC	94ºC	94ºC	
Annealing	0.75	55ºC	43ºC	52ºC	40
Extention	1.50	72ºC	72ºC	72ºC	
Final Extention	10.0	72ºC	72ºC	72ºC	1
	5.00	25ºC	25ºC	25ºC	1

Gel Electrophoresis

The PCR products were run on 2% agarose gel with 100 bp DNA Ladder as a marker. The gels were stained with ethidium bromide and visualized under UV transilluminator. Due to differences in length of VP1 genes in different HEV serotypes, the PCR product size varied from 350-400 bp.

Gel Purification and nucleotide sequencing

Expin™ for purify DNA fragment kits (GeneAll, Korea) were used for gel purification. The kit recovered DNA fragments with silica membrane that bind to DNA at high salt condition and washed impurities with buffer contained ethanol. DNA were released from membrane with low ionic strength buffer.

The PCR products bands of VP1 gene were excised from the gel and dissolved in GB buffer at 50 °C. The solution were transfer to SV column, contained silica membrane, and centrifuged* for 1 minute to remove impurities. The column was washed with 700 µl of NW buffer which contained ethanol to remove impurities and unwanted oligos. The column was further centrifuged* for another 1 minute to remove any residual ethanol that could interfere with further application. The DNA was eluted with low salt and weakly alkaline EB buffer. EB buffer were pipetted to the center of the membrane and incubated for 1 minute for efficient elution before centrifuge*.

*all centrifuge were performed at 30,000 rpm

Gel electrophoresis was performed on purified product as a confirmation. Purified DNAs were sequenced at First Base laboratories SDN BHD with Sanger method.

Sequence analysis

Sequences were edited through Seqman program as a part of DNASTAR Software (v5.0). Multiple sequence alignments with other strains were performed using CLUSTAL W. Different partial VP1 sequences were analyzed and nucleotide identities were calculated with BioEdit Sequence Alignment Editor package (v7.0.9.0). The phylogenetic trees were constructed with the neighbor-joining (NJ) method and Kimura's two-parameter distance model. The 1,000 random sampling of bootstrap analyze was use to estimate the phylogenetic tree strength with strong tree topology supported by ≥ 70% bootstrap value. Pair-wise deletions was implemented for missing data, all was achieved on MEGA software package (v5.0)

Results

Detection of HEV with CODEHOP primers

The study performed enterovirus detection on 584 clinical specimens, a subset of 1221 clinical specimens of HFMD and HA confirmed or suspected cases. The specimens were preiviously examined for EV71, CV-A16, CV-A6, CV-A8, CV-A10, pan-enterovirus and were reported as untypable enterovirus infection or negative. 115 out of 584 clinical specimens were tested positive for enterovirus with CODEHOP technique where 61 specimens were previously reported as panEV and 47 were reported negative. Among 115 specimens identified with CODEHOP technique, we identified 26 different serotypes all belong to HEV. Within these serotypes, at least one member of each specie from all 4 species of the HEV were detected. Within HEV species A; CV-A2, -A4-6, -A8, -A10, -A12, -A16, and EV71 were detected, HEV species B; CV-A9, CV-B1- B5, E-11, E-6, E-16, E-18, E25 were detected, HEV species C; CV-A21, poliovirus 1, poliovirus 2, rhinovirus A40, rhinovirus A57 were detected and HEV species D; enterovirus 68 were detected. The summarized data is demonstrate in figure 3. With partial VP1 sequences, HEV can be phylogenetically seperated into 4 species as show in figure 4. Enterovirus species A was reported highest prevelance among patients diagnosed with HFMD and HA of all together 71 specimens (61.7 % detected in this study) follow by enterovirus species B with 37 specimens (32.2%), species C of 6 specimens (5.22%) and species D of 1 species (0.870%).

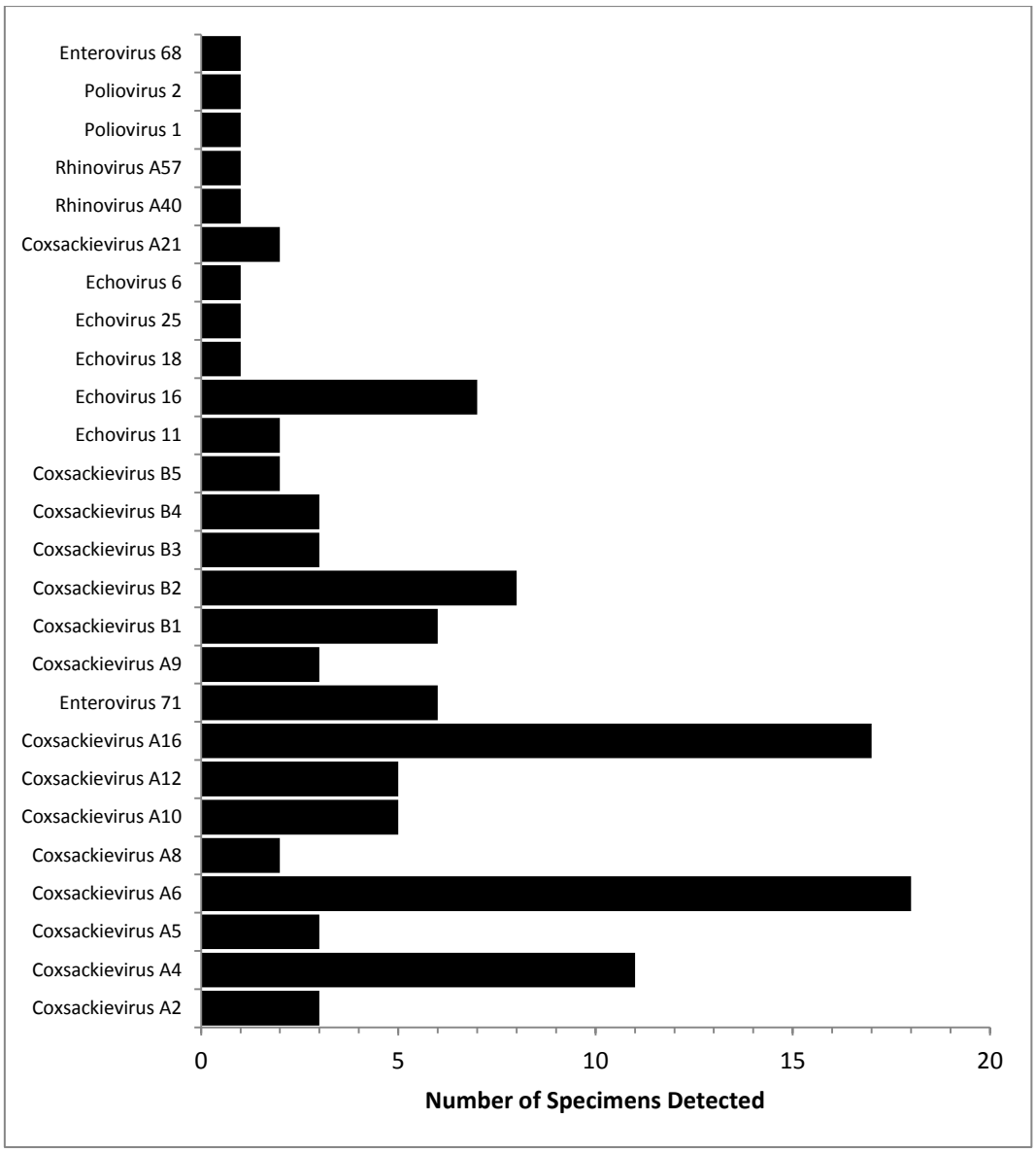


Figure 3: Column graph representing verious serotype of enterovirus detected using CODEHOP technique.

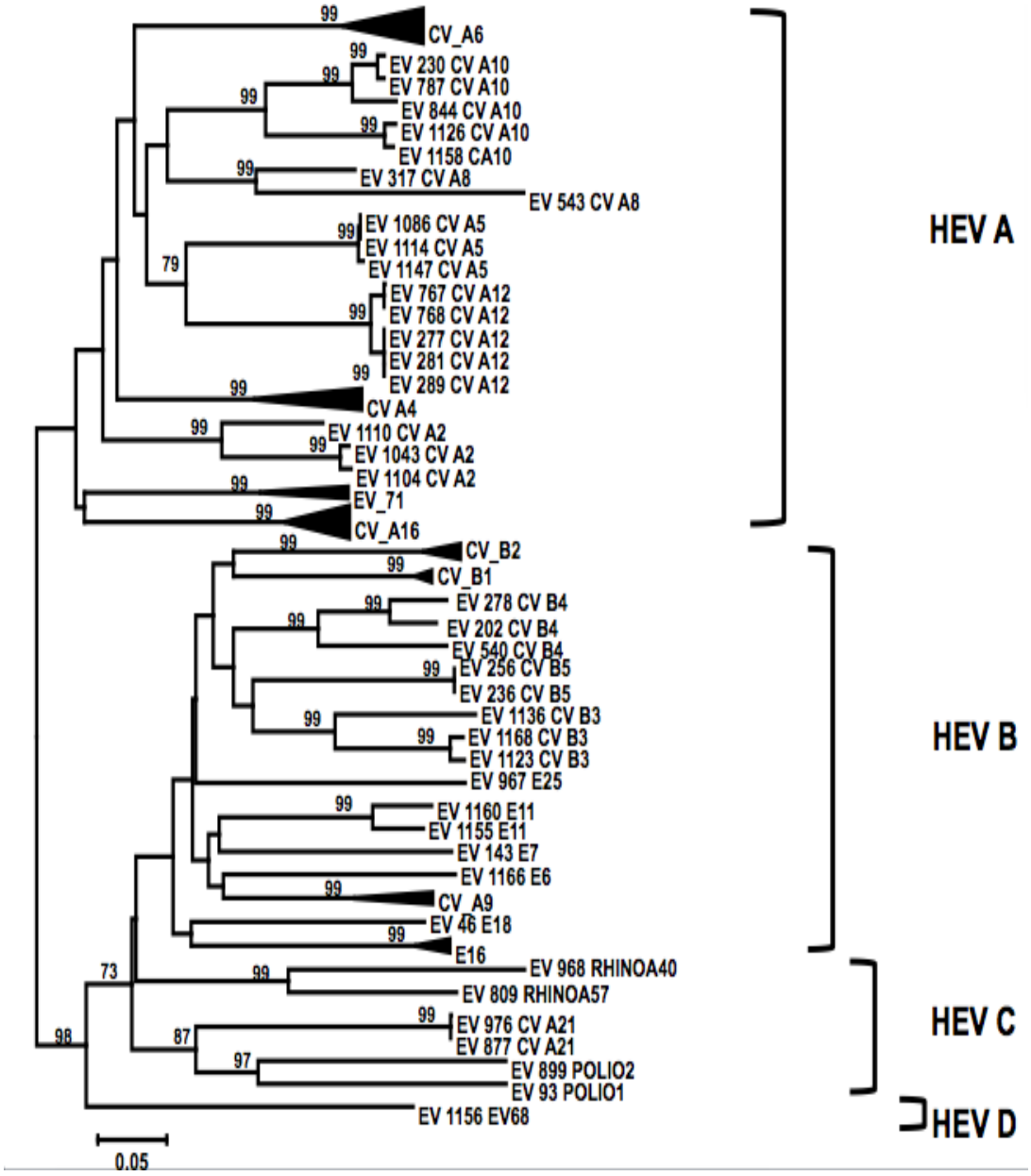


Figure 4: *Phylogenetic tree representing HEV detected and identified from negative and untyped specimens. The partial VP1 sequences were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.*

Nucleotides identities

Based on partial VP1 sequence, the nucleotides identities range from 34.1-100% among all enterovirus detected. Within HEV-A, HEV-B and HEV-C the nucleotides identities range increased to 52.3-100% ,54.3-100% and 41.3-99.5%, respectively. When multiple isolates were detected, the nucleotide identities of each serotypes ranges from 80.4-100% within each serotype. High nucleotides identities with minimum range over 90.0% was noted among CV-A5, A9, A12, B1, B2, B5, echovirus 11 and 16 while lower nucleotides identities within serotype were noted in CV-A2, A4, A10, B3 and B4. The phylogenetic relationship was examined between isolates within the same serotypes with low nucleotide identities. The Phylogenetic tree of isolates with the reference strains obtain from Genbank was constructed as show in figure A1. The phylogenetic tree showed multiple isolates of CV-A2, A4, A10, B3 and B4 were from different lineages.

Undetected specimens

Throughout the period of study, we detected a total of 47 specimens serotyped as EV71, CA16, CV-A6, CV-A8 and CV-A10 which were neglected when detected with specific primers. Out of these specimens CV-A6 was detected highest follow by CV-A16, EV71, CV-A10 and CV-A8 which account for 5.81%, 14.2%, 5.13%, 31.1% and 3.92% from all positive specimens detected of each serotype respectively. Of these specimens, 22 specimens (46.8%) were not detected by specific primers but positive for pan-enterovirus.

Prevalence of HEV

When combined collected data from CODEHOP primers with previously collected data, the data show exceptionally high prevalence of HEV-A followed by B, C and D. Among all 647 identified specimens; 93.5% (n=603) were grouped into HEV-A, 5.74% (n=37) were in HEV-B, 0.930% (n=6) were HEV-C and 0.155% (n=1) were in HEV-D. Within these population, 67.8% (n=437) were diagnosed with HFMD while 13.8% (n=89) were diagnosed with HA and the remaining 18.4% (n=119) of clinical diagnosis data were incomplete.

During the period of study, from 2008 to 2013, the graph, show in figure 5, demonstrate an outbreak in 2012 with 16-folds increased of positive specimens when compared to number of positive specimens detected from 2008 to 2011. From 2008 to 2011 an average of 32 specimens were detected each year ranging from 30 to 33 or 3 specimens per months. The same consistancy continue at the beginning of 2012 but a dramatic increase was reported in June, July and August of that year. The number drop in 2013 but ramain relatively high incompare to specimens from 2008 to 2011, with 48 positive specimens detected that year. The positive specimens were either from Bangkok or Khon Kean.

The correlation between diversity of enteroviruse detected and the number of positive specimens were reported in the study. From 2008 to beginning of 2012, maximun of 3 different enterovirus serotypes were detected each months, with majority of the viruses detected were EV71, CV-A16 and CV-A6. Highest diversity of enteroviruses were reported during the outbreak with 11 and 12 different enteroviruses reported in June and July, respectively. During the rainy season, June, July and August, the diversity of enterovirus detected show to be higher incomparison to the rest of the year for 2010, 2011, 2012 and 2013. During the outbreak in 2012, highest number of specimens were reported in July with with greatest diversity in that paticular month of 12 enterovirus serotypes detected. The majority serotype detected in July were CV-A6 of 49.0%. Other viruses detected included EV71 and CV-A8 both account for 11.5% and CA16, which consist of 9.5%. The remaining 6.9% are other serotypes included CV-A4, CV-A5, CV-A9, CV-A10, CV-A12, CV-B1, CV-B2 and CV-B4.

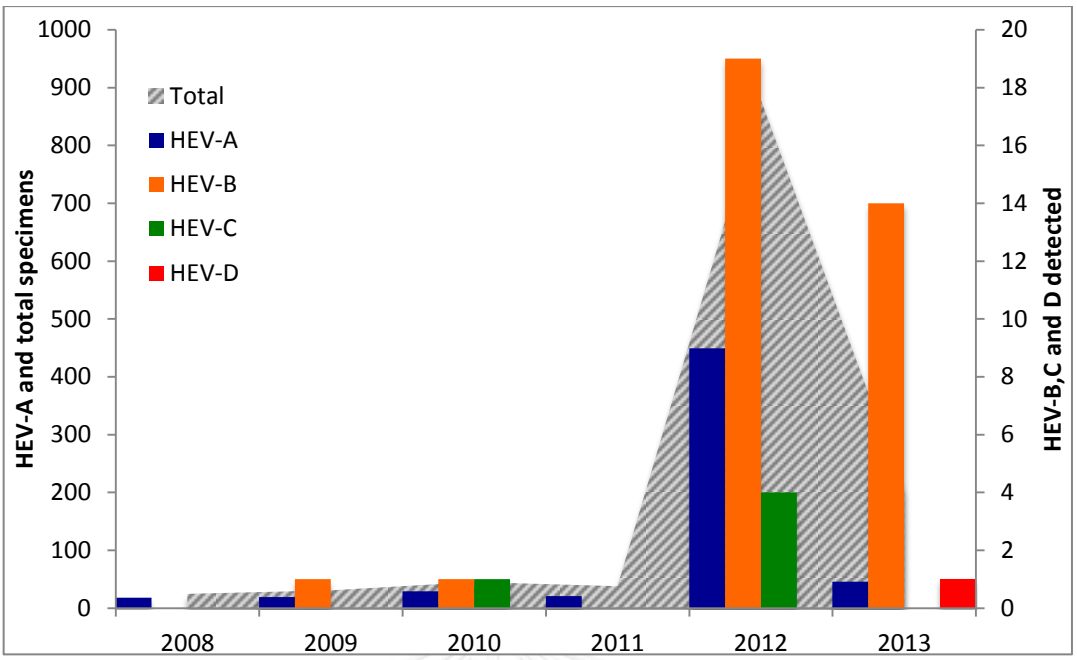


Figure 5: Prevalence of HEV according to species separated by year from 2008-2013

Seasonal distribution

During the 6 years period of study an increased in number of enterovirus detection were demonstrated during rainy season, coresponded to June to September, as show in figure 6. In most years, number of enterovirus detection start increasing in June except for 2013 where specimens increases in July. High detection rate usually occupied a month or two except for 2012 and 2013 where high detection rate extended for over three months. In addition to rainy season high detection of enterovirus was also exhibited during winter espeacially in January and Febuary in 2010 and 2013.

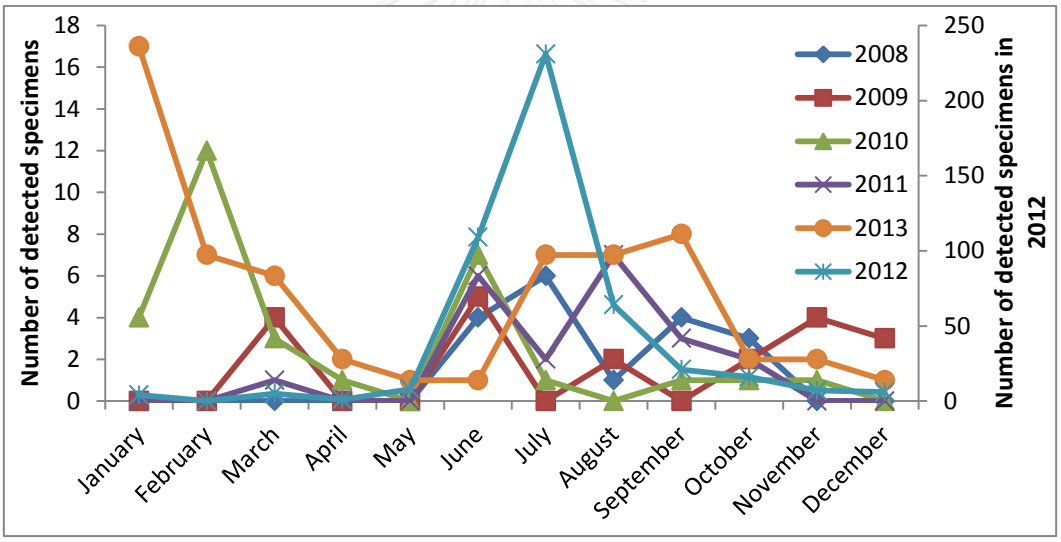


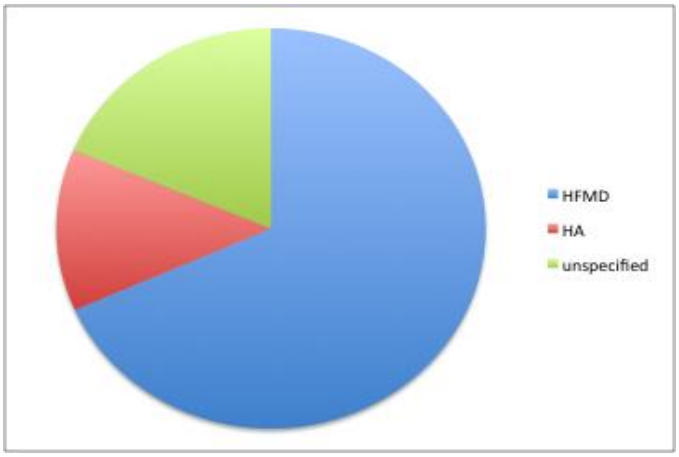
Figure 6: Seasonal distribution of detected HEV from 2008 - 2013

HEV-A distribution

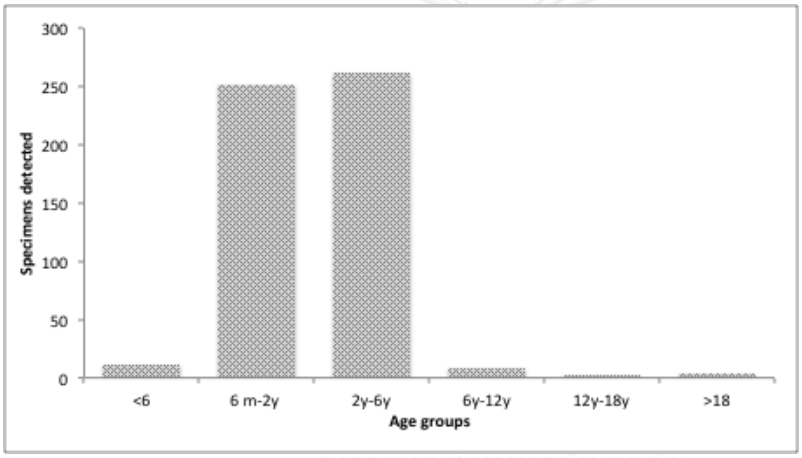
Throughout 6 years surveillence, HEV-A was highest circulating species among all enterovirus in Thailand of 93.5% (n=603). Three major circulating serotypes of HEV-A are CV-A6, EV71, and CV-A16 which contributed to 42.3% (n=273), 17.2% (n=111) and 17.2% (n=111), respectively and all three serotypes was prevalent since 2008. The prevalence of other members of HEV-A species was not detected untill 2012 except for CV-A10 which was first detected in 2009 in this study. High detection of CV-A8 and CV-A10 was noted in the last two years of study of 7.75% (n=50) and 2.33% (n=15), respectively while other, CV-A2, -A4, -A5 and -A12, still remain at low prevalence. Partial VP1 gene sequence were used in construction of a phylogenetic tree show in figure 8.

Among all patients detected with HEV-A enrolled in this study, 68% (n=412) were diagnosed with HFMD, 13% (n=79) were diagnosed with HA and the remaining 19%(n=112) were unspecified, show in figure 7(A). The age distribution of HEV-A infected patients are high within 6 months to 2 years old and 2 to 6 years old when conbining two groups result reflect 94.8% (n=514) of the infected patient, show in figure 7(B). Age range of HEV-A infected patient are from 1 months to 36 years old. No specific sesonal distrubution were noted for HEV-A, show in figure 7(C).

(A)



(B)



(C)

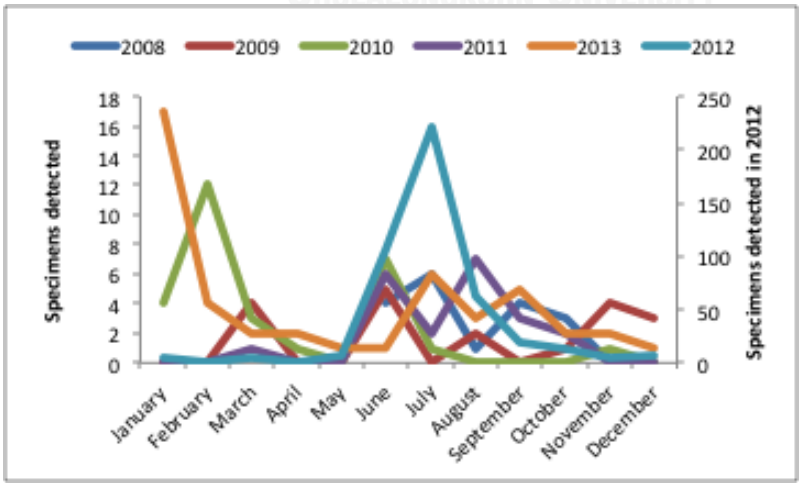


Figure 7: *Prevalence of HFMD vs HA (A), age distribution (B), and seasonal distribution (C) of all patients infected with HEV-A*

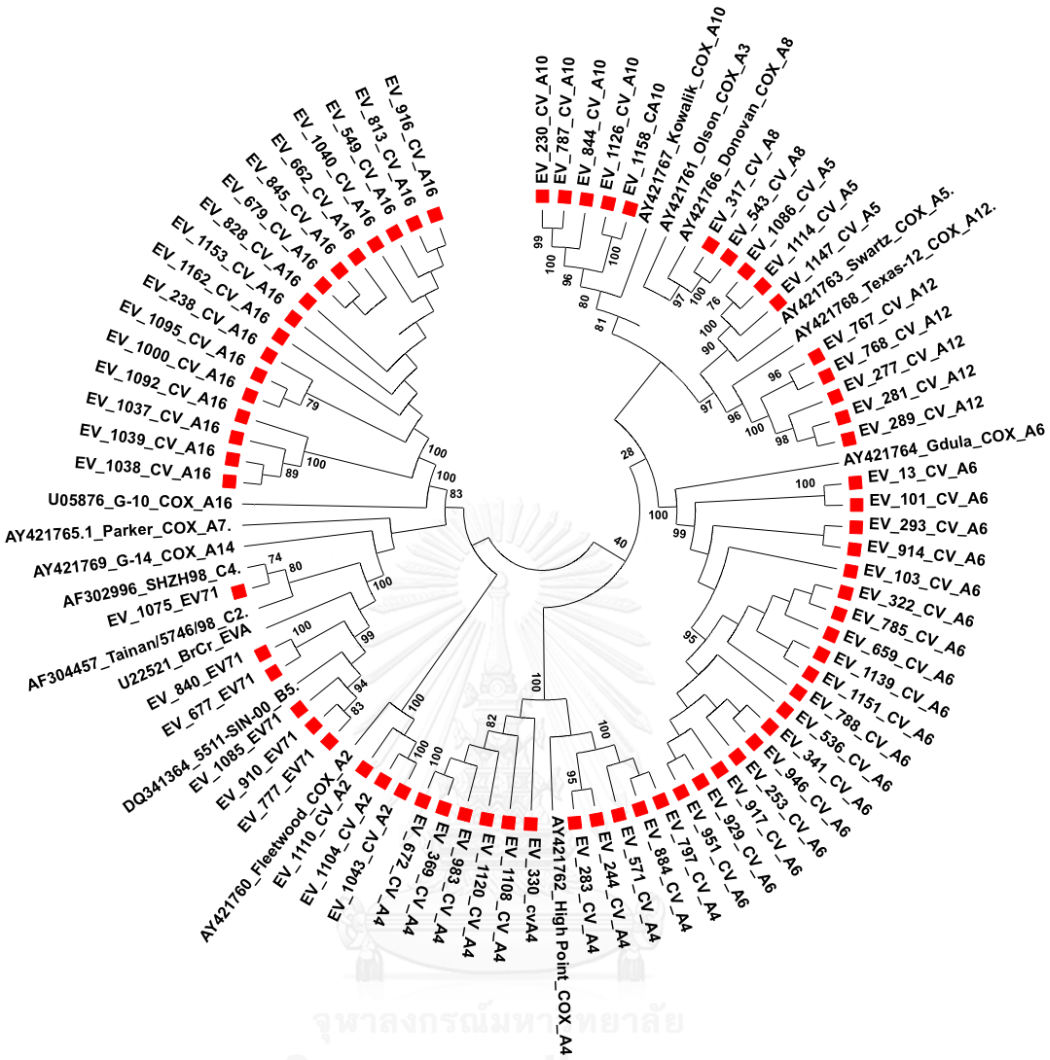


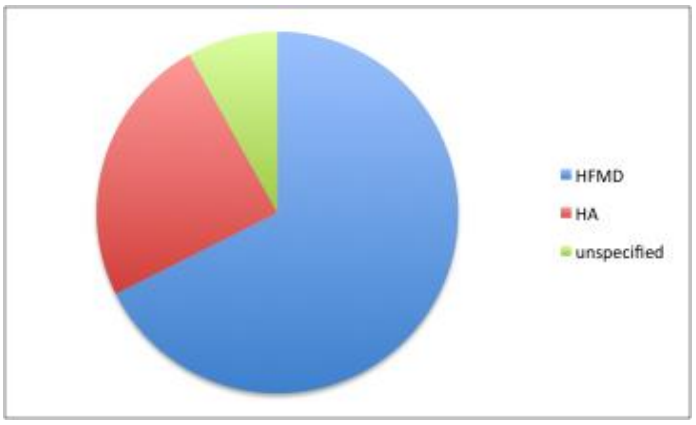
Figure 8: Phylogenetic tree representing HEV species A detected and identified from negative and untyped specimens (red squared). The partial VP1 sequences were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.

Prevalence of HEV-B

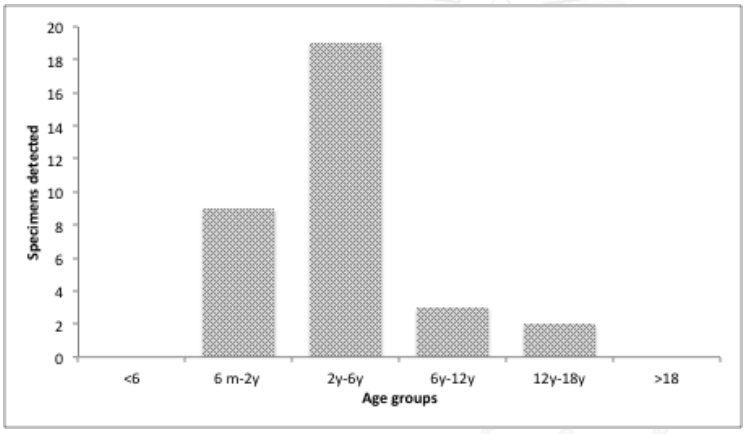
The circulation of HEV-B were predominantly in year 2012 and 2013 with only one CV-A9 detected in 2010. HEV-B contribute to 5.74% (n=37) of all detected enterovirus over 6 years period but in year 2013 alone HEV-B rate increase to 18.2% (n=14) among all enterovirus. CV-B2 were detected highest among HEV-B with 9 isolates all from June to November of 2012 follow by 7 isoates from echoviruses 16 all within Febuary and March of 2013. Other member of HEV-B, CV-A9 (n=4), -B1 (n=3), -B3 (n=3), -B4 (n=2), –B5 (n=1) and echovirus 6 (n=1), 7 (n=1), 11 (n=2), 18 (n=1), 25 (n=1). Partial VP1 gene sequence were use in construction of a phylogenetic tree show in figure10.

Among all patients enrolled in this study who were detected with HEV-B 68% (n=25) were diagnosed with HFMD while 24% (n=9) were diagnosed with HA and remaing 8%(n=3) were unspecified , show in figure 9(A). The age distribution of HEV-B infected patients shows 55.9% (n=19) are within 2 to 6 years old with highest infected patient aged 16, show in figure 8(B). No specific sesonal distrubution were noted from HEV-B, show in figure 9(C).

(A)



(B)



(C)

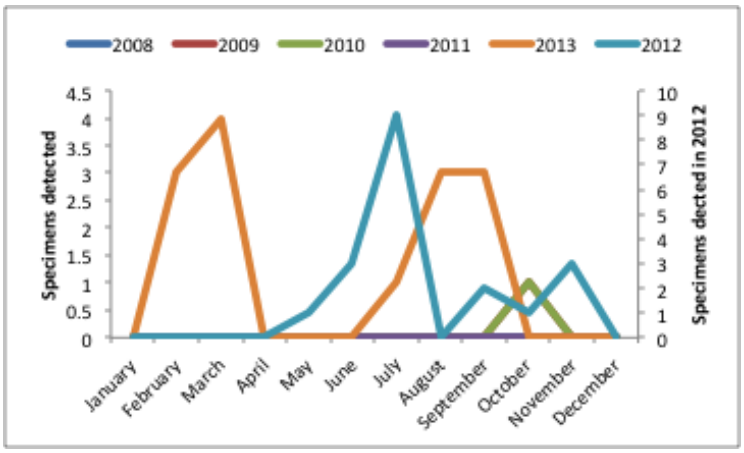


Figure 9: *Prevalence of HFMD vs HA (A), age distribution (B), and seasonal distribution (C) of all patients infected with HEV-B.*

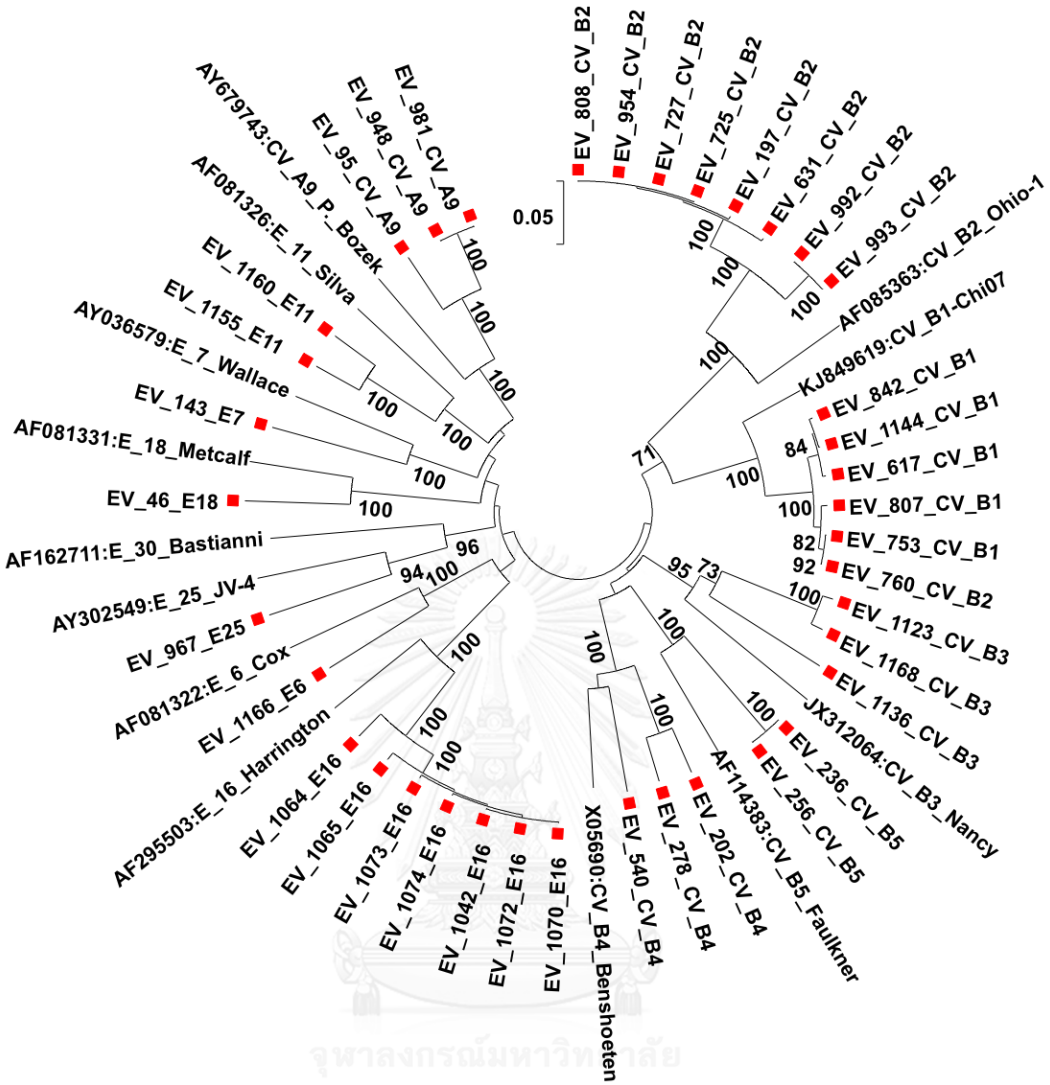


Figure 10: *Phylogenetic tree representing HEV species B detected and identified from negative and untyped specimens (red squared). The partial VP1 sequences were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.*

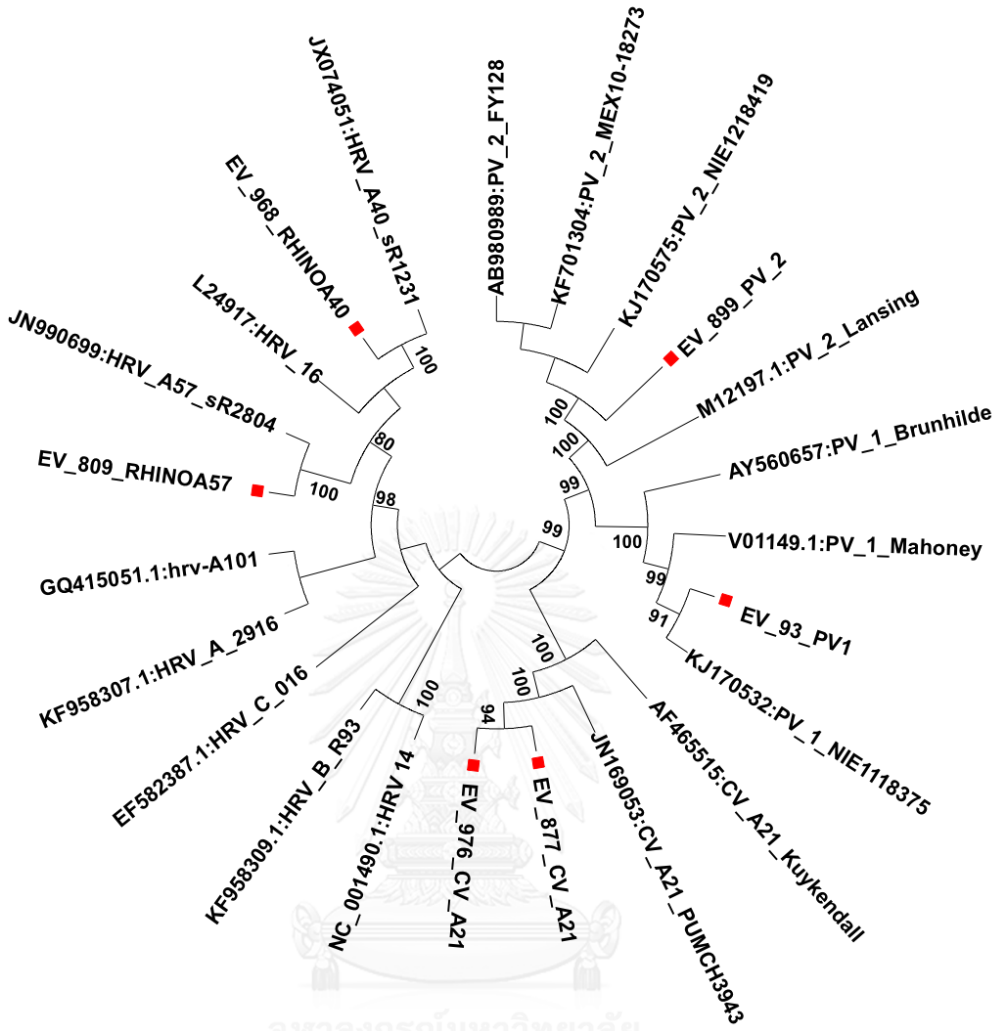


Figure 11: Phylogenetic tree representing HEV species C detected and identified from negative and untyped specimens (red squared). The partial VP1 sequences were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.

Discussion

EV71 and CV-A16 was widely known as a major causative agent of HFMD until recent HFMD outbreak in Thailand, CV-A6 was identified as a new major causative agent. Due to high number of negative molecularly detected specimens and untypeable detected enterovirus and to avoid misidentification of HFMD outbreak causative agent in the future, this study further examines other enteroviruses that could circulating in patients diagnosed or suspected with HFMD or HA. We are able to further detect and identify enteroviruses in 19.3% of previously negative and unidentified specimens from pervious study using CODEHOP techniques. Overall detection rate from 2008 to 2013 increases from 54.2% to 61.0% and further identified 32.2% of previously unidentified enterovirus detection.

The prevalence rate of enterovirus in HFMD and HA patients from 2008 to 2013 were 49.4% (n=603) were grouped into HEV-A, 3.03% (n=37) were HEV-B, 0.491 % (n=6) were HEV-C and 0.0819% (n=1) were HEV-D with age distribution highest among 2 to 6 years old. Similar age distribution were reported in previous study where high infection rate correspond with low antibody detected that gradually increase with age while infant under 6 months was still protected by maternal immunity(27). It is interesting to note the high diversity of enterovirus serotypes detected in July, August and September in comparison to January, February and March, though both periods were high in specimens.

Increasing members of enterovirus species A were identified responsible for HFMD outbreak worldwide in recent years. Our study demonstrated highest prevalence of HEV-A in HFMD and HA patients when compare to other enteroviruses species or 93.8% specimens with enterovirus detection (6, 34). In this study we further detected

CV-A2, -A4, -A5, -A6, -A8, -A10 and -A12 in both patient diagnoses with HA and patients diagnosed with HFMD relatively low among patients infected with EV71 and CA16 of 4.17% and 5.88%, respectively. Higher numbers of patients diagnosed with HA were report among patient infected with CV-A6, 10 and 8 of 7.29%, 22.2 %, and 47.6%, respectively. Similarly in Japan the study report CV-A10 in combination with CV-A6 was most prevalence in patients diagnosed with HA since 1994 in Aichi (35) and Kanakawa prefecture (36). The infection involving CV-A6 and CV-A10 was reported to be less virulent but more infectious in comparison to EV71. In Singapore and Finland a fast spread outbreak as a result of high transmissibility of HFMD found CV-A6 and CV-A10 as prominent detected enteroviruses (6, 37).

Enterovirus species B consist of coxsackievirus and echovirus contribute to 5.5% of all positive specimens. In this study, we detected coxsackievirus B, infections are generally self-limited with symptoms common among other enteroviruses such as fever or rashes but the viruses can cause a severe inflammatory disease leading to myocarditis. Coxsackievirus B2 expressed highest prevalence among all of the CV-B with all patients was detected in 2012 among patients diagnosed with HFMD and HA. Numerous studies reported link higher association between CV-B3 and CV-B4 with myocarditis. The cardiovirulence determination was observed in VP2 and VP3 region of the capsid proteins where antibody escape mutant of such gene resulted in a reduced virulence. In northern Taiwan, patients infected with coxsackievirus B were reported of having longer duration of fever, more CNS involvement and elevated rate of hospital admission (38). A HFMD outbreak in Linyi City, China reported a 78.6% rate of HFMD patients infected with CV-B5 displayed neurological manifestations (39). Multiple echovirus belong to HEV-B were also detected in this study including echovirus 6, 11, 16, 18 and 25. Multiple serotypes of echovirus were linked to a cause of an outbreak of aseptic meningitis. In Korea patients with aseptic meningitis during the epidemics were

commonly presented with echovirus 6 infection (40). 70% of meningitis patients during the outbreak in Canada were also found a presence of echovirus 25 antibodies (41). However, a study in Shenzhen, China HEV-B species was detected highest among other HEVs species in stool specimens isolated from healthy children (42).

Species C and D of enterovirus were detected least among the 4 species. Multiple members detected from these species were highly correlated to causative pathogen of respiratory tract infection. CV-A21 was detected in a 16 years old patient with herpangina. The strain showed high nucleotides identities with CV-A21 of Thai patient with influenza like illness and Chinese strains, such strain were reported at high prevalence among HEV-positive adult over 15 years old with acute respiratory tract infection (43, 44). Rhinoviruses are common causative agent of upper respiratory tract infection, in this study, only rhinovirus species A, A40 and A57, were detected. Previous report suggested that in contrast to rhinovirus species C which often associate with respiratory illness, rhinovirus species A are found in high prevalence among healthy children (45, 46). Human poliovirus 1 and 2 were each detected in clinical specimens with high nucleotides identities to the Sabin strain therefore they are most likely due to recently administration of oral polio vaccine these patients.

3.2 Complete genome genetic characterization of enterovirus 71

Research methodology

Complete Genome

Enteroviruses that characterized as EV71 were selected for virus isolation. The original clinical specimens were inoculated in rhabdomyosarcoma cells. The cells with cytopathic effect (CPE) observed were harvested.

Virus Isolation

Culture medium and reagents preparation

- DMEM (Dulbecco's Modified Eagle Medium 1x) (Wisent Inc, Canada)
- Medium for cell culture
    - 10% of Fetal Bovine Serum
    - 2% of Penicillin-Streptomycin (P/S) (10,000 u/ml)
    - Add DMEM make up the final volume
- Medium for viral culture
    - 2% of Fetal Bovine Serum
    - 2% of Penicillin-Streptomycin (P/S) (10,000 u/ml)

- Add DMEM make up the final volume

- PBS
  - 8.07 g/l of NaCl
  - 0.200 g/l of KCl
  - 1.42 g/l of Na_2HPO_4
  - 0.240 g/l of KH_2PO_4

dH_2O were used to make up the final volume and autoclave at 121 °C for 30 min.

Culture tube preparation

After cells reached confluence growth medium were removed from the flask and wash with 10 ml of PBS. 1 ml of pre-warmed trypsin-EDTA (0.05% trypsin, 0.02% EDTA) was added to the cells monolayer and incubation at 37°C until all cells are detached. 5-10 ml of pre-warmed growth medium was added to the flask and cells are separated into single cells through pipetting. Viable cells were counted using hemacytometer. Cell numbers were adjusted to $1x10^5$ cells/ml using DMEM growth medium. 1 ml of cells was transfer to each culture tube and incubated at 35 °C, CO_2 for minimum 24 hours.

6-wells plate preparation

The growth and morphology of cells were observed and 6-wells plates were prepared for viral inoculation. Cells were washed and separated into single cells using the same method as mentioned above. Cell counting was performed and cell numbers were adjusted to $1.5x10^5$ cells/ml using DMEM growth medium. 2 ml of adjusted cell were added to each well, making seeding density of $0.3 x10^5$ cells per well. Cells were incubated for 18-22 hours at 35 °C with 5% CO_2 and were observed constantly and use when it reached confluence.

Viral inoculation and observation of CPE

Once cell reached confluence, growth medium were removed and washed with 1ml of PBS. 4 drops of diluted specimens were added to the culture tube followed by one hour incubation at 37 °C. 1 ml of viral culture medium was added and further incubated at 37 °C. The CPE was observed every day in comparison to control. If low CPE level is noted after one week, cell supernatant were passage again for maximum of 2 times. Cell supernatant of sample with CPE noted was harvested.

Complete genome sequencing

Primers from Puenpa et al. (8) were used as a primary for amplification of EV71 and some of the primer designed by Chen et al. (46) were used for amplification CA6. If multiple alignments of EV71 and CA6 strain detected were BLAST with genomes available on Genbank database and differences were shown, new primers were designed.

Principle of PCR Primers Design

- Primer should be approximately 18-24 nucleotides by length
- G/C content is limited to 40 – 60%
- G/C content of forward and reverse primers should roughly be the same, to facilitate binding of primers and template at the same temperature.
- Melting temperature (T_m) of primer should be around 55-62 °C (T_m= 2 °C x (A+T) + 4 °C x (G+C))
- To prevent primer dimmer following were avoid:
    - Complementarity of 2 or 3 base at the 3' ends of primer pairs
    - Mismatches between the 3' end of the primer and target template sequence
    - T at 3' end
    - Repeating Gs or Cs of 3 or more at the end

- Complementary sequence within a primer sequence and between primer pair.

RNA extraction performed on cell supernatant harvested. The extracted RNA was reverse transcribed with random hexamer primer, generated cDNA and PCR were conducted with primers designed, primers used in this study are in table 4 and 5, and gel electrophoresis was performed on PCR product, which is visualized under UV light. The spot of interest were excised and sequenced.

Table 4: *Primers for EV71 complete genome amplification.*

	Primer	Sequence (5' to 3')	Position	Regions
1	HEV71_F62 *	CTC GGT ACC TTT GTG CGC CTG	62-82	5'UTR -VP4
	HEV71_R1122 *	GGG CGC GTT GGT TTA TCC ACT	1102-1122	
2	HEV71_ F843	ATT ACA AAG ATT CCT ATG CTG CG	843-866	VP2
	HEV71_ F895 *	TCC AGA CAA GTT TGC AAA TCC TGT	895-918	
	HEV71_R2089 *	GGA TCC AGT AAA CAT RAA GGT G	2068-2089	
3	HEV71_F1818	TTC ACA TAC CCG GTG AAG TCA G	1818-1840	VP3
	HEV71_ F1931	GGT CTC AGC ACA AGC AGG GA	1931-1951	
	HEV71_R2400	CAG TTT CAT GGT AAA ATT CTT CTG	2376-2400	
4	HEV71_ F2210 *	GTC ACC CTT GTV ATA CCA TGG AT	2210-2232	VP1
	HEV71_ F2339 *	GCC CAA YAC AGC YTA YAT AAT AGC	2341-2364	
	HEV71_R3453 *	AGT CGC GRG AGC TRT CYT CCC	3432-3456	
5	HEV71_ F3196	CCA AAT TAT GCT GGC AAC TCC A	3272-3293	2A
	HEV71_F3272 *	CCA AAT TAT GCT GGC AAC TCC A	3272-3294	
	HEV71_R4140	CCA CTC TAA ACC CTT GGC GG	4120-4140	
6	HEV71_ F3500 *	TGC AAY TGY CAR ACA GGR GTG TA	3500-3522	2B
	HEV71_ F3560	CTT CGT RGA RGC YAG YGA GTA TTA	3560-3584	
	HEV71_R4437 *	CGG TGT TTG CTC TTG AAC TGC A	4416-4437	
7	HEV71_ F4030	ATC TTA GGT ATC CCT ATC GCT C	4052-4073	2C
	HEV71_ R5115	TGG CTT CTC CTC AGG ACT GAT	5115-5136	
	HEV71_R5140 *	GAG ATC GCT AAT AGC GTC TGG	5120-5140	

8	HEV71_ F4883 *	AAA CTG TGC TCT GAA AAC AAC AC	4883-4905	3ABC
	HEV71_ F6491	TGT GAC ATT GAA GTG ACA GAC TC	4817-4839	
	HEV71_R6314	ATG CCA AGG GCG CTG TAT GG	6314-6334	
9	HEV71_ F5782	GCC AAC ACA TMG VAC VAT GAT GTA	5782-5806	3D
	HEV71_ F5932	AAG TAT RCA RGG DGA GAT CCA	5932-5953	
	HEV71_R6857	CTA ATA ATG ATG TTR TTG ATC ATT G	6832-6857	
10	HEV71_ F6486	TGA CTC TGT CTA CCT CAG AAT G	6486-6508	3'UTR
	HEV71_ F7406	GTT ATA ACA AAT TTA CCC CCA CC	7406-7429	
	HEV71_R7413 *	TTT TTT GCT ATT CTG GTT ATA ACA A	7388-7413	

*Prmiers designed by Puenpa et al.(8)

Sequence analysis

The VP1 gene was analyzed though BLAST program on NCBI website and complete genome sequence with highest score was used as a reference strain for editing. The sequence was assembled into contigs through overlapping DNA sequences through Seqman program as a part of DNASTAR Software (v5.0). Multiple sequence alignments with other EV71 retrieved from Genbank were performed with MAFFT version 7 program. Different parts of the genome were selected, analyzed and nucleotide identities were calculated with BioEdit Sequence Alignment Editor package (v7.0.9.0). The phylogenetic trees were constructed with the neighbor-joining (NJ) method and Kimura's two-parameter distance model. The 1,000 random sampling of bootstrap analyze was use to estimate the phylogenetic tree strength with strong tree topology supported by ≥ 70% bootstrap value. Pair-wise deletions was implemented for missing data, all was achieved on MEGA software package (v5.0)

Recombination Analysis

To demonstrate recombination between EV71 and CV-A16, the sequences were aligned and similarity plot and bootscan analysis of Thailand C4 subgenotype against different EV71 genotype and CV A16 were constructed with Simplot program (version 3.5.1; Stuart Ray, John Hopkins University, Baltimore, Maryland, USA). The EV71 sequences obtained in this study were aligned against other HEV-A to determine their genetic relationship. Phylogenetic tree of different regions of the genome was constructed with neighbor joining method. The trees were examined manually to determine any changes in the tree topology throughout the genome. Similarity plots were then constructed. Suspected recombination was further analyzed with RDP4 program to determine recombination breakpoint. Default setting was used in the analysis and detection with three or more detection method implemented in RDP4 was counted as a significance evidence of recombination.

Results

The study conducted a complete genome sequencing on 14 isolates from EV71 infected patients, obtained from 2012 HFMD outbreak till 2014; 8 EV71 were detected during the HFMD outbreak from July to August 2012, 1 in December of 2012, 2 in 2013 and 3 in 2014. From 14 patients, 12 patients showed symptoms of mild HFMD and 2 patients reported a clear evidence of CNS infection. Most patients are under 6 years of age with an exception of one 26 years old patient. The complete genome sequences consist of 5'untranslated region, the open reading frame (ORF) containing 6582 nucleotides without insertion or deletion, which translate into 2194 amino acids assembled into single polyprotein, and 3'untranslated region.

Phylogenetic analysis and Nucleotide and Amino Acid identities of Thailand EV71

The complete genome sequences obtained from the studied were aligned with 305 other complete genome sequences retrieved from Genbank database. The phylogenetic analysis, from phylogenetic tree in figure 12, indicated that almost all strains clustered into subgenotype B5 except one, which belongs to subgenotype C4 (EV1219). Among the subgenotype B5, the EV71 strains were separated into two cluster; with one cluster is strains found during the 2012 outbreak while the other strains was detected post-outbreak from December of 2012 till October of 2014. The EV71 strains detected during the 2012 HFMD outbreak found to cluster with THA-EV71-019 strain detected in Thailand and NUH/SIN/08 strain. The strains detected post-outbreak cluster with subgenotype B5 strain isolated from Taiwan. The C4 subgenotype cluster into C4a linage with the FY17.08-6/AH/CHN/2008 strain isolated from Mainland China in 2008.

The aligned 14 complete genome sequences showed lower nucleotide homology in the open reading frame when compare to 5'UTR, range from 81.1% to 100% and 85.8% to 100%, respectively, with higher identities after translated to amino acids of 94.8% to 100%. High nucleotide homology was reported among the EV71 strains detected during the 2012 HFMD outbreak of 98.5% to 99.9%. In comparison to the EV71 prototype, BrCr strain, highest homology was reported in the group of B5 strain detected post outbreak of 80.3% and lowest in the C4 strain. The amino acid identities in the structural protein region are higher when compare to non-structural protein region range from 96.7% to 100% and the lowest identities were reported in the P3 region of 93% to 100%, especially in the 3B genes, 90.9% to 100%.

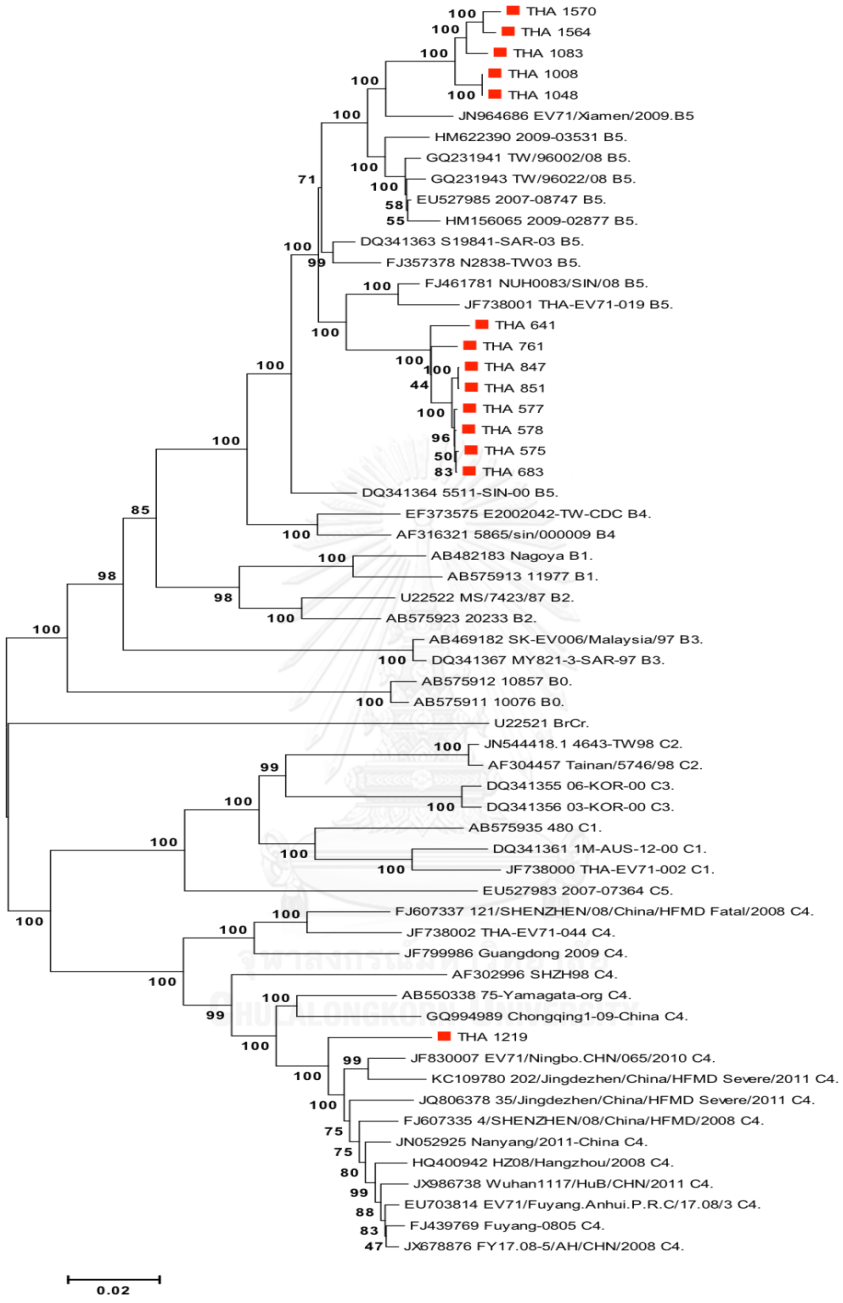
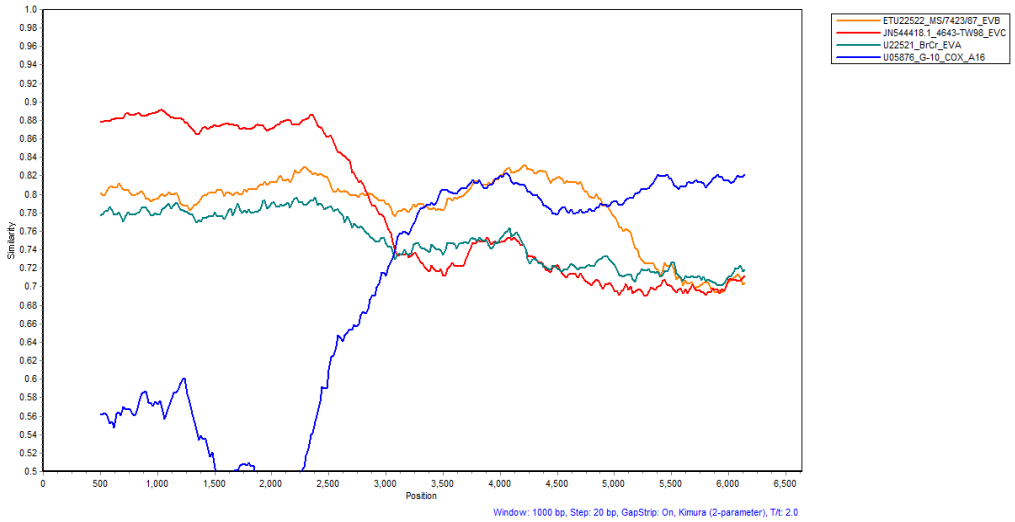


***Figure 12**: Phylogenetic tree representing Thailand EV71 constructed using complete genome sequence. The complete genome sequence were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.*

Recombination Analysis of C4 subgenotype detected in Thailand

To determine the recombination between EV71 and CVA16, Simplot and Bootscan of C4 subgenotype was plotted against EV71 prototype strain, BrCr, representative B genotype, MS/7423/87, representative of C genotype, 4643-TW98, and, CVA16 prototype strain, G-10. The similarity plot in figure 13a shows highest similarity to 4643-TW98 from 5'UTR region till 2832nd position after the ORF around 2A gene. From 2832nd till 4900th position after the ORF both MS/7423/87 and G-10 show high similarity to the Thailand C4 subgenotype before similarity to MS/7423/87 decrease after 4900th position, leaving highest similarity with only G-10. Similar result was reported when bootscaning was performed in figure 3b. Bootscan suggest two breakpoints at approximately at the 3008th and 4856th position after the ORF, which corresponded to 2A/2B junction and the middle of 3D gene, respectively. MS/7423/87 express a highest percentage of permuted trees between 3008th till 4856th position when compare to other references.

(A)



(B)

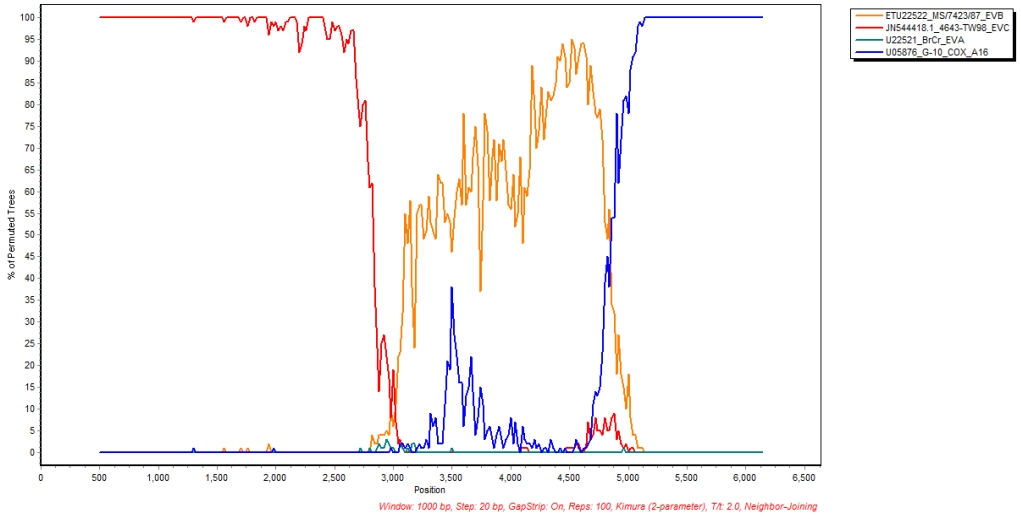


Figure 13: *Similarity plot (A) and bootscaning (B) of Thiland C4 subgenotype EV71 strain against BrCr, MS/7423/87, 4643-TW98 and G-10 with Y-axis represent percentage of identity and percenatgge of permutated tree, respectively. The graph was generated using kimura distance model within sliding window of 1000bp and step size between plots of 20 bp.*

Phylogenetic relationship of EV71 detected in Thailand and other HEV-A

The complete genomes of EV71 strains detected in Thailand were further aligned with prototype strain of other HEV-A species, MS/7423/87 and 4643-TW98 uses complete genome available on Genbank database. Genetic relationships were examined through construction of phylogenetic trees, as represent in figure 14, between the complete genome (a), 5'UTR (b), P1 (c), P2 (d), and P3 (e) regions of EV71 Thailand strains and other representative strains. At the 5'UTR region, the EV71 Thailand strains separated into two clusters away from EV71 prototype strain; the C4 subgenotype cluster with 4643-TW98 and CV-A8 with bootstrap value of 78 % and the entire B5 subgenotypes cluster with MS/7423/87 while the EV71 prototype strain shifted away from other EV71s and form a cluster with CV-A3. The phylogenetic tree constructed using P1 region resembled one constructed using the complete genome with All EV71 Thailand strains cluster together with EV71 prototype strain with high bootstrap value of 100 %. CV-A16 demonstrates to share the same linage with EV71 in the P1 region tree with high bootstrap value of 100%but not for the complete genome tree. At P2 region, all of the EV71 Thailand strains and MS/7423/87 cluster together and share the same lineage with 100% bootstrap value with CV-A5, -A16, -A4 and -A14, however, 4643-TW98 segregated away to cluster with CVA8 and the EV71 prototype strain form a cluster with CV-A12, -A2, -A6, and -A10, both with 100 % bootstrap supported. The EV71 detected in Thailand split again at P3 region and formed a cluster of EV71 B genotype and CVA5 with bootstrap value of 75 % and the Thailand C4 subgenotype detected in Thailand with CV A16, A4 and A14 support with high 100 % bootstrap value. The BrCr strain showed to form another cluster with CV-A3, -A12, -A6, -A10 and -A2 and supported with strong bootstrap value of 100 %.

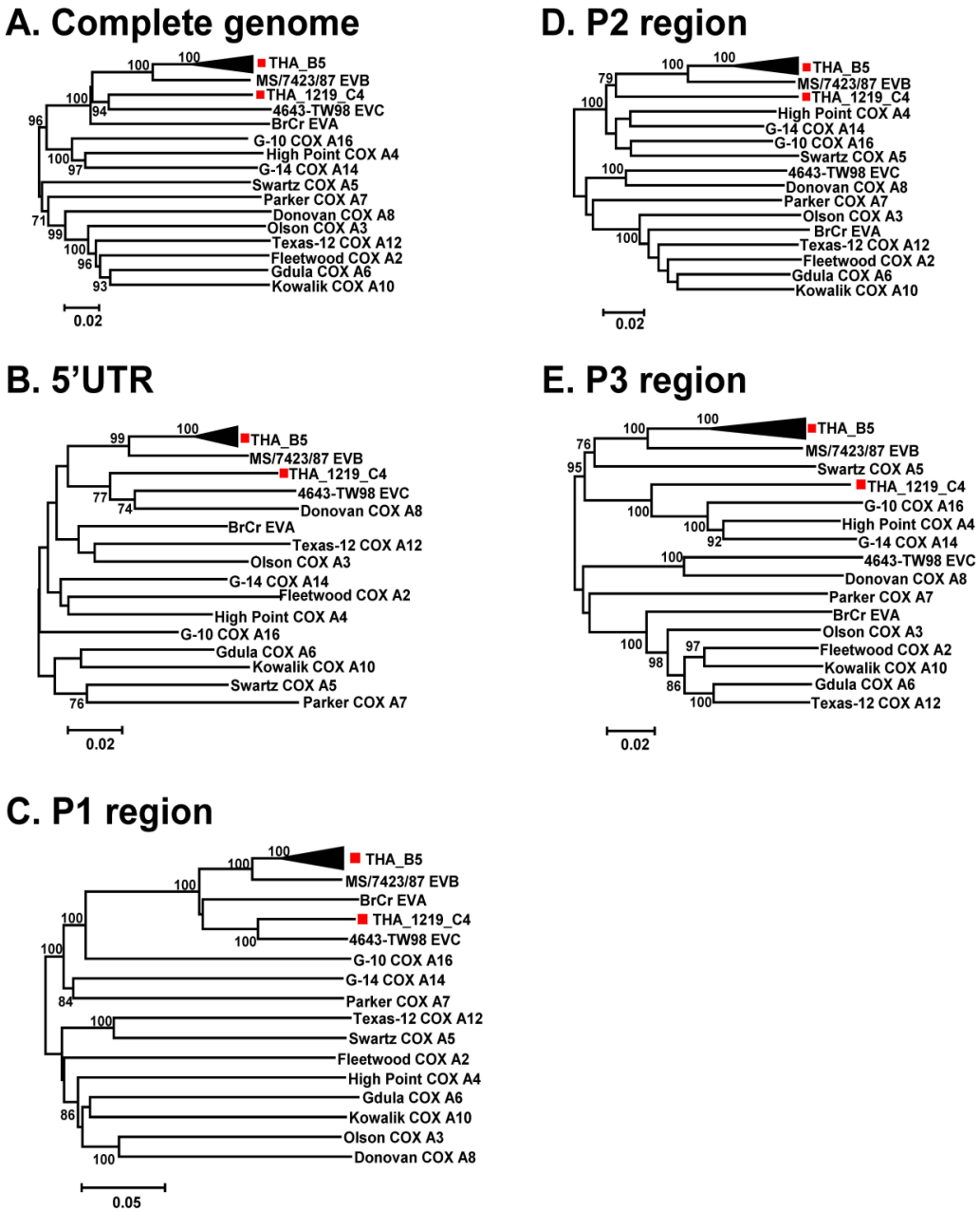


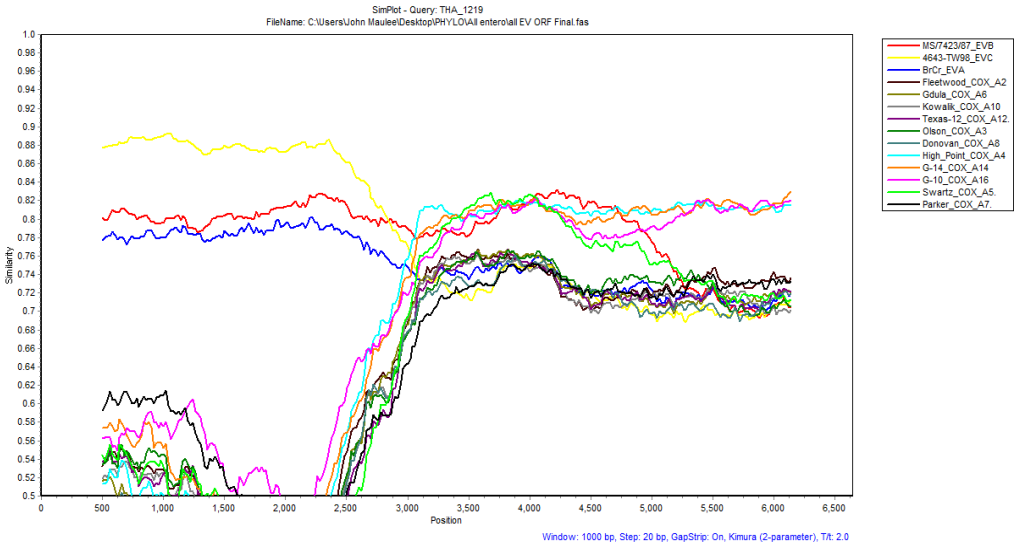
Figure 14: *Phylogenetic trees representing relationship of Thailand EV71 and other HEV-A in complete genome (A) and different region of the genome 5'UTR(B), P1(C), P2(D) and P3(E). Different regions were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates of over 70% supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.*

Recombination Analysis of EV71 detected in Thailand and other HEV-A

Simplot software was used to analyze the genetic relationship and support recombination suggested by the phylogenetic trees between EV71 found in Thailand and other human enterovirus species A. Simplot of a representative complete genome of subgenotype B5 and C4 detected in Thailand against HEV-A are show in figure 15a and 15b respectively. Excluding high similarity range from ~82 % to ~90% of Thailand B5 to MS/7423/87 throughout the genome, in figure 15b, similar pattern is noted for Thailand B5 and C4 subgenotype. Both plots shows high similarity to all HEV-A in the 5'UTR that decreases in the structural region leaving only high similarity to EV71 of around 80% but the similarity of other HEV-A increases back in the non-structural region, resulting in a pool of HEV-A with similarity fluctuates at a range from 70% to 82%. Among pool of HEV-A in the non-structural region, Simplot demonstrate a dominant group that corresponds to the phylogenetic tree.

When compare similarity of B5 to other human enterovirus A species, CV-A4, -A5, -A14 and -A16 reported higher similarity in the P2 region in comparison to other strain. In P3 region, all strains are group together with CV-A5 report the highest similarity in most part. In contrast to B5, the C4 express two distinct group in the non-structural region, with one group show a similarity range from 80% to 83% and the other group show similarity of 69% to 76% with crossover point at around 5550th position. In the non-structural region, higher similarity was noted with MS/7423/87, CV-A4, A5, A14 and A16, however, at 5500th position similarity of MS/7423/87 and CV-A5 start to decrease to around 72%. Left behind high similarity to only CV-A4, A14 and A16 at the P3 region

(A)



(B)

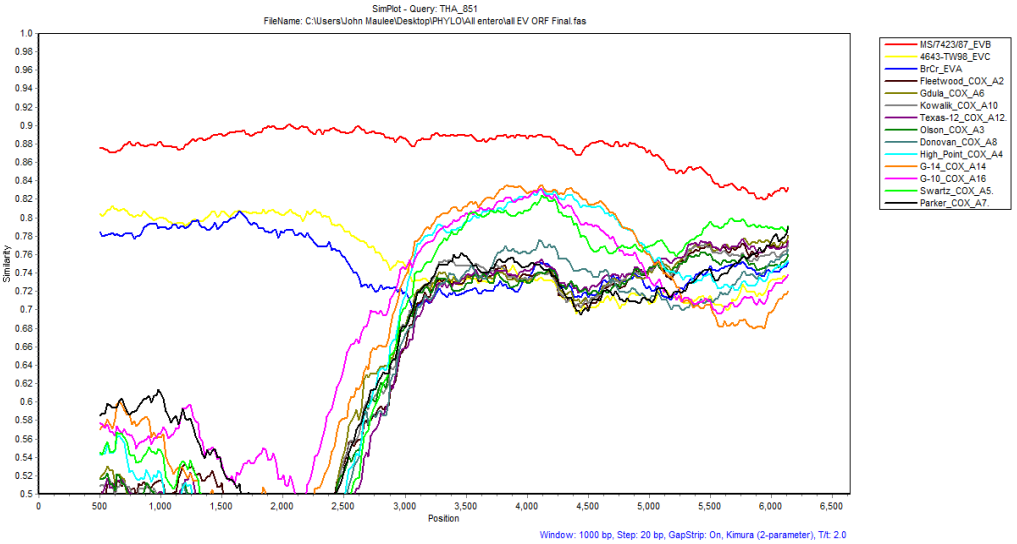


Figure 15: *Similarity plot analyses on the complete genome of the representative strain of C4 (A) and B5 (B) subgenotype of EV71 detected in Thailand against other HEV-A. The sliding window of 1000bp was use with step size between plots of 20 bp.*

The intertypic recombination of Thailand EV71 C4 is support by the analysis with RDP4 program on the complete genome sequences, which revealed recombination between a major parent strain, G-10 of CV-A16, with 83.4% similarity and a minor parent strain, 4643-TW98 of EV71 genotype C, with 88.4% similarity. The analysis suggest breakpoint beginning at position 500 which is approximately the end of 5'UTR region and ending at 3372 around the 3' end of 2A gene.

Table 5: Overview of recombination detected with RDP4. Detection of recombination in Thailand C4 strain when analyze using aligned complete genome sequence of HEV-A.

Breakpoint position in sequence alignment	**Beginning**	504
	Ending	3468
Major Parent strain (% similarity)		G-10 Coxsackievirus A16 (83.4%)
Minor Parent Strain (% similarity)		4643-TW98 Enterovirus 71 C genotype (88.4%)
Method of Detection (Av. P-Value)	**RDP**	1.659×10^{-50}
	BootScan	4.888×10^{-56}
	Maxchi	1.715×10^{-27}
	Chimaera	7.294×10^{-33}
	SiScan	1.500×10^{-58}
	3Seq	2.983×10^{-55}

Discussion

Since first identification of EV71 in Thailand by positive serology in 1998, EV71 has been in a spotlight for both public health and research field(48). Main concern of EV71 can be separated into two categories, the severity of the disease once it infected and it transmissibility among population. The complete genome of EV71s performed in the study reported a genetic insight into EV71 circulating in Thailand from 2012 till 2014. The study examines complete genome of EV71 detected during and after the HFMD outbreak, genome of patient with severe EV71 infection, and recombination analysis of strain detected during the period of study.

The phylogenetic analysis indicates changes in clustering of EV71 subgenotype B5 from 2012 to 2014. EV71 detected during the 2012 HFMD outbreak cluster together with close relation to THA-EV71-091, Thailand strain detected in 2008, suggesting virus was circulating for number of years. The degradation of maternal immunity could influence the prolonged circulation of the virus due to constant emergence of venerable age group as study report an undetectable immunity at the around age of 12 months(49). The NUH/SIN/08, which also clustered with EV71 subgenotype B5, was detected in Singapore 2008 outbreak. The nature of an outbreak described highly similar to 2012 HFMD outbreak in Thailand of “high transmissibility and low fatality (50). Strains detected after the outbreak form another cluster with B5 strains from Taiwan. The group of strain show cluster of patient with mild and severe EV71 infection include TW/96016/08, a strain isolate from patient diagnose with encephalitis. This formation of new cluster may due to high exposure of the old B5 strain during the outbreak which consequently results in an increase in population immunity enforcing a selective pressure for new variant in order to avoid host immunity.

Enteroviruses are known to cause variety of diseases, EV71 is distinguished from other enteroviruses through it ability to cause neurological complications causes major concerns in multiple countries therefore numerous efforts were put in attempting to determine the cause of these differences in clinical phenotype. The study performed whole genome sequencing on two cases reported with clear evidence of central nervous system (CNS) infection. Both patients were presented with HFMD and encephalitis with one isolate, EV_1564, was from 4 months old patient and another isolate, EV_1219, was a fatal case of 3 years old patient. The phenotype of disease form EV71 infection can be a contribution of both viral and host factor. Phylogenetically, B5 strain of the severe case cluster with to B5 EV71 detected after 2012 and a group of Taiwan strains with patient reported with encephalitis. The B5 strain reported in a youngest patient of all patients conducted in this study. Previous study suggested that infant under 6 months showed lower infectious rate in comparison to 6-10 years old children, however, if the infection occur infant risk a high chance of complication, with 5 fold increase in severity, and death, with 25 fold increases in fatality (51-53). The ineffectiveness of response to EV71 infection in infant may due to insufficient level of required specific antibody and innate and cellular immune response. Specific pattern of innate immune response are gradually established during childhood leaving infant vulnerable to specific pathogens and once infected unable to efficiently control the viral replication and generation of preferential pathogenic immune response (54). Another case of CNS infection was phylogenetically categorized in the C4 strain and subgenotyped into C4a, which was reported in China to be highly associated with neurovirulence in comparison to C4b, where no severe or fatal case has been reported. The molecular analyses of previously reported mutation, which are suspected to be a virulence factor, were performed. Mutation of C→T and A→T at position 241 and 571, respectively, in the 5'UTR region is correlated to the mutation of a fatal strain,

SDLY107(55). Position 241 and 571 are located in domain IV and VI of the IRES element, a highly variable domain in contrast to domain II and III that is relatively conserved. It was suggested that these variation might relate to the virulence (55).

Recombination plays an important role in evolution of the virus and is commonly known as a mechanism that viruses use in generation of the genetic variant resulted in a diversity among its population, in combination with point mutation, this could result in an avoidance of host immune response and emergence of greater virulence variants. In enteroviruses, the recombination is commonly found in the nonstructural region in comparison to structural region as the structural region contains essential coding required in receptor recognition that in turn facilitates viral entry into cell where recombination that occur in such region may produce a less fit variant and may not survive. By altering the non-structural region, virus alter it pathogenic potential allowing possibility of replicative advantages. The recombination analyses demonstrate intratypic and intertypic recombination in C4 strain detected in Thailand from patient with clear symptom of CNS infection. The first breakpoint suggests an intratypic recombination between genotype B and C of EV71. The strain shows to be related to EV71 genotype C strain in the P1 region of the genome that changes at around nucleotide position 3000th after the ORF to genotype B of the EV71. The recombination breakpoint corresponds to the 2A-2B gene junctions located in the P2 region. 2A gene was frequently reported as a hotspot for recombination in enterovirus and intratypic recombination between B and C genotype at 3' terminus of 2A gene was previously reported in Taiwan suggesting the recombination could be a result of co-circulation of both genotype that is favorable by high degree of similarity (56, 57). Higher sequences homology influence the rate and frequency of recombination and was demonstrated that frequency of intratypic recombination of poliovirus are 100 times higher than that of intertypic recombination (32, 58, 59). Moreover, further research shows recombination

in the P2 region of enterovirus C species are found to occur more between strains that are closely correlated in the P1 region(60). The analyses also suggest an intertypic recombination between EV71 and CA16 G-10 strain located in the nucleotide position 4904th after the ORF or in the 3C gene. Both EV71 and CA16 is common causative agent of HFMD commonly found to co-circulation and causing similar clinical manifestation(34, 61, 62). The "double recombinant" strain between genotype C and B of EV71 and CA16 G-10 strain was previously reported in children with HFMD from Shenzhen, China in 2008 and was proposed to be considered as a new novel genotype D(63).

Further recombination analysis was performed of EV71 detected in Thailand against prototype strain of Human enterovirus species A (HEV-A). The phylogenetic tree and similarity plot analysis further support an evidence of recombination occurred between the Thailand C4 subgenotype and other HEV-A as, in contrast to P1 region, 5'UTR, P2 and P3 region did not cluster with the EV71 prototype. P1 region consist of capsid proteins genes, a part of genome widely use in characterizing the virus identity, the region contain serotype-specific information that showed through phylogenetic analysis to be highly correlated to serotyping with neutralization tests, especially in the VP1 gene(24). The phylogenetic tree constructed using P1 region also show to resembles tree constructed with complete genome sequences even though it is only one third of the genome, emphasizing the importance of viral capsid proteins property in relation to it identity. In the non-structural part of the genome (P2 and P3 region), C4 strain demonstrates to be more related to CV A4, A14 and A16 in comparison to EV71 prototype strain. Similar result was reported in Yoke-Fun and AbuBakar, 2006 and Zheng et al., 2013. In Zheng et al., 2013, the C4 strains found in China showed recombination in both subgenotype C4a and C4b, where these recombinant strains were noted to be circulating in Mainland China before 1998 and were responsible for

national wide outbreak with high morbidity and mortality (31). The study further on proposed that the recombination are unlikely to be coxsackievirus A4 as it usually associated with different clinical outcome and uses different cellular pathway to infect cell(4, 31). In addition to coxsackievirus A4, A14 and A16, C4 strain also show to be related to coxsackievirus A5 and B subgenotype of the EV71 in the P2 region but not P3 support evidence of double recombination. In contrast to C4 strain detected, the B5 stain in Thailand show consistently high homology with other B genotype of the EV71. The high similarity only account for B genotype but not C or A genotype, the prototype strain, of EV71 which suggest an evidence of intertypic recombination which occurred in the past before it independently evolve. The P2 region of B5 strain show to be related to coxsackievirus A4, A14, A16 and A5 which was similar to what was observed for C4, which may suggest a characteristic of modern EV71 strain where it evolves away from the BrCr prototype strain. The Simplot analysis of Thailand EV71 B5 indicated higher similarity to other prototype strains of HEV-A, especially coxsackievirus A5, when compare to it prototype.

The differences in origin between different regions of the genome demonstrated in this study might be explanation of the variety of clinical manifestations and severity of the disease detected from the same EV71 serotype. The capsid proteins may be best representatives in identifying the virus; nevertheless, it represents only a single region of a genome and with constant evolution through mutation and recombination producing various variants, the rest of the genome could be unpredictable. Typing of an enterovirus might require a new approach that would examine different parts of the genome to better identify the virus and avoid misidentification of the viral genetic.

CHAPTER 4

Summary

This thesis focus on examined prevalence of multiple enterovirus serotypes, a major causative agent of HFMD, in Thailand from 2008 to 2013 and a complete genome characterization of EV71.

HFMD and HA are frequently associated with enterovirus infection in many Asia Pacific countries and infection could resulted in major public health issues. In the last decade there are an increasing numbers of reports on HFMD outbreaks. Due to absent of proof reading mechanism in enterovirus, the virus evolves rapidly resulted in an outbreak occurring at regular cycle regulating with continuous intra- and intertypic shift of different subgenotype and serotype of enterovirus. Among all 647 positive specimens 93.5% (n=603) were grouped into HEV-A, 5.74% (n=37) were in HEV-B, 0.930% (n=6) were HEV-C and 0.155% (n=1) were in HEV-D. The seasonal distribution of enterovirus could be seperated into two period during January to March and July to September.

EV71 is a unique member of enterovirus as it could potentially infect the central nervous system resulted in neurological complication. Study performed complete genome sequencing on 14 isolates of EV71. The complete genome analyses show distinct separation molecularly between B5 subgenotype of EV71 detected during HFMD outbreak and after HFMD outbreak in 2012. Study further demonstrate both subgenotype C4 and B5 are able to cause severe EV71 infection in patients. Recombination events contribute to diversity in virus population increasing higher potential for more virulent strain to arise. The recombination analyses demonstrated

recombination in one strain of the C4 subgenotype from a fatal case of enterovirus infection. The analysis suggests higher similarity to CV-A4, 14, 16 in P2 and P3 region.

Multiple serotypes of enterovirus, which are now circulating at low prevalence in Thailand, has ability to cause an epidemic therefore all enterovirus should be monitored closely in order to rapidly and effectively control the outbreak. Recombination is a common phenomenon in enterovirus, identification of multiple genes in the viral genome could help us better understand the virus true identity. Together these data are crucial and contribute to prevent and control the outbreak.

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APPENDIX

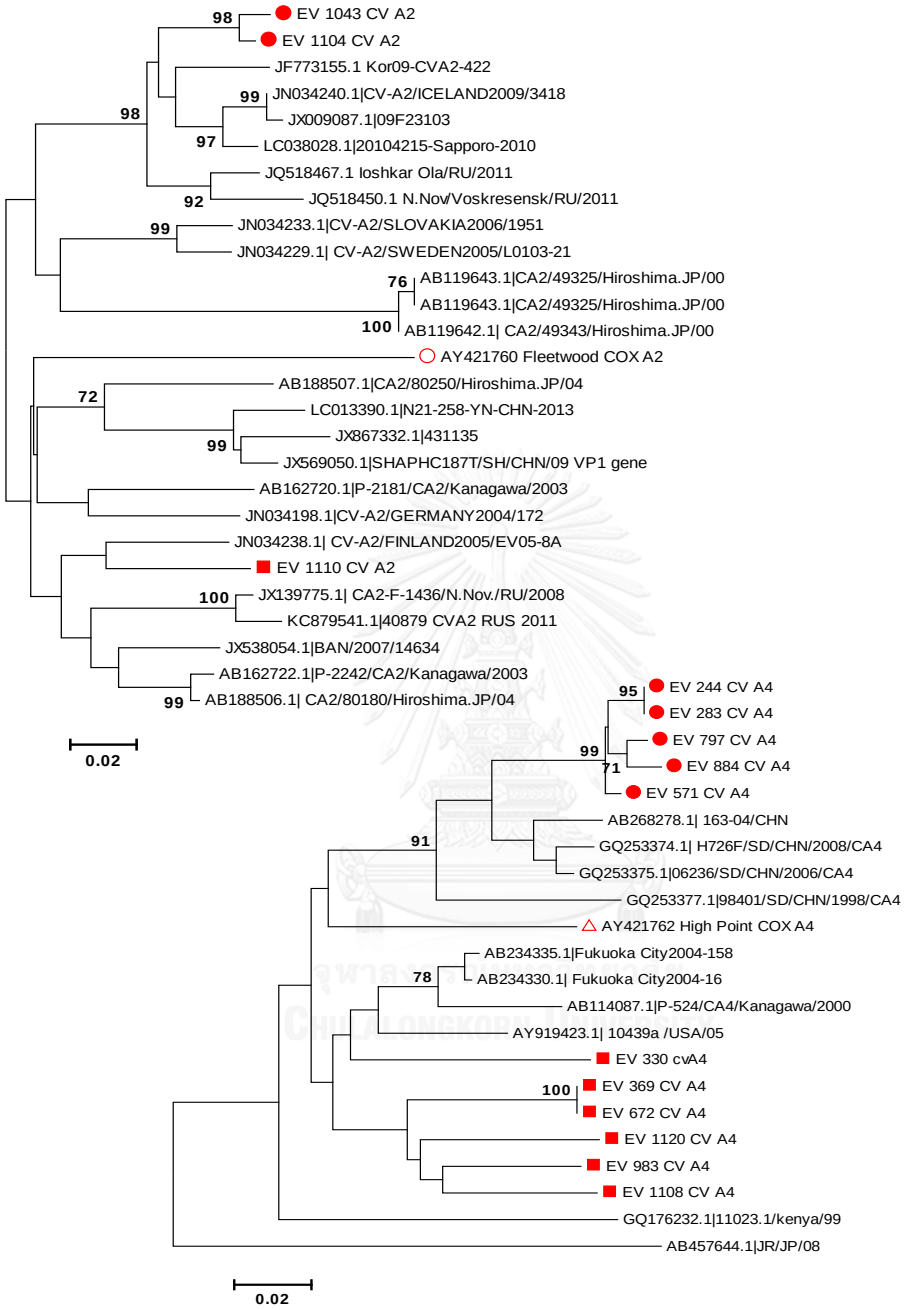

Figure A1: *Phylogenetic tree representing clustering of CV-A2 and CV-A4 isolates with reference strains obtained from Genbank. The isolates are labeled in circle and square with prototype strain labeled in triangle The partial VP1 sequences were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.*

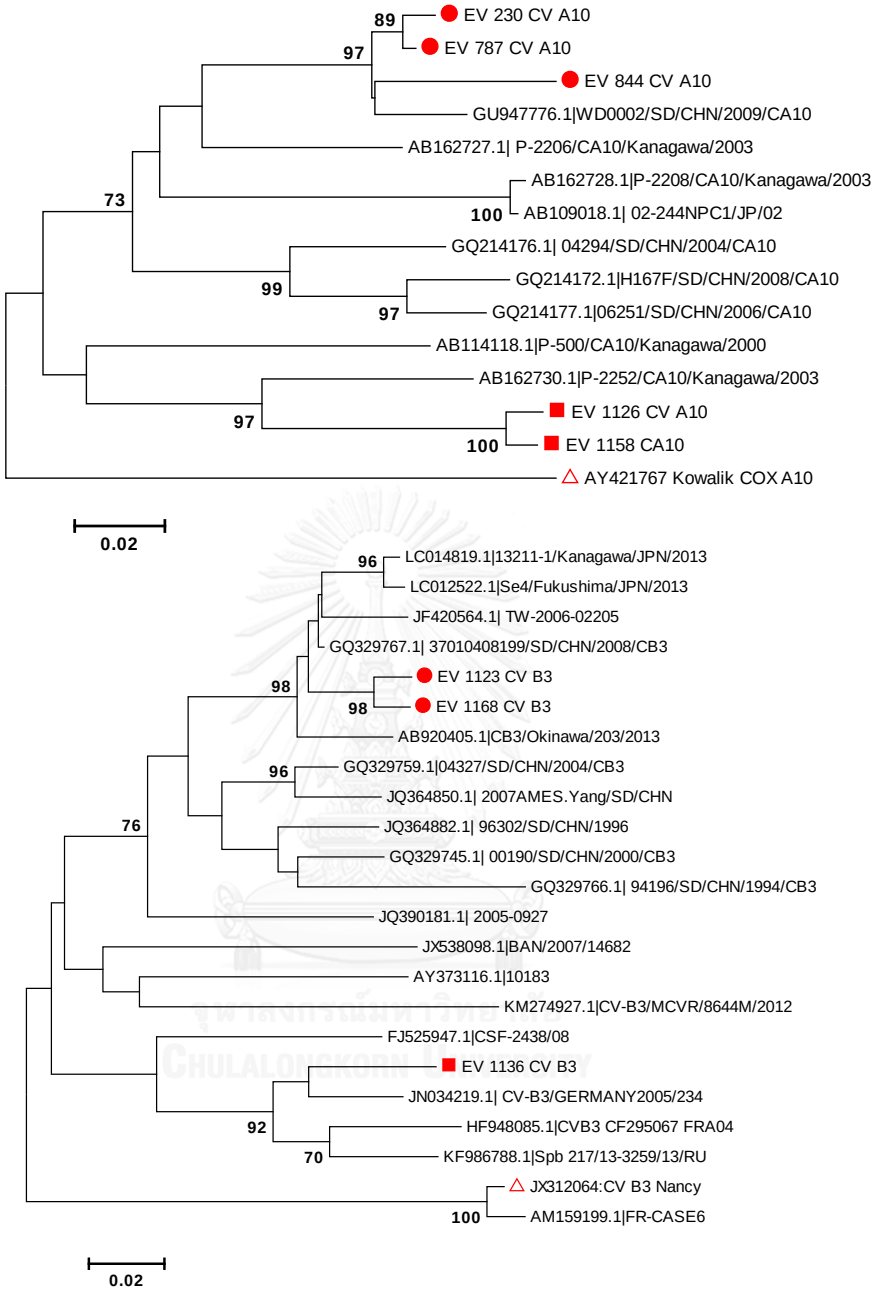

Figure A2: *Phylogenetic tree representing clustering of CV-A10 and CV-B3 isolates with reference strains obtained from Genbank. The isolates are labeled in circle and square with prototype strain labeled in triangle The partial VP1 sequences were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.*

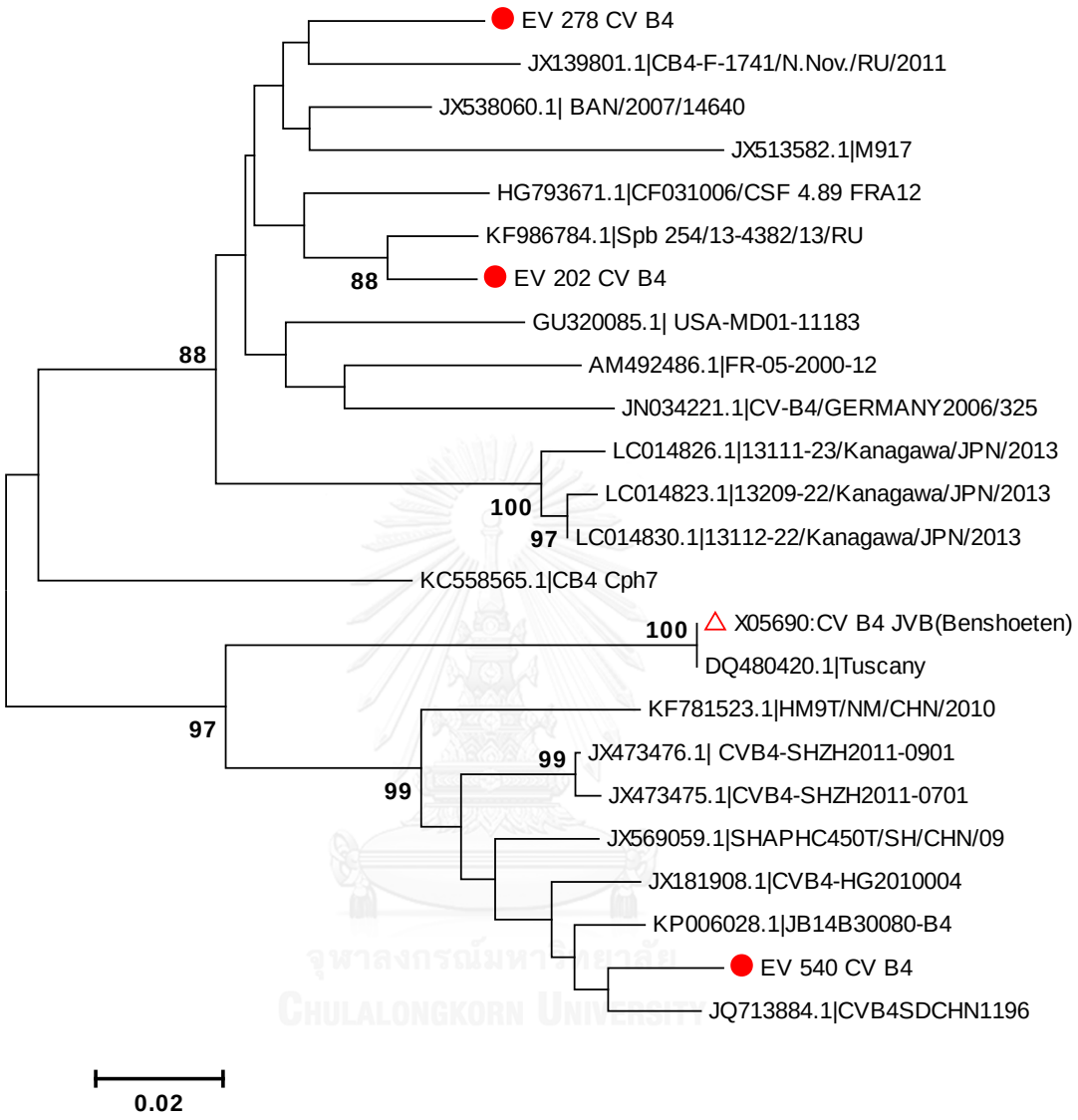

Figure A3: *Phylogenetic tree representing clustering of CV-B4 isolates with reference strains obtained from Genbank. The isolates are labeled in circle and square with prototype strain labeled in triangle The partial VP1 sequences were aligned to construct a neighbor-joining tree with percentage of bootstrap replicates supporting the tree indicate at the nodes. The branches lengths are proportional to genetic distances corrected using Kimura-two-parameter substitution model.*

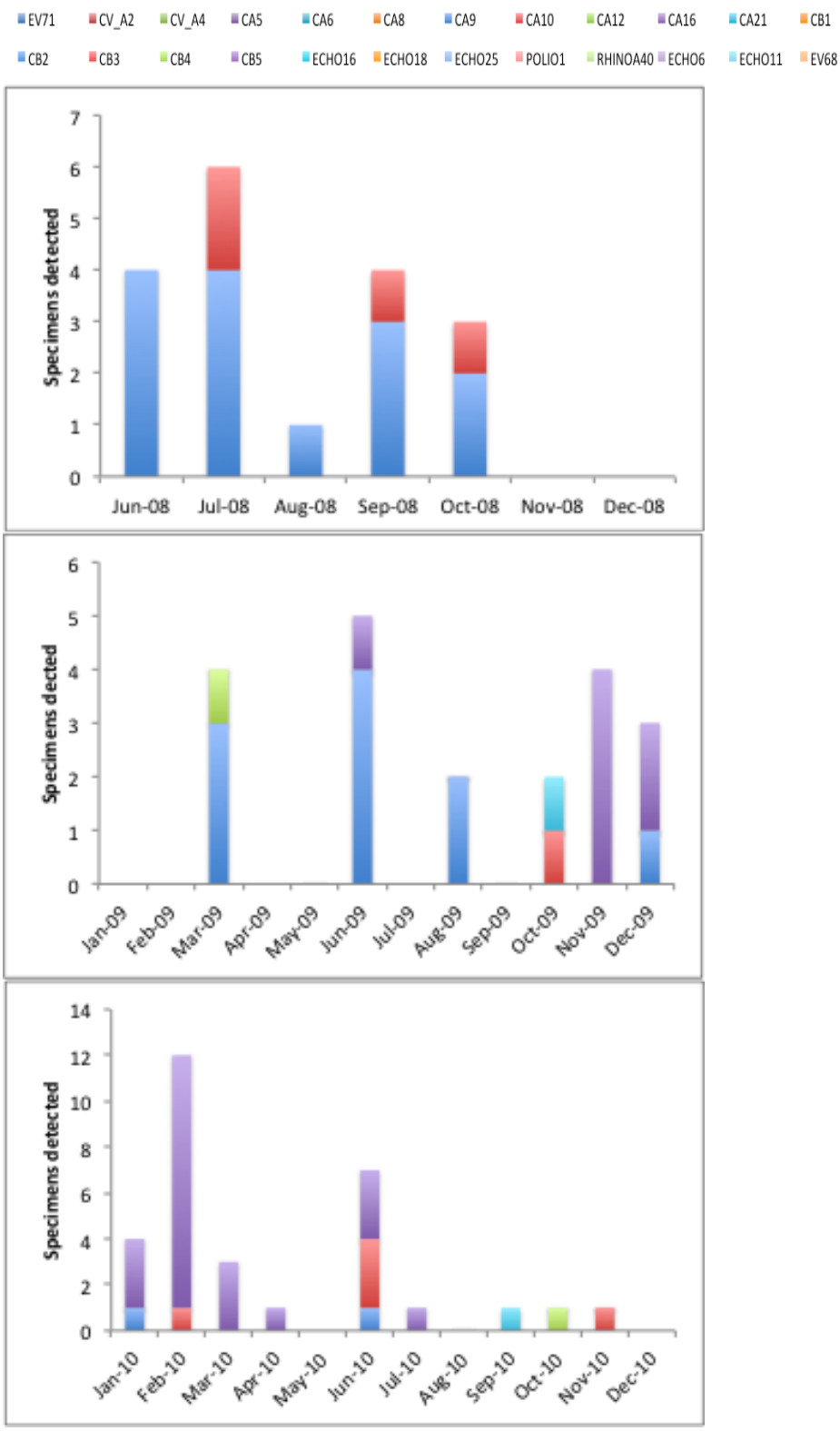

Figure A4: *Graph representing detected enterovirus serotypes from 2008-2010*

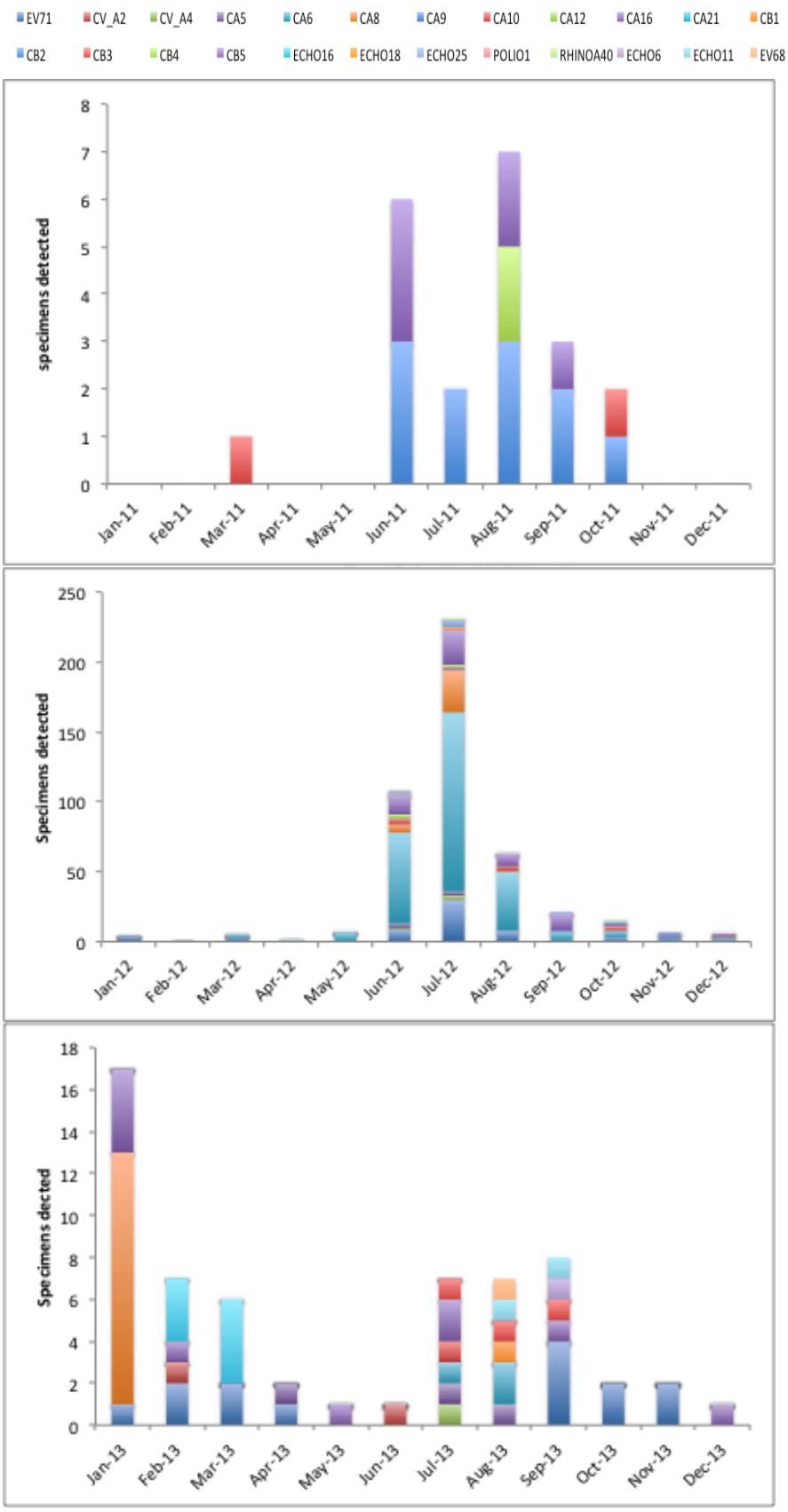

Figure A5: *Graph representing detected enterovirus serotypes from 2011-2013*

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