ระบาดวิทยาเชิงโมเลกุลของเชื้อฮิวแมนเอนเทอโรไวรัสในตัวอย่างทางคลินิกที่สงสัยว่าติดเชื้อเอนเทอโรไวรัส

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MOLECULAR EPIDEMIOLOGY OF HUMAN ENTEROVIRUS IN VARIOUS CLINICAL SPECIMENS WITH SUSPECTED ENTEROVIRUS INFECTION

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	MOLECULAR EPIDEMIOLOGY OF HUMAN ENTEROVIRUS IN VARIOUS CLINICAL SPECIMENS WITH SUSPECTED ENTEROVIRUS INFECTION
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Field of Study	Medical Science
Thesis Advisor	ProfessorYong Poovorawan, M.D.

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จิระ จันท์แสนโรจน์ : ระบาดวิทยาเชิงโมเลกุลของเชื้อฮิวแมนเอนเทอโรไวรัสในตัวอย่างทางคลินิกที่สงสัยว่าติดเชื้อเอนเทอโรไวรัส (MOLECULAR EPIDEMIOLOGY OF HUMAN ENTEROVIRUS IN VARIOUS CLINICAL SPECIMENS WITH SUSPECTED ENTEROVIRUS INFECTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ.ยง ภู่วรวรรณ, 125 หน้า.

โรคติดเชื้อหลายโรคที่ส่งผลกระทบต่อปัญหาด้านการสาธารณสุข มีสาเหตุมาจากการติดเชื้อเอนเทอโรไวรัสหลากหลายจีโนไทป์ มีหลายงานวิจัยจากทั่วโลกที่ได้รายงานถึงการอุบัติใหม่และอุบัติซ้ำของเชื้อไวรัสนี้ ซึ่งในปัจจุบันยังไม่มีวัคซีนและยารักษาที่มีประสิทธิภาพ อีกทั้งข้อมูลด้านระบาดวิทยา, ความเกี่ยวข้องกับอาการทางคลินิก และข้อมูลวิวัฒนาการของไวรัสนี้ยังมีอย่างจำกัด โดยเฉพาะในภูมิภาคเอเชีย เพื่อที่จะได้รวบรวมข้อมูลดังกล่าวให้ชัดเจนยิ่งขึ้น การศึกษานี้จึงมีวัตถุประสงค์ที่จะศึกษากลุ่มประชากรที่เป็นโรคต่างๆที่เกิดจากการติดเชื้อเอนเทอโรไวรัส อาทิเช่น โรคตาแดง, โรคแผลในช่องปาก, โรคมือ เท้า ปาก, โรคท้องร่วงเฉียบพลัน, การปนเปื้อนของเชื้อโปลิโอไวรัส, โรคเยื่อหุ้มสมองอักเสบ โดยทำการตรวจวิเคราะห์ตัวอย่างเพื่อตรวจหาเชื้อเอนเทอโรไวรัสสายพันธุ์ต่างๆ ด้วยวิธี reverse transcription-polymerase chain reaction และ sequencing เพื่อให้ได้ข้อมูลเชิงระบาดวิทยา และวิเคราะห์ข้อมูลทางรหัสพันธุกรรมของไวรัสในการศึกษาวิวัฒนาการที่เกิดขึ้นในระดับชีวโมเลกุล จากการศึกษาพบว่าเชื้อเอนเทอโรไวรัสแต่ละสายพันธุ์ มีความสัมพันธ์ในการก่อโรคที่แตกต่างกัน โดยผลที่ได้พบว่าเชื้อคอกซากี่ไวรัส A24 เป็นสาเหตุในการเกิดโรคตาแดง, เชื้อเอนเทอโรไวรัส สายพันธุ์ A เป็นสาเหตุในการเกิดโรคแผลในช่องปาก และโรคมือ เท้า ปาก, เชื้อเอนเทอโรไวรัสสายพันธุ์ B เป็นสาเหตุในการเกิดโรคเยื่อหุ้มสมองอักเสบ, และเชื้อเอนเทอโรไวรัสหลากหลายชนิดที่เกี่ยวข้องในการเกิดโรคท้องร่วงเฉียบพลัน ซึ่งจากข้อมูลที่ได้สามารถนำมาใช้เป็นข้อมูลสำหรับการตรวจเชื้อเอนเทอโรไวรัสสายพันธุ์ต่างๆที่แพร่กระจายอยู่ในประเทศไทย งานวิจัยนี้ยังได้ทำการศึกษาวิวัฒนาการของเชื้อโปลิโอไวรัสและเชื้อเอนเทอโรไวรัสที่ก่อโรคในผู้ป่วยเยื่อหุ้มสมองอักเสบ ผลการวิจัยพบว่า อัตราการเปลี่ยนแปลงทางวิวัฒนาการของเชื้อโปลิโอไวรัสเท่ากับ $3.7x10^{-4}$ s/s/y ซึ่งเป็นค่าที่ต่ำกว่าอัตราการเปลี่ยนแปลงในเชื้อโปลิโอไวรัสสายพันธุ์วัคซีนก่อโรค จึงเป็นการสนับสนุนว่าการเปลี่ยนนโยบายการให้วัคซีนโปลิโอแบบหยอดมาเป็นแบบฉีดนั้นมีประสิทธิภาพ และใกล้ที่จะสามารถกวาดล้างโปลิโอไวรัสจากประเทศไทยได้ ส่วนในโรคเยื่อหุ้มสมองอักเสบ ผลอัตราการเปลี่ยนแปลงทางวิวัฒนาการอยู่ที่ $1.33x10^{-3}$ s/s/y และผลการวิเคราะห์ทาง phylogeographic แสดงให้เห็นว่าประเทศไทยได้รับการแพร่กระจายของไวรัสสายพันธุ์นี้มาจากทางทวีปยุโรป ในขณะที่ไม่มีการรับสายพันธุ์ที่มาจากทางทวีปเอเชีย ดังนั้นจึงสามารถเป็นตัวอย่างในการศึกษารูปแบบการแพร่กระจายจากทั่วโลกของไวรัสที่มีผลกระทบต่อการเกิดโรคต่างๆได้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: ENTEROVIRUS / MOLECULAR EPIDEMIOLOGY / EVOLUTION

JIRA CHANSAENROJ: MOLECULAR EPIDEMIOLOGY OF HUMAN ENTEROVIRUS IN VARIOUS CLINICAL SPECIMENS WITH SUSPECTED ENTEROVIRUS INFECTION. ADVISOR: PROF.YONG POOVORAWAN, M.D., 125 pp.

Many infectious diseases have been well recognized caused by several genotypes of human enterovirus species which significantly in public health. Publications worldwide have reported on the emerging and re-emerging of enteroviruses. Until now, epidemiological surveillance data, clinical complication and evolutionary history regarding these viruses in actual on the Asia continent are still limited. To address these concerns, this study aimed to establish comprehensive population-based surveillances, and provided evidence for the evolution in many diseases such as acute haemorrhagic conjunctivitis, herpangina, hand foot mouth disease, acute gastroenteritis, poliovirus and meningitis caused by enteroviruses. Employing reverse transcription-polymerase chain reaction and sequencing approaches for detection, our study revealed that each enterovirus has a specific to cause different disease. Results displayed that infections by coxsackie A24 caused acute haemorrhagic conjunctivitis, enterovirus species A caused herpangina and hand foot mouth disease, enterovirus species B caused meningitis, various of enterovirus species associated with acute gastroenteritis. These represent the surveillance data of multiple enteroviruses circulate in Thailand. Moreover, this study also investigated the evolutionary relationship based on the analysis of the VP1 gene of poliovirus vaccine strain and meningitis. For poliovirus surveillance, this study did not found positive selective pressure and evolution rate is $3.7x10^{-4}$ s/s/y which lower than rate of vaccine derived poliovirus strain. This study supported that transition of oral to inactivated poliovirus vaccine is effective and nearly complete eradication. For meningitis cases, the evolution rate is $1.33x10^{-3}$ s/s/y. The phylogeographic analysis revealed that this virus transmitted from Europe and did not found strain from Asia. This study revealed the potential of etiologic agent and transmission pattern that circulated worldwide.

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

Academic Year: 2017 Advisor's Signature .....

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List of Abbreviations

Abbreviation	Full name
α	Alpha
β	Beta
µ	Micro
µg	Microgram
µl	Microliter
%	Percent
◦C	Degree Celsius
3’UTR	3’ untranslated region
5’UTR	5’ untranslated region
aa	Amino acid
ADV	Adenovirus
AFP	Acute flaccid paralysis
AGE	Acute gastroenteritis
AHC	Acute hemorrhagic conjunctivitis
BBB	Blood-brain barrier
BEAST	Bayesian Evolutionary Analysis Sampling Trees
BLAST	Basic local alignment search tool
bp	Base pairs
CAR	Coxsackie-adenovirus receptor
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CODEHOP	Consensus degenerate hybrid oligonucleotide primer
CNS	Central nervous system
CSF	Cerebrospinal fluid
Ct	Threshold cycle
CV-A	Coxsackievirus A
CV-B	Coxsackievirus B
DAF	Decay accelerating factor

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
Echo	Echovirus
ESS	Effective sample size
EV	Human Enterovirus
EV-A	Human enterovirus group A
EV-B	Human enterovirus group B
EV-C	Human enterovirus group C
EV-D	Human enterovirus group D
FEL	Fixed-effects likelihood model
g	Grams
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCC	Global Commission for the Certification of Poliomyelitis Eradication
GIS	Geographic Information System
GPEI	The Global Polio Eradication Initiative
HA	Herpangina
HFMD	Hand, foot and mouth disease
HPD	Highest posterior density
hr	Hour
HRV	Human rhinovirus
HTUs	Hypothetical taxonomic units
ILI	Influenza-like illness
IPV	Inactivated polio vaccine
IRB	Institutional Review Board
IRES	Internal ribosome entry site
IVIG	Intravenous immunoglobulin
kb	Kilobase
LRTs	Likelihood ratio tests
M	Molar
MCC	Maximum Clade Credibility
MCMC	Markov Chain Monte Carlo

MEGA	Molecular evolutionary genetics analysis
NCBI	The National Center for Biotechnology Information
NESS	National Enterovirus Surveillance System
NGS	Next Generation Sequencing
NJ	Neighbor-joining
NPEV	Non-polio enterovirus
NS	Non-structural protein
nt	Nucleotide
NTP	Nucleotide triphosphate
OPV	Oral polio vaccine
ORF	Open reading frame
OTUs	Operational taxonomic units
p-value	Probability value
PCR	Polymerase Chain Reaction
Poly(A)	Polyadenylated
PV	Poliovirus
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNAse	Ribonuclease
RT	Reverse transcription
RV	Rotavirus
SAGE	Strategic Advisory Group of Experts on Immunization
SLAC	Single likelihood ancestor counting model
SPREAD	Spatial Phylogenetic Reconstruction of Evolutionary Dynamics
ss	Single-stranded
tMRCA	The most common recent ancestor
tOPV	trivalent oral polio vaccine
VDPV	vaccine-derived poliovirus
VLPs	Virus-like particles
VPs	Viral proteins
WHO	World Health Organization
WPV	Wild poliovirus

CHAPTER I
GENERAL INTRODUCTION

Background and rationale

Human enterovirus (EV) can cause various diseases. They usually cause mild infections ranging from subclinical such as cutaneous, visceral and mild gastroenteritis but can also affect the heart, pancreas and central nervous system (CNS). In some cases, it can cause severe systemic inflammatory disease. It can be fatal or result in lasting organ dysfunction. It can causes of morbidity in all ages. Children less than five years old are the major sector of the affected population.

Due to the remaining significant gaps of knowledge in EV, this study will focus on the molecular epidemiology of EV associated with several distinct diseases herpangina (HA), acute gastroenteritis (AGE), acute hemorrhagic conjunctivitis (AHC), meningitis, encephalitis and surveillance of escape poliovirus vaccine in stool. These data will provide a better understanding of the prevalence of human enterovirus and will be critical for the successful implementation of new strategies for disease control and prevention. Moreover, human enterovirus has a complexity of the evolutionary processes associated with its geographical expansion and the occurrence of a number of recombination events each involving replacement at varying times since the founder recombinant form. The understanding of the recombination and evolution pattern will help ascertain their possible origin and predict the pandemic in the future. Hence, this study will reveal the recombination, mutation and evolution of human enterovirus variants. Data from this study will help predict the evolutionary pattern of human enteroviruses that may affect the re-emergence in epidemic region. As human enterovirus are driven by immunological and host adaptive factors, this study will assist in the understanding of human enterovirus evolution and the prediction of associated clinical outcomes.

Objectives

From hypothesizes that there are other enteroviruses play an important role in each disease. Therefore the objectives of this study were to:

(i) Characterize epidemiological profile of enterovirus in each disease.

(ii) Achieve the evolutionary history of these distributed viruses.

Hypothesis

Seemingly, different types of EV can cause the same disease with specific clinical manifestations. Categorizing viruses into genotypes and groups most often found in association with particular diseases will enable us to predict the pathogenesis of infection. It is possible that the dominant genotype will drive the epidemic events. Moreover, the recombination or variation events may happen from the accumulation of evolution in the previous epidemic areas, which results in clinical manifestation or re-emergence.

This study plan to achieve the overall objective by pursuing these studies as follows;

Part 1: Molecular epidemiological study of enterovirus among patients with acute haemorrhagic conjunctivitis.

Part 2: Molecular epidemiological study of enterovirus among patients with herpangina.

Part 3: Molecular epidemiological study of enterovirus among patients with hand foot mouth disease and acute gastroenteritis.

Part 4: Surveillance of poliovirus vaccine strain contaminate and evolution dynamic of poliovirus.

Part 5: Molecular characterization, evolution dynamic and phylogeographic of enterovirus among patients with meningoencephalitis.

Significance of the study

At the present, EV is still one of the problems worldwide especially in developing countries. Hence, increasing evidence indicates that epidemiology and evolution analysis may play a crucial role to help prevention of enterovirus infections. A detailed knowledge of the epidemiology and observation of evolution rates may lead to better understanding of viral pathogenesis and the development of new therapeutic approaches for the treatment of each disease. Continued surveillance of probable virus-associated infection is essential in assisting awareness and facilitating disease prevention and control.

Conceptual framework

EV in each disease has a different proportion of enterovirus species. Therefore, the identification of possible proportion in each species may be useful. The re-emergence

may be caused by evolution events, sequencing variation analysis will help to improve knowledge and predict pandemic in the future.

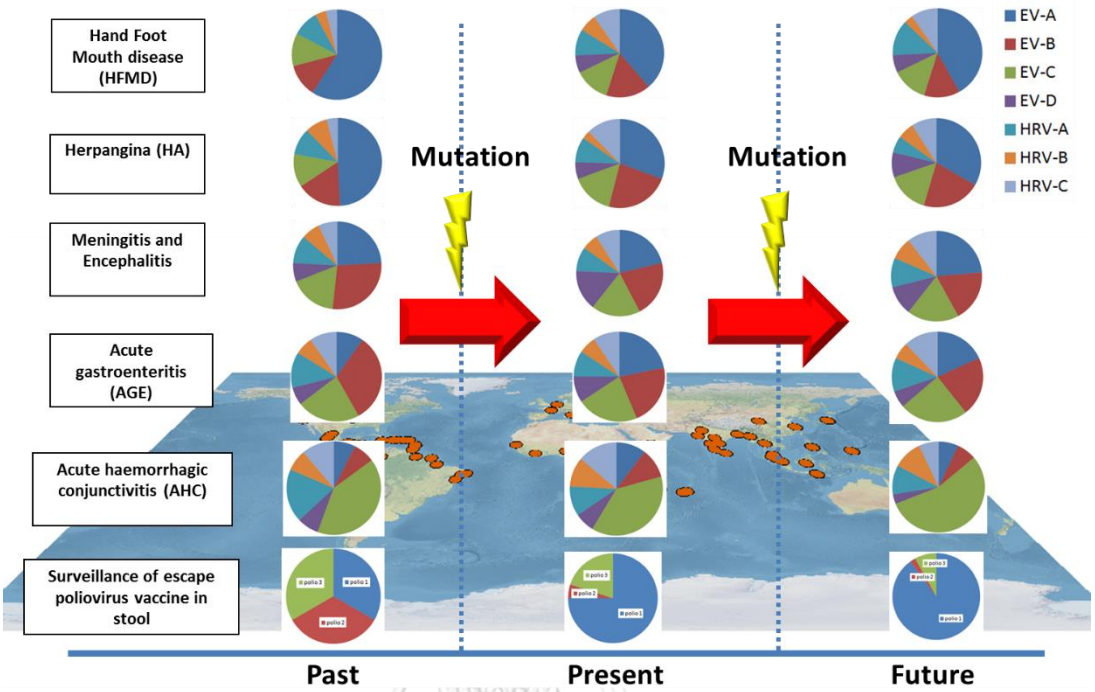

Figure 1 The variation of EV distribution in different timelines.

Experimental design

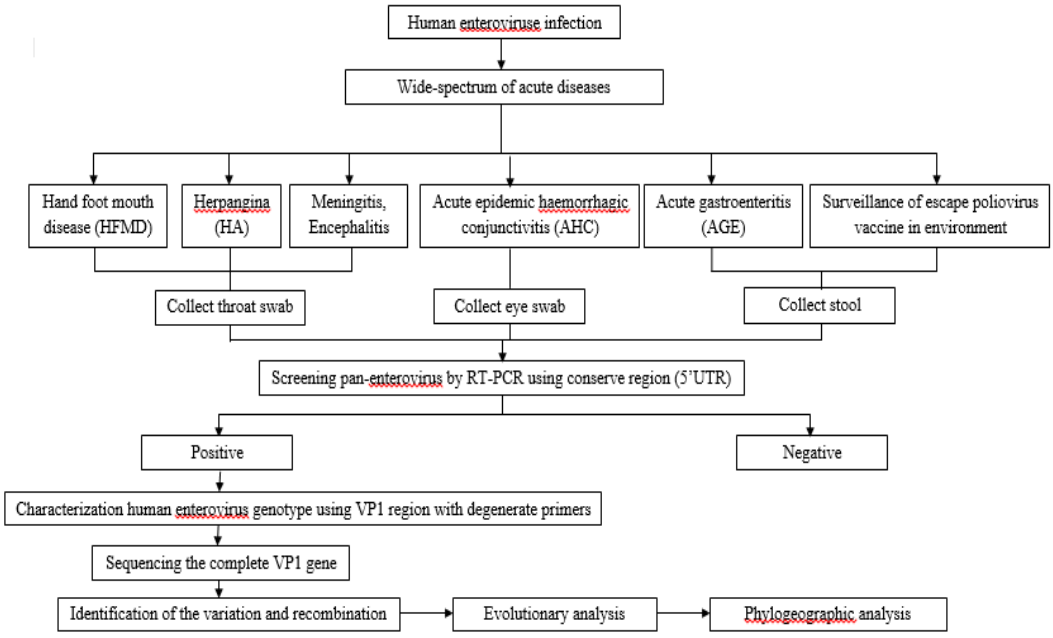

Figure 2 Procedure framework of the present study.

Ethical Consideration

This study have been approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University. The Belmont Report identifies three fundamental ethical principles for any human subject research:

1. Respect for persons: This study will use specimens which remaining from routine check-up, not involving patients. No informed consent for participants. The patient's data will be miscellaneous, unable to track data back. Investigator known only sex, age and clinical manifestation.
2. Beneficence and non-malfeasance: The patients will not have directly benefit. This study will provide epidemiological data for surveillance of human enterovirus in Thailand. Moreover, it may provide understanding for evolution of human enterovirus for predicting the re-emergence in the future.
3. Justice: This study will not select specimens. This study involving not greater than minimal risk for patient's specimen.

Limitations

The current study was limited by some of specimens which remaining from routine check-up will not sufficient for this study and some participant's information is not complete.

CHAPTER II
LITERATURE REVIEW

2.1 Introduction

Enteroviruses are members of the *Picornaviridae* family, are non-enveloped viruses which genome about 7400 – 7500 nt length differ for strains. Their genome has a large 5'UTR (about 750 nt) and 3'UTR (about 100 nt) which harbours secondary structural elements essential for RNA replication and cap-independent initiation of translation. The RNA accounts for 30% and protein coat about 70% of the weight of the virion. The genome is translated as a single large polyprotein then is cleaved co- and post-translationally by viral protease into four capsid proteins (VP1-VP4) and seven non-structural proteins. Firstly, researchers believed that alimentary tract was a natural habitat for these viruses, therefore, the name enterovirus. When more viruses were identified and association with human diseases was not known, they were grouped as enteric cytopathogenic human orphan or "ECHO" viruses. The enterovirus genus includes over 70 serotypes. Recently, EV was re-classified into four species (A-D), based on their phylogenetic relations of viral polypeptide capsid (1). In each specie, their share >70% amino acid identity in P1, >70% amino acid identity in non-structural protein 2C+3CD, a limited range of host cell receptors, a limited natural host range, a genome base composition (G+C) which varies by no more than 2.5% and significant degree of compatibility in proteolytic processing, replication, encapsidation and genetic recombination. A new type of EV is normally defined by less than 75% nucleotide and 85% amino acid sequence identity across VP1 region (2).

EV infections are asymptomatic approximately 50% while young age is more frequency of symptomatic. EV cause a wide range of clinical manifestations (3). A majority infections are subclinical, common cause of self-limiting febrile illness in infants and young children but also can cause other disease, including hand foot mouth disease, acute haemorrhagic conjunctivitis. However, EV can occasionally cause severe neurological disease including meningoencephalitis, myelitis, myocarditis, sepsis which also associated with outbreak resulting in significant

morbidity and mortality. Therefore, age is an important factor of susceptibility and clinical outcome.

EV is ubiquitous viruses that primarily transmitted via the fecal-oral or respiratory route and symptoms may develop after an incubation period of 3-21 days. Infection is acquired orally and viral replicates which different replication capacity in different cells. EV resist lipid solvents, ether, chloroform and alcohol. EV is retain activity for several days at room temperature, inactivate at temperatures above 50°C, can be stored indefinitely at freezer and also stable at the low pH and grow rapidly when adapted to susceptible host systems. The primary site of replication is in the intestinal mucosa of the gastrointestinal tract or respiratory tract (Peyer patches, mesenteric nodes, tonsils) (4). Their genome possess higher percentage of average guanine and cytosine (G+C) composition which are suggested to be an essential genomic factor for virus adaptive capability to replicate in various parts of the human body such as respiratory tract, gastrointestinal tract and central nervous system (5). Virus entry into susceptible host cells via several processes, including viral surface attachment, receptor binding and through into host cell by endocytotic pathway. Subsequently, viral is uncoated and viral RNA is released into the cytoplasm. EV can invade the CNS through three main mechanisms: 1. Retrograde axonal transport, infect the peripheral nerve and gain access into the CNS via retrograde axonal transport and trans-synaptic spread; 2. Blood-brain barrier (BBB) penetration, cross the BBB through disrupted tight junctions during viremia; 3. “Trojan-horse” invasion, invade the CNS through virus-infected immune cells which act as carriers to deliver virus (6-8).

Virus can shed for up to several weeks in oral or several months in the feces by symptomatic and asymptomatic persons. Therefore, should be collect sample as soon as possible after symptoms onset according to clinical manifestations where the type of specimens would be most suitable to detect. Moreover, patient age should be taken into account when considering the value of diagnostic sampling because young age is increased risk of severe outcome (9). Proper and early detection, combined with genetic characterization in appropriately specimens is essential (Table 1). Prompt laboratory diagnosis may reduce antibiotic usage, costly investigations, shorten hospitalization and minimize the risk of complications. In addition, it enables health

care providers to respond in a timely fashion with infection control measures as well as to evaluate the usefulness of potential therapies.

Table 1 Type of clinical specimens for EV diagnosis in different clinical manifestations

(Modified from Harvala, H. et al., J Clin Virol, 2018 (10, 11))

Clinical manifestations	**EV genotype most often implicated**	**Clinical features**	**Type of specimen**	**EV diagnosis**
Meningitis/meningoencephalitis	CV-B, echovirus	Fever with meningeal signs, mild CSF pleocytosis	CSF, stool and respiratory	EV RNA detectable in CSF by PCR but inconsistently.
Nonspecific febrile illness	All types	Febrile illness with nonspecific upper respiratory and gastrointestinal tract symptoms	Respiratory, stool	Consider testing both HRV and EV.
Neonatal sepsis	CV-A, EV-A71	Fever, shock	CSF, stool, blood and respiratory	Viral load can be higher in blood than in CSF.
Acute flaccid paralysis (AFP)/myelitis	CV-A, EV-A71	Paralysis but less severe illness and less bulbar involvement than poliovirus	CSF, stool, blood and respiratory	In case of classical AFP (without myelitis), consider also possibility of polio.
HFMD/rash, Herpangina	CV-A, EV-A71, echovirus	Fever with vesicles in the mouth, hands and feet	Vesicle fluid, stool and respiratory	Usually high viral loads in vesicle fluid.

Clinical manifestations	EV genotype most often implicated	Clinical features	Type of specimen	EV diagnosis
Respiratory disease	All types	Fever for 3-4 days, cough, dyspnea, wheezing	Respiratory, stool	Consider testing both HRV and EV.
Myocarditis	CV-B	Myopericarditis presenting with heart failure or arrhythmias	Stool, respiratory, blood, heart biopsy	Tissue biopsy, chest radiography can be used to confirm diagnosis.
Poliomyelitis	Poliovirus	Non-paralytic polio; fever, sore throat, vomiting and fatigue	Stool	Consider wild type poliovirus, vaccine derive poliovirus
		Paralytic polio; sudden paralysis		
Acute haemorrhagic conjunctivitis	CV-A24, EV70	Sudden onset of eye pain with subconjunctival haemorrhage	Eye swab	Viral haemorrhagic conjunctivitis is highly infectious.

EV has distribute worldwide. Epidemics may be localized and sporadic. National Enterovirus Surveillance System (NESS) of the Centers for Disease Control and Prevention (CDC) has been collecting data since the 1960s, has reported that EV detection were found to have remarkable seasonality. More prominent EV detection in summer-fall season (11). The major point for prevention is to block viral transmission and prevent severe complications and death. Although similar types and sublineages of enterovirus are slightly different in their genetic background, clinical manifestation and severity are different and therefore, hamper the differentiation between specific virus infections and their clinical consequences based on the sole observation of clinical signs. Treatment of enterovirus infections include supportive care for the affected individual and prevention for others, aimed at relieving symptoms because

there is no specific therapy for enterovirus infection and vaccination is not available now, so practicing good personal hygiene is good prevention. Meanwhile, intravenous immunoglobulin (IVIG) treatment has been used therapeutically and prophylactically in chronic EV meningoencephalitis or life-threatening cases (12).

In theory, inactivated or live, attenuated vaccines can be developed against non-polio enterovirus (NPEV) such as EV-A71 vaccine that is currently marketed in China (13) or combining 50 inactivated rhinovirus type into single vaccine elicited neutralizing antibodies against 49 types will be developed (14). Another encouraging approach is the use virus-like particles (VLPs), which assemble co-expression of capsid protein and $3CD^{pro}$. Although EV VLPs are differ slightly in their structure, antigenic properties and stability but these problem can be avoided by introducing specific stabilizing capsid mutations (15).

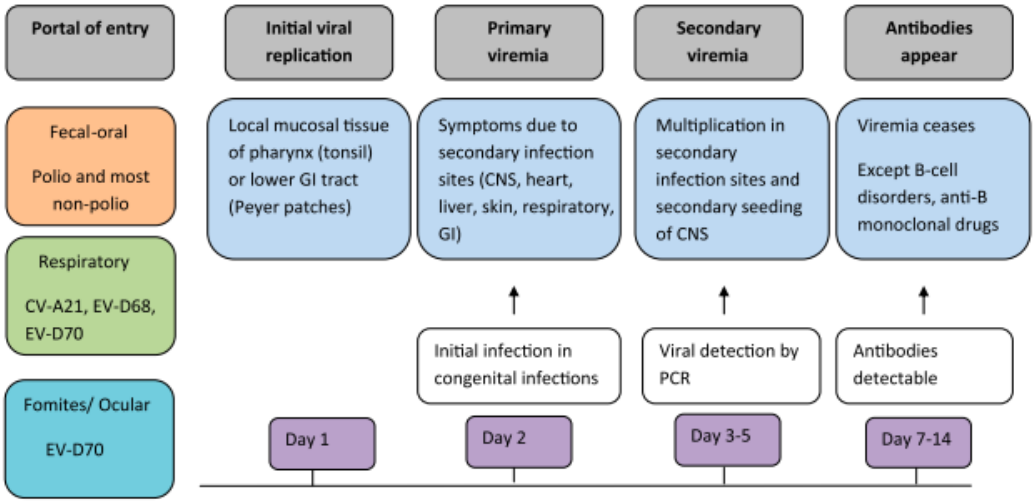

Figure 3 Timeline of events in enterovirus infection (11).

For the EV detection, classical serotyping methods such as serum neutralizing are not sufficient to specify all genotypes. Molecular techniques such as RT-PCR has been shown to be more sensitive and successful EV isolated to the corresponding genotype in clinical samples. It mainly target the highly conserved 5'UTR which suitable for screen all EV types due to their sensitivity, specificity and short turnaround time while other region use for defined EV types only, while VP1 has been suggested for EV genotyping (Table 2) (10, 16, 17).

Table 2 Diagnostic and typing assays for EV
(Modified from Harvala, H. et al., J Clin Virol, 2018 (10))

Test	Target	Characteristics
Screening	5'UTR	Primary assay for EV detection which detected all EV types.
	VP4, VP1	Rapid screening for defined EV types specific, most likely to be used in outbreak situations.
Virus typing	VP1	A minimum of 350 nt in VP1 sequence is required for surveillance by reference laboratories while complete VP1 is necessary when assign new genotypes.
	VP4, VP2	In case of VP1 region fails to amplify.
	3D	Identification of new recombination events and the emergence of recombinant EV.
	Whole genome	Next Generation Sequencing (NGS) methods will also enhance the detection of mixed infections.
Virus isolation	Whole virus	Competency in virus culture is required but not suitable as a front line screen.
Virus neutralization	Isolated virus	Typing antisera are increasingly unavailable and limited in range.
Serology	IgM, IgG	IgM antibody response is not always detectable during acute disease. However, the diagnostic utility of antibody detection using these available assays should also be explored.

One of the key mechanisms in EV is recombination which is a frequent event between EV types within the same species and usually occurs between structural and non-structural regions in EV species A-C. Circulating EV naturally recombine every few years within a species and capsid encoding genome region evolves virtually independently from the genome region that encodes non-structural proteins.

2.2 Clinical manifestation

2.2.1 Acute hemorrhagic conjunctivitis (AHC) was first described in 1969. The enterovirus 70 and coxsackievirus A24 are the common etiologic agents during epidemics. They are highly contagious and are transmitted via direct or indirect contact with eye secretions. Symptoms of conjunctivitis include ocular pain, swelling of the eyelids, irritation and eye discharge. Recovery within 7 – 10 days. Outbreaks are often associated with close contact in community settings, such as schools, prisons and swimming pools. Many countries have reported extensive outbreaks of AHC due to CV-A24v (18, 19), a member of the enterovirus species C initially isolated during an epidemic in Singapore. Viral conjunctivitis in Thailand occurs throughout the year, but increases during the rainy season. Previous AHC outbreaks appear to be cyclical.

2.2.2 Hand Foot and Mouth Disease (HFMD) was first identified in New Zealand in 1957 and subsequently has been reported across the Asia-Pacific region where it is now endemic in Malaysia, Taiwan, China, Singapore and Japan. Symptoms are usually mild. Multiple enterovirus species A strains infect young children under 5 years old (20). The common manifests as a fever, rash and vesicular skin rash on hands and feet along with vesicles in the oral cavity, hands are involved more commonly than feet. It most commonly affects school-age children. The most recognized virus associated with HFMD is EV-A71, which is responsible for more severe outcomes. EV-A71 was first isolated in California, 1969. It caused several large outbreak in Asia while has also been detected in the United States and Europe, but incidence and lethal cases are occasionally reported. It responsible for severe neurological and cardiopulmonary outcomes which develop as complications of HFMD. CV-A16 is another genotype within EV-A species which characterized in HFMD infection but usually presents with only mild symptoms. Other viruses associated with HFMD are CV-A6 and CV-A10. Infection generally resolves within few weeks. The systematic review summarized the risk factors involved (21).

2.2.3 Herpangina (HA) is a primarily pharyngeal infection in children caused by human enterovirus, mainly by coxsackievirus A. It is mucous membrane disease, characterized by multiple oral ulcers predominantly on the soft palate and the posterior of the oral cavity. These lesion start as papules, become vesicles and ulcerate in a short period of time. The most common site is the anterior tonsillar

pillars. The onset is sudden, with high temperatures. Although symptoms often spontaneously resolve within 1-2 weeks, infection contributes significantly to childhood morbidity around the world. Previous reports in other countries have continued to report outbreaks of HA associated with CV-A2 and CV-A4 including Taiwan in 2008, mainland China in 2009-2014, and Korea in 2009.

2.2.4 Acute gastroenteritis (AGE) caused by viral infection contributes significantly to childhood morbidity and is the third leading cause of death in young children annually. Epidemiologically, certain viruses are more commonly associated with childhood diarrhea. Although viruses most commonly associated with acute gastroenteritis are human rotavirus, norovirus, and adenovirus, the etiology of a significant proportion of gastroenteritis remained undiagnosed. Even so, the etiology of a significant proportion of gastrointestinal illness remains undiagnosed, especially in developing countries. Many human enterovirus are also responsible for a significant proportion of diarrhea-related diseases and are often examined when stools are negative for all common diarrhea viruses in children. The previous cohort studies revealed that enterovirus have been detected in 6-38% of children with acute and persistent diarrhea and throughout the year. The prevalence is highest in the rainy season.

2.2.5 Poliomyelitis caused by poliovirus. Although polioviruses are clustered in EV-C, but they assigned to a distinct species in recognition of their biological uniqueness as agents of poliomyelitis. Polioviruses colonize and replicate in the intestine and commonly excreted from the gut into stool for several weeks after vaccination. Therefore, it is the main source of contamination in the stool. Most infections are asymptomatic or mild. Acute paralytic disease, rapidly onset of paralysis after minor febrile illness. The paralysis is asymmetric and affects the proximal muscles more than the distal muscles and lower limbs are more frequently affected. Poliomyelitis outbreaks were widespread since the end of the 19th century until middle of the 20th century. Anti-poliomyelitis vaccines was developed and widely used which decreased the incidence level by 99%. World Health Organization (WHO) launched the Global Polio Eradication Initiative (GPEI) to interrupt wild poliovirus (WPV) transmission worldwide through the use of extensive immunization campaigns with live attenuated oral polio vaccine (OPV). Until 20th September 2015,

the Global Commission for the Certification of Poliomyelitis Eradication (GCC) certified that the WPV type 2 has been eradicated worldwide (22). Then, WHO endorsed a plan that calls for the ultimate withdrawal of OPV from all immunization programs globally. The withdrawal would begin in a phased manner with the removal of type 2 component of OPV in May 2016 through a global switch from trivalent OPV to bivalent OPV (containing only types 1 and 3). To mitigate risks associated with immunity gaps after OPV type 2 withdrawal, the WHO Strategic Advisory Group of Experts has recommended that all 126 OPV-only using countries introduce at least one dose of inactivated polio vaccine into routine immunization programs by the end of 2015, before the trivalent OPV-bivalent OPV switch. The introduction of inactivated polio vaccine would reduce the risks of reintroduction of type 2 poliovirus by providing some level of seroprotection, facilitating interruption of transmission if outbreaks occur, and accelerating eradication by boosting immunity to types 1 and 3 polioviruses (23, 24). In present, four of the six regions of the WHO have been certified polio-free: America in 1994, Western Pacific in 2000, Europe in 2002 and South East Asia in 2004.

2.2.6 Aseptic meningitis is the most common neurologic disease which common caused by virus especially EV and usually seen in infants and young children (25). It has a high tropism for CNS and various neurological disorders, particularly in infants and children. The most common cause is non-polio enteroviruses, coxsackie A, B and echoviruses. It usually onset with low-grade fever, headache, sore throat, muscle aches, stiff neck, anorexia, nausea, vomiting, photophobia, meningeal signs and CSF pleocytosis. Kerning and Brudzinski signs are often present. The acute illness usually resolves in 1 week. In addition, when clinical evidence of brain involvement is called “encephalitis”, usually occur in newborn. It results from damage of the parenchyma with cytolytic infection of neurons. World Health Organization (WHO) indicated that this disease is responsible around 12% of deaths/year worldwide in children (26). Diagnosis with PCR in CSF considered as the gold standard. The most outbreaks belong to group B enteroviruses (Coxsackie B viruses and various echoviruses) (27).

2.3 Viral genome

The viral genome is comprised of an open reading frame (ORF) flanked by 5' and 3' UTR with poly-A. It covalently linked to small viral peptide at the 5'-uridine of the genome. The 5'UTR length 600-700 nt approximately. This region contains highly structured secondary elements with cloverleaf like motif and internal ribosome entry site (IRES) which necessary for RNA synthesis. The 3'UTR forms the stem loops which can be a putative recognition motif for termination factors and promoted replication (28). It is translated into a large polyprotein with single ORF in the cytoplasm. Their polyprotein is cleaved by viral protease into precursor for structural protein P1 and precursor for non-structural protein P2 and P3. The first cleavage reaction is catalyzed by $2A^{Pro}$ at N-terminus of P1 adjacent to P2 and cleaved into a single protein by $3CD^{Pro}$. The P1 region is encoded for capsid VP1 to VP4 while P2 and P3 regions are encoded for viral protease ($2A^{Pro}$ and $3C^{Pro}$), helicase, membrane associated factor (2C) and RNA-dependent RNA polymerase (RdRp) (4). Viral protein 2B contains two hydrophobic regions, which is crucial for multimerization, and integrates into the membrane of the host Golgi and ER complex to produce virus-induced vesicles and form viral-protein complex. The 2C protein is a multifunctional protein of 329 amino acid residues and contains many inter-species conserved motifs including membrane, RNA, and NTP binding sites, which are crucial for decapsidation process, host cell membrane rearrangement, genome replication and virus particle encapsulation. Protein 3A is a membrane binding protein that plays a role in inhibiting cellular protein secretion. Viral protein 3B is a small peptide (21-23 aa) covalently linked to the 5' end of the positive and negative strand of the viral genome. It primes RNA synthesis. The viral RNA-dependent RNA polymerase 3D is a major component in the viral RNA replication complex (29, 30)

The virions are spheroidal particles 22-30 nm in diameter consisting of internal RNA core surrounded by protein shell or capsid. The RNA accounts for about 30% and the protein coat about 70% of the weight of the virion. Their particles are constructed of 60 repeated protomers which each protomer consist of four structural proteins (VP1-VP4). They form icosahedral shell to encapsidates viral genome. The surface of the particle is composed of VP1, VP2 and VP3 which each adopt the typical wedge-shaped eight-stranded, antiparallel β-barrel fold whereas VP4 is located

on the inside of the virion (4). A large twisted beta sheet comprising of four strands B, D, I and G form the front and bottom sections of the barrel while a flatter sheet comprising four strands C, H, E and F form the rear section. Their capsids serves multiples functions in many stages of the virus life cycle such as cell surface receptor binding, capsid internalization and destabilization. Each capsid protein taking the form of four loops (BC, HI, ED and FG) connect with these eight strands (31). Most EV has a deep, circular surface depression or canyon encircling each fivefold axis of symmetry which serves as the receptor binding site. The several highly variable loops on surface of the capsid are accessible to the host immune system and account for the high antigenic diversity (32).

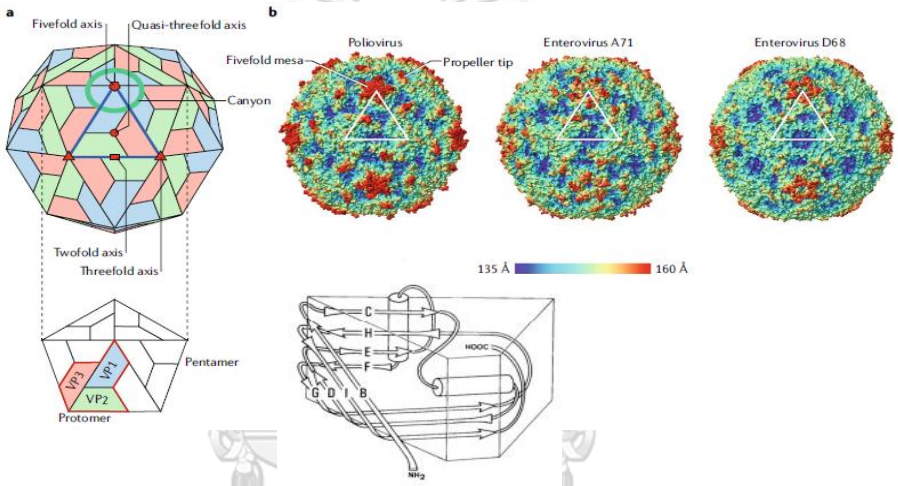

Figure 4 Enterovirus structure.

A.) Schematic represent a enterovirus particle, showing the asymmetric unit (blue), different symmetry axes (red) and location of the canyon (green). Sixty protomers consist of surface proteins constitute the capsid. B.) Comparison of capsid surfaces between different EV genotype. Surfaces are colored according to their distance from the centre of the virion. (Modified from Baggen, J. et al., Nat Rev Microbiol, 2018 (4, 33))

EV can establish persistent infections which associated with many syndromes. For example, CVB persistence was shown to contribute significantly to the occurrence of chronic myocarditis and dilated cardiomyopathy, through direct effects of viral replication (34). Viral and cellular host factors involved in these mechanism. For viral factors, the selection of virus mutants that are less cytopathic for cells or that

result in low-level viral replication. Some mutations, amino acid substitutions affect the binding properties of the virus such as weak interaction with the coxsackie and adenovirus receptor (CAR) and strong binding to the decay accelerating factor (DAF) (35). Furthermore, the deletion in the 5'UTR which harbor the genomic determinants of EV replication, can have a lower replication rate and persist in host cells over a prolonged period (36, 37). Latent EV might be reactivated either spontaneously or in response to exogenous stimulations (38).

EV is highly genome plasticity which high mutation and recombination rates, their significant errors usually introduced during genome replication. Several studies have shown that circulating EV recombine only within species. EV recombinants arise frequently in the 5'UTR/VP4 junction and within the non-structural genomic regions while does not occur frequently in the P1 region. However, recombination hot spots appear to differ among species. For example, EV-B recombination mostly occurred in the P2 region. EV-C (non-polio) recombines is the P3 region and rarely in the 2A (39-41).

2.4 Molecular evolution

2.4.1 Phylogenetic trees

Phylogenetic tree is a diagrammatic for describe relationships between gene family, history, evolution, epidemiological dynamics among species in systematics and taxonomy (42). It has become an indispensable tool for genome comparison which helps identify similarity and differences among genome. A tree containing nodes which connected by branches, each branch represents the persistence of a genetic lineage through estimate time called "topology", each node represents the birth of a new lineage. One branch can connect only two nodes, the terminal nodes represent the operational taxonomic units (OTUs) whereas the internal nodes represent hypothetical taxonomic units (HTUs). The best tree score should be identified by comparing all possible trees. Many methods are feasible to analyze large data sets; maximum parsimony, distance matrix, maximum likelihood and bayesian inference

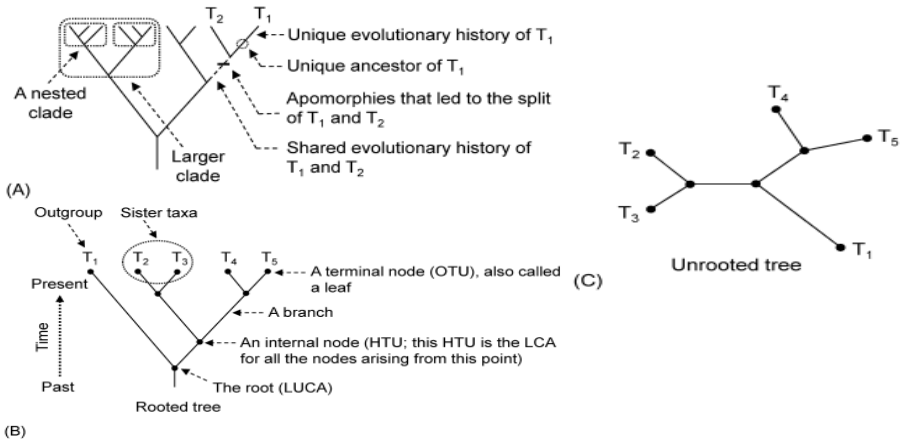

Figure 5 Conceptual of phylogenetic tree.

A and B showed rooted trees while C showed unrooted tree (Modified from Choudhuri, S. 2014 (43)).

Table 3 A summary strengths and weaknesses of different tree reconstruction methods

(Modified from Yang, Z. et al., Nat Rev Genet, 2012 (42))

Method	Strengths	Weaknesses
Parsimony methods	Simplicity and intuitive appeal	Assumptions are implicit and poorly understood
		Lack of a model makes it nearly impossible to incorporate our knowledge of sequence evolution
		Branch lengths are substantially underestimated when substitution rates are high
		Maximum parsimony may suffer from long-branch attraction
Distance methods	Fast computational speed	Most distance methods do not consider variances of distance estimates
	Can apply to any type of data	Distance calculation is problematic when sequences are divergent and involve many alignment gaps
Likelihood methods	Can use complex substitution model to approach biological reality	Maximum likelihood iteration involves heavy computation
	Powerful framework for estimate	The topology is not a parameter so that it is difficult to apply

Method	Strengths	Weaknesses
	parameters and hypotheses	maximum likelihood theory for its estimation. Bootstrap proportions are hard to interpret
Bayesian methods	Can use realistic substitution models, as in maximum likelihood	Heavy computation
	Prior probability allows the incorporation of information	Hard to identify or rectify
	Prior probabilities for trees and clades have easy interpretations	Posterior probabilities often appear too high

The aim of phylogenetic inference is to estimate the tree topology and possibly based on four criteria to judge tree reconstruction methods.

1. Consistency: A tree reconstruction method is consistent if the estimated tree converges to the true tree when the number of sites in the sequence grows.
2. Efficiency: A smaller variance is more efficient than larger variance. It may be measured by the probability of recovering the correct tree or subtree given the number of sites.
3. Robustness: The rapid accumulation of sequence data, sampling errors in tree reconstruction are considerably reduced, thus robustness of the method become more important.
4. Computational speed: This property is easy to assess. Considerable advancements in computational algorithms have made likelihood-based methods feasible for the analysis of large data sets.

2.4.2 Model of evolution

Evolutionary models are models of assumptions about the nucleotide or amino acid substitution process which important role in analysis molecular sequence data. The different probabilities of change can be implicit in many methods which may change the results of a phylogenetic analysis. All statistical models are based on certain assumptions that each position evolves independently (43). There are many models for nucleotides as following;

1. Jukes-Cantor one-parameter model (JC69): The simplest substitution model for nucleotides is which assumes all nucleotides occur in equal frequency (25%), equal substitution probability and requires a single parameter.
2. Kimura's two parameter model (K80): All nucleotides occur equal frequency (25%) but transition mutations provide a better estimate of evolutionary divergence than transversion mutations and requires two parameters.
3. Felsenstein model (F81): Extension of the JC69. Nucleotides occur at different frequencies and transitions and transversions occur at different rates.
4. Hasegawa-Kishono-Yano model (HKY85): Combining the extension of Kimura80 and F81 models which nucleotides occur at different frequencies and transitions and transversions occur at different rates.
5. Tamura and Nei model (TN93): Nucleotides occur at different frequencies. Transversions are assumed to occur at the same rate while two different types of transition is allowed to have a different rate.
6. General time reversible model (GTR): This is more complex model. The nucleotide frequencies are estimated by the observed in the alignment data and different rates of substitution for each pair of nucleotides.

If the model assumed is incorrect, branch lengths, transition/transversion ratio and sequence divergence may be underestimated, whereas the strength of rate variation among sites may be overestimated. Normally, more complex models will fit the data better than simpler one. However, it has several disadvantages; analysis becomes computationally difficult, more parameters need to be estimated and requires significant time. In practically, it would be advisable to incorporate as much complexity as needed which enough to explain the data. The best-fit model of evolution can be selected through statistical testing. The several fit model can be performed through statistical test, "likelihood ratio tests (LRTs)" to select the best-fit model within a set of possible ones (44). The LRTs compare the likelihoods of two nested models of evolution with preset parameters to determine whether positive selection occurred.

For determining the reliability of the tree, bootstrapping were used. It is a computationally statistical analysis by repeated resampling from the original samples to create many novel subsets of pseudosamples that are subjected to the matching

analysis as the original samples to obtain many bootstrap tree. It can be applied to all tree-construction methods. The higher number of resampling, the greater is confidence interval level of the estimate, usually recommended perform 200 – 2000 resampling from the origin sequences. In general, branch is considered accurate when the bootstrap values is greater than 70% which means that 70% of bootstrap trees support the topology at the branch obtained in the original phylogenetic tree.

2.4.3 Phylodynamic

Many organisms, especially viruses, accumulate genetic variation during environmental change and evolve on the same time scale. It also be affected on transmission dynamics and phenotypes. To infer this complexity, the formulation and simulation of dynamical interaction has integrated ecological, epidemiological and evolutionary processes called “Phylodynamic”. The goal of phylodynamic is to make inferences of epidemiological processes from phylogenies which it can also highlight the evolution through time of mutations that may reflect adaptations. Thus, it can apply for investigate selective pressures, circulation patterns, dating origins and spreading. Models for analyses phylodynamic commonly use molecular clock models to represent the relationship between genetic distance and time. A second is phylogepgraphic to discern the route of transmission and the rate of geographic spread (45).

Several methods have been developed to specifically address problems related to phylodynamics and epidemiological parameters such as coalescent theory and birth-death models. Coalescent theory is a mathematical model that assumes each variant is equally (no recombination, no selection and no gene flow). The model looks backward in time from the present where two lineages coalesce in their most recent common ancestor, merging into single ancestral according to a randomly sampling. Birth-death models is a continuous-time Markov process where state transitions in two types: births (increase the state variable by one) and deaths (decrease the state by one). This model use for represent the current size of a population where the transitions are literal births and deaths.

2.4.3.1 Molecular clock

The molecular clock is tools to investigate the timing of phylogenetic events by genetic data which raised the possibility of inferring evolutionary rates, estimate historical timescales and also substitution process. A wide range of molecular clock models and methods are available in various statistical setting. In practically to place an absolute timescale, various factors need to be considered including the size of data set, calibration using independent temporal information (46). For calibration, can be done by setting the rate to a known value or constraining the age of at least one node. There are two major clock models as following:

1. Strict clock model is the simplest model which has a rate of evolution for assume homogenous rates among branches of the phylogeny. It expressed in substitution per site per year. It often used as a null model for first steps in a molecular dating analysis to analyze of sequence data sampled at the intraspecific level which expected has a low rate of variation among branches. The unknown times of divergence could be estimated by applying the equation of the regression line to genetic distances estimated from the taxa. Recently, strict clock has been incorporated into Bayesian phylogenetic method for permitting greater flexibility of uncertainty in the topology (47).
2. Relax clock model is depending on their statistical assumptions about how rates vary among branches and the extent of other parameters that each branch can have a distinct evolutionary rate. There are two major forms: autocorrelated relaxed clocks, which the rate is assumed to be correlated between neighboring branches and uncorrelated relaxed clocks which the rates along different branches are assumed to be drawn independently from a single distribution (48). It can distinguish on the basis of whether they are implemented in rate-smoothing methods or as parametric models of rate variation in Bayesian and likelihood method. There is uncertainty about whether forms are more appropriate for explaining patterns of rate variation among lineages but can select the best-fitting clock model by using statistical approaches such as Bayes factors (49).

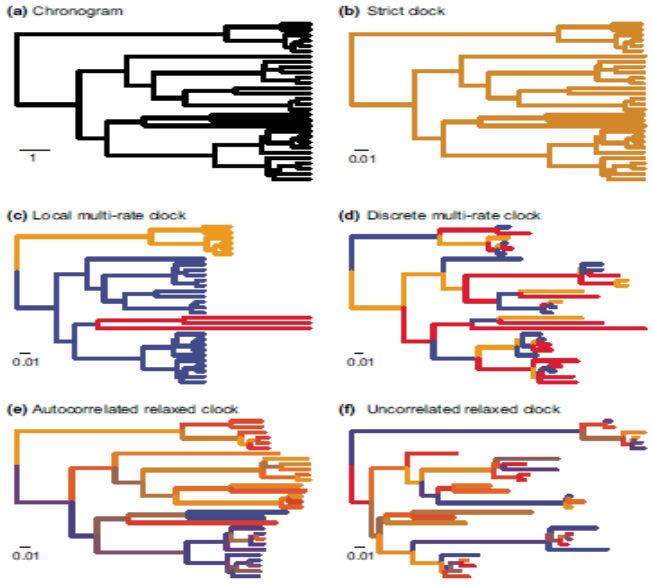

Figure 6 Phylogenetic trees with differences models of rate variation.
(a) chronogram with branch lengths measured in time units; (b) strict clock model with a constant rate among branches; (c) local multi-rate clock with a distinct rate in each of three groups of branches; (d) discrete multi-rate clock with a small number of branch-specific rates distributed throughout the tree; (e) autocorrelated relaxed clock with a distinct rate along each branch that is correlated with parent branch; (f) uncorrelated relaxed clock with a distinct rate along each branch drawn from a chosen probability distribution (46).

2.4.3.2 Phylogeographic analysis

Phylogeographic was developed to complement the phylodynamic framework by providing means to statistically identify origin and reservoirs of genetic diversity. It seeks to infer the origin of geographical structuring of genetic variation within and among closely related species across the landscape. The usual approach is to build a phylogenetic tree from sequences and then represent the lineages geographically. When combined with their population frequency and geographic distribution on the phylogeny, this provides a strong basis for inferences on the evolutionary history of taxa (50). Phylogeographic approaches can identify historical hybridization events, occurrences of introgression and the geographic determinants of isolation. It is instantly becoming one of the most integrative disciplines in biology, which many analytical tools have been developed to evaluate spatial and temporal congruence or

incongruence. Geographic Information System (GIS) technology is becoming friendly available tool for provide a variety of integrative approaches that useful for illuminating phylogeographic patterns and processes. However, in reality, the abundance of available geospatial tools offers a rich resource for incorporating GIS into phylogeography as following table 4.

Table 4 Summary of some spatially explicit methods of use to biogeographers. (Modified from Chan, LM. et al., Mol Phylogenet Evol, 2011(51))

Method	**Software**	**Summary**	**Genetic data required**	**Spatial data requires**
Data exploration/pattern visualization	GenGIS, GeoPhyloBuilder	Projects a phylogeny onto a 2D or 3D map	Phylogenetic tree	GPS data
Matrix corrections	GenAlex, Alleles in Space, R-packages	Regression analysis of pairwise matrices. Test significant isolation by distance.	Genetic distance matrix	Geographic distance matrix
Delineating biogeographic units/identifying barriers	Barrier (Monmonier's algorithm), Alleles in Space, TESS, Geneland, Landscape Genetics GIS Toolbox	Elucidates geographic positions of biogeographical boundaries, Spatially explicit assignment method that estimates the number of populations, Calculates genetic landscape	Genetic distance, Single or multi-locus genetic data	Species composition data at each localities, GPS data
Source-sink dynamics	RAMAS, GIS	Estimates source-sink dynamics of populations	Population information	Landscape and GPS data

Method	Software	Summary	Genetic data required	Spatial data requires
Dispersal route analyses	Path Matrix (Least-cost paths), GrassGIS, Circuitscape, SPLATCHE 2	Computes distance among samples given habitat heterogeneity, predicts geographic connectivity based on habitat heterogeneity and circuit theory, Incorporates connectivity based on the simulation of migration	Species, subspecies or populations, defined taxonomic groups, phylogenetic data	GPS data, relevant friction layer and ancestral distribution
Ancestral distribution	Phylomapper, DIVA (dispersal-vicariance analysis)	Uses a maximum likelihood framework or parsimony to estimate ancestral	Phylogenetic tree	GPS data, specified geographic units

Therefore, approaching phylogeographic studies from multiple independent perspectives can help to highlight some of the potential mechanisms underlying diversification so that more thoroughly consider relevant and testable alternative hypotheses that might not otherwise be apparent. Moreover, combination with phylodynamic methods can be used to extrapolate the rates of movement of viral lineages between geographic locations and reconstruct the geographic locations of ancestral lineages. For visual summaries, a Bayesian inference of spatio-temporal dynamics incorporating phylogenetic and mapping were converted with divergence time and spatial estimate to keyhole markup language (KML), compatible with virtual globe software.

In virus evolution, sequence evolution is occurring simultaneously with geographic dispersal by using phylogeographic analysis. It can be recovered genomic data for characterized a spatial phylodynamic process. Beyond a historical

perspective, it potentially help to predict the emergence of infectious diseases by identifying a key reservoir and geographic areas which are likely to emerge and spread. To estimate the ancestral locations in a phylogenetic tree conditional, it may depend on the sampling scheme and hypothesizes. The discrete distributed traits are used when sampling from different countries or cities, which are useful for study the history of cross-species transmission further demonstrate the generality of phylogenetic diffusion models, while continuous distributed traits are used when latitude and longitude coordinates as spatial locations, which may provide a natural framework for studying antigenic evolution which is inherently associated with genetic history (52, 53).

2.5 Evolution of enterovirus

EV is considered as high infectivity and transmissivity pathogens. EV show extensive genetic and antigenic heterogeneity reflecting in the identification more than 100 genotypes among them. Their genetic variations can also be increased by selection mechanisms in order to control their replication capacity from several pressures. These result allow for the virus adaptation dynamically to different hosts and environments (54, 55) and also impact on fitness and virulence of the viruses.

Recombination and mutations caused by error-prone proof-reading mechanism, have been recognized as a key mechanism for the evolution and adaptation of EV. These event consist of intra-species (recombination occurring between the strains of the same species) and inter-species (recombination between strains of different species). The copy choice model is the most popular model of recombinant RNA genomes, where two viruses infect the same cell and replicate simultaneously their genomes and sharing the same replication intracellular complex. During replication, viral polymerase can switch viral RNA templates with similar nucleotide sequence (56). Another model, their RNA are cleaved and their exposed ends are rejoined, regardless of the presence of enzymatic activity (57). Recombination process significantly generates the new EV progenies that continually contribute to the development of many diseases. The intra-species recombination occurred nearly 100 times more often than inter-species recombination. The majority of recombination events take place in the non-structural region, especially 2A-2C.

Recombination rate is not a selectively determined trait in itself but rather the natural outcome of particular genome structure. The rates of recombination can be measured by the rate of template switching that occurs during replication and rate amongst the population level. Point mutation is introduced into viral genome by errors in replication fidelity, high error-prone RdRp combined with the absence of proofreading and post-replicative repair mechanisms. These factors increase the number of mutations incorporated into the viral genomes overtime with the frequency of 10^3-10^4 nucleotides per replication cycle (58). After generation of mutant genomes, the process to define new genotypes are genetic drift (fixation or elimination of the mutations) and diversifying selection based on the consequent caused by mutation. The neutral mutations can become fixed in the population by genetic drift. To diversifying selection, the increasing the frequency of advantageous mutations called "Positive selection" while decreasing the frequency of deleterious mutations called "Negative selection".

In phylogenetic studies, the circulating EV strains were most similar within capsid region while recombination frequently occurred in the 5'UTR/VP4 junction and within non-structural regions (59), its differ among EV species. Recombination events have been observed more frequently detected in strains from EV species B (60) but recombination does not occur frequently in the P1 region. Since VP4 protein is located on the internal side of the capsid, it is not subjected to immunological pressure and thus has an increased evolutionary flexibility. Meanwhile, VP2-VP1 region intra-species genetic exchanges can occur more often than inter-species. Furthermore, accumulation of mutation in the binding pocket affects many biochemical properties which may turn binding affinity of antiviral drug such as changing the hydroxyl function and enhancing conformation flexibility (61). Enhanced detection of recombinant strain is important to understand their evolution and emergence but the main problem remains the absence of full genome sequences because of inter- and intra-species recombination events. Identification in 2C, 3D non-structural and 3'UTR region can be used as a marker of detection these events (62, 63). The sequence analysis will provide more valuable information for viral evolution and fitness change in enterovirus surveillance and might lend insights into pathogenesis and host interaction in the future (64). VP1 protein interacts with cell surface receptor at

specific amino acid residues. Thus, mutations in this region may significantly affect the binding sites for entry to cell, which rapidly allow evolution. It leads to functional relevance of receptor to viral pathogenesis and provide significant insight into vaccine development (65).

For example, most studies of recombination in human enterovirus species A concern EV-A71, which often cause large epidemics. The main force for evolution of EV-A71 is from recombination events in the VP1 region, which has produced four lineages (A to D) and sublineages (genogroups B1-B5 and C1-C5). The epidemiological history showed that EV-A71 changed sublineage in each outbreak (66).

CHAPTER III
EXPERIMENTAL RESULTS

Part 1: Epidemic outbreak of Acute Hemorrhagic Conjunctivitis caused by Coxsackievirus A24 in Thailand, 2014

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Part 1: Epidemic outbreak of Acute Hemorrhagic Conjunctivitis caused by Coxsackievirus A24 in Thailand, 2014.

Acute hemorrhagic conjunctivitis (AHC) is highly contagious and transmitted via direct or indirect contact with eye secretions (67). Symptoms of conjunctivitis include ocular pain, swelling of the eyelids, irritation and eye discharge. Viral conjunctivitis generally persists for three to seven days before resolving spontaneously. Major outbreaks of AHC are often attributed to adenoviruses, enterovirus 70 (EV70) and coxsackievirus A24 variant (CV-A24v) (68, 69). Many countries have reported extensive outbreaks of AHC due to CV-A24v (18, 19, 70-72). CV-A24v belongs to the species C group initially isolated during an epidemic in Singapore (73, 74).

CV-A24v is a non-enveloped plus-strand RNA virus with a genome of approximately 7400 bp (75). The virus belongs to the genus Enterovirus in the family *Picornaviridae*. The genomic RNA is translated into a single polyprotein, which is catalytically processed by the viral protease into 4 structural capsid proteins and 7 nonstructural proteins (76). The capsid proteins (VP1-VP4) assemble to form an icosahedral virion. The external VP1 capsid protein is under constant evolutionary pressure to induce changes in the neutralization epitope for evasion of the host immune response.

Traditionally, antisera are used for viral neutralization detection of enterovirus serotypes, but this assay is time-consuming, costly, and requires large sample volumes. Moreover, new strains are often untypable due to accumulated changes on the capsid protein. Molecular methods, such as polymerase chain reaction (PCR) and RT-PCR, are feasible diagnostic tools that may replace conventional cell culture methods (70). For molecular epidemiological analysis of enteroviruses, VP1 and 3C protease regions can be used to identify distinct genotypes, which would facilitate accurate and rapid determination of the virus species involved in outbreaks.

During the rainy season of 2014, an outbreak of AHC occurred throughout Thailand. The Ministry of Public Health documented a significantly greater than usual number of AHC cases, beginning in July. By August, > 100000 individuals had been affected. The number of affected patients (from January 1st through to October

30th) with infectious conjunctivitis from all 77 provinces of Thailand increased to 417824 cases. The cause of this outbreak was investigated using epidemiological data and molecular methods to determine disease etiology in the current study.

Materials and Methods

Clinical samples

The study protocol was approved by the Ethics Committee of the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB 418/57). In total, 119 conjunctivitis swabs were collected from patients with clinical diagnosis of AHC who sought medical care two to five days after the onset of symptoms. Specimens were randomly collected from 50 males and 69 females at Bueng Kan Provincial Hospital, (Bueng Kan), Chum Phae Hospital (Khon Khen), Thai Health Promotion Foundation of Roi Et (Roi Et), Thonburi 2 Hospital (Nakhon Pathom) and Bangpakok 9 International Hospital (Bangkok) during September, 2014. Samples were obtained from individuals of all ages (infants to the elderly). The affected eyes were swiped with sterile cotton swabs that were subsequently placed in 1 ml of viral transport medium containing antibiotics (2 x 10^6 U/L penicillin G and 200 mg/L streptomycin).

Adenovirus detection

Viral genome extraction was performed using the Exgene Viral DNA/RNA kit (GeneAll, Seoul, South Korea) according to the manufacturer's instructions. A 956 base-pair fragment of the human adenovirus (HAdV) hexon gene was identified using nested PCR. Primers for first- round PCR were ADV_FO (5' AYG CYA MCT TYT TYC CCA TGG C 3') and ADV_R1 (5' GTR GCG TTR CCG GCN GAG AA 3'). Primers for second-round PCR were ADV_F2 (5′ TTY CCC ATG GCN CAC AAC AC 3′) and ADV_R2 (5′ GYY TCR ATG AYG CCG CGG TG 3′). PCR conditions were 94^0C for 3 min, followed by 40 cycles of 94^0C for 30 s, 50^0C for 30 s and 72^0C for 1.45 min, with final extension at 72^0C for 10 min. A stool sample containing adenovirus genotype 8 served as a positive control (77).

Pan-Enterovirus detection

Extracted RNA samples were subjected to cDNA synthesis using random hexameric primers and the ImProm-II Reverse Transcription System (Promega, Madison, WI). Pan-enterovirus real-time PCR was used for initial screening (78).

Additional pan-enterovirus semi-nested PCR was employed to amplify the highly conserved 5' UTR using the first primer pair, CU-EVF2760 (5'ATG GKT ATG YWA AYT GGG ACA T 3') and CU-EV3206 (5' CCT GAC RTG YTT MAT CCT CAT 3'), and second primer pair, CU-EVF3029 (5' TTC ATG TCR CCW GCS AGT GC 3') and CU-EV3206. Both amplification reactions were performed under the following conditions: 95^0C for 3 min, followed by 40 cycles of 95^0C for 1 min, 55^0C for 1 min and 72^0C for 1 min, and final extension at 72^0C for 10 min.

Additional characterization of coxsackievirus A24

Specimens that tested positive for pan-enterovirus 5'UTR were sequenced to identify the enterovirus genotype. Coxsackievirus A24-positive samples were further characterized by additional PCR and sequencing of full-length VP1 and 3C regions. The PCR primer sets used were CA24_VP1_F (5' CACAGAGAACTTTGTTTGCG 3') and CA24_VP1_R3417 (5' CCTCCAAAAGTATTAATGTTTTC 3') for VP1 and CA24_3C_F (5' ACCATTAGAACAGCAAAGGTG 3') and CA24_3C_ R6047 (5' CTTTTGATGGTCTCATCCATT 3') for 3C. Both amplification reactions were performed under the following conditions: 94^0C for 3 min, followed by 40 cycles of 94^0C for 30 s, 55^0C for 45 s and 72^0C for 1.30 min, and final extension at 72^0C for 10 min.

Sequence and phylogenetic analyses

Sequencing results were analyzed with Chromas Lite (version 2.01) and BioEdit (version 7.0.4.1) and subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/) to identify the viral sequence. Nucleotide sequences were submitted to the GenBank database under accession numbers KP121936 – KP122019 for 5'UTR, KP122020 – KP122090 and KP137044 – KP137046 for the VP1 gene, and KP122091 – KP122162 and KP137042 – KP137043 for the 3C gene.

Phylogenetic trees were generated using Clustal W alignments of nucleotide sequences. The neighbor joining method was implemented in MEGA (version 5) with bootstrap resampling values of 1000 replicates.

Results

AHC occurs yearly throughout Thailand and its incidence often peaks during the rainy months of July to October. As of July 2014, the Ministry of Health in Thailand reported an unusually higher than expected monthly incidence of AHC,

compared to previous records in 2006 and 2009 (Figure 7). In these years, monthly incidences were below 10000, and peaked between July and October. However, this year, the number of AHC cases in August increased dramatically, exceeding 160000 in September. Although individuals of all ages were susceptible, the majority of patients were between 7 and 54 years of age (Figure 8). The highest incidence was found in the age group of 5-14 years (29.09%), followed by 35-44 years (12.24%) and 15-24 years (11.27%).

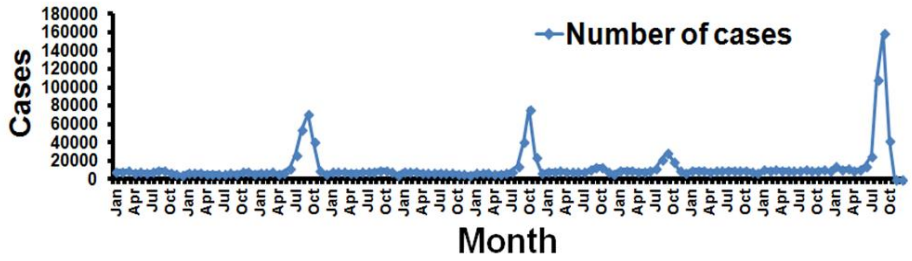

Figure 7 Incidence of acute hemorrhagic conjunctivitis (AHC) in Thailand between 2004 and 2014.

Data compiled by the Ministry of Health Thailand were obtained from physicians and healthcare workers from all 77 provinces. The majority of AHC cases were reported between August and October. July–October coincide with the local rainy season

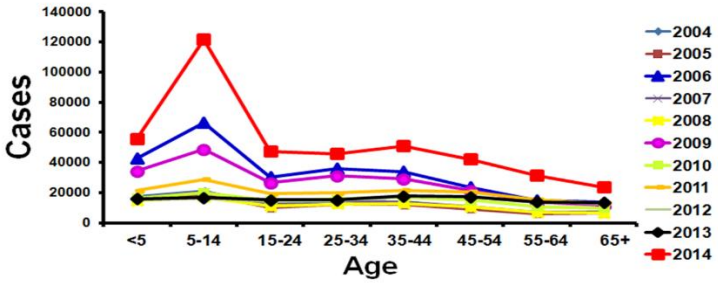

Figure 8 Reported cases of AHC in Thailand between 2004 and 2014 classified by age group.

The highest incidence was observed among patients between 10 and 54 years.

In view of its rapid spread, combined with previous epidemiological data on acute outbreaks, viral conjunctivitis was suspected. Eye swab samples were obtained from several hospitals within and outside Bangkok for analysis. Sequence-specific PCR of the 119 samples did not lead to detection of adenovirus nucleic acids. However, 71.43% (85/119) tested positive in pan-enterovirus PCR. Subsequent enterovirus species-specific PCR analyses led to the identification of coxsackievirus A24 (CV-A24) in 84 specimens and echovirus in 1 specimen. CV-A24-positive samples were further confirmed by full-length amplification of VP1 and 3C genes and comparison with the viral genomes available in the GenBank database.

Phylogenetic analysis of the VP1 and 3C genomic sequences from Thai outbreaks and comparison with other clinical isolates and reference strains for which sequences were available led to the grouping of CV-A24 into four genotypes (I-IV) and genotype clusters. The VP1 phylogenetic tree consisted of the prototype strain (genotype I, EH24/70), strains from 1987 to early 1990s (genotype II), late 1990s (genotype III), and those isolated between 2003 and 2010 (genotype IV) (Figure 9). Genotype IV (GIV) was further subdivided into several clusters, depending on the isolation dates (GIV C2 for 2003–2005 and GIV C5 for 2006–2010).

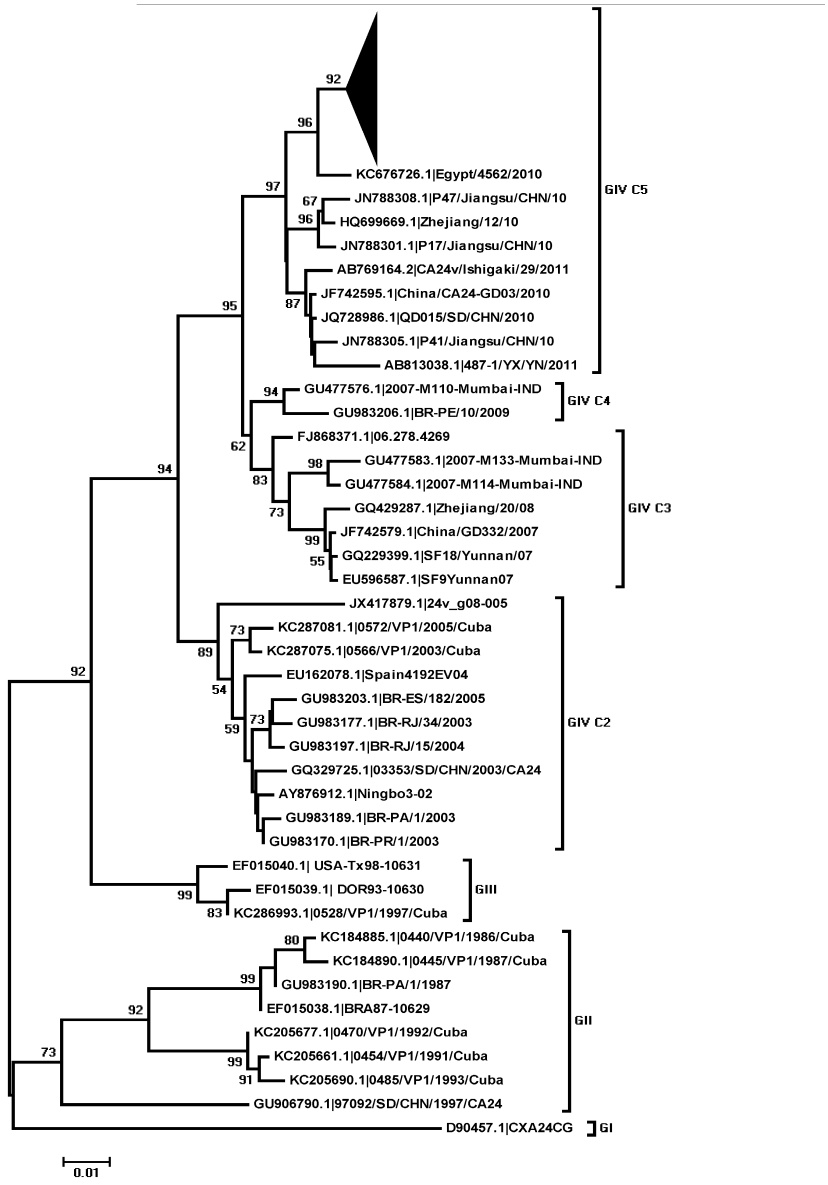

Figure 9 Phylogenetic analysis of full-length VP1 nucleotide sequences of CV-A24.

Phylogenetic trees were produced using Clustal W alignments and the neighbor-joining method implemented in MEGA (version 5). Strains identified in this study are shown as one cluster located on the top of the tree (black arrowhead). Bootstrap resampling values are indicated at the nodes. The scale bar indicates the number of substitutions per site.

The phylogenetic tree of the 3C nucleotide sequences also showed four distinct genotypes (Figure 10). In addition to the genotype I reference strain, EH24/70 (Accession Number D90457, (79)) (73), and genotype II strains from Singapore and Thailand identified in 1975, genotype III included isolates from 1985 identified in Asia, Africa and France (80). Genotype IV is a cluster of a diverse group of isolates from recent years. In both VP1 and C3 trees, the Thai isolates grouped together and shared highest sequence identities with the C5 lineage. Although all the Thai isolates belonged to genotype IV, they clustered with the C5A and C5B variants isolated in recent years.

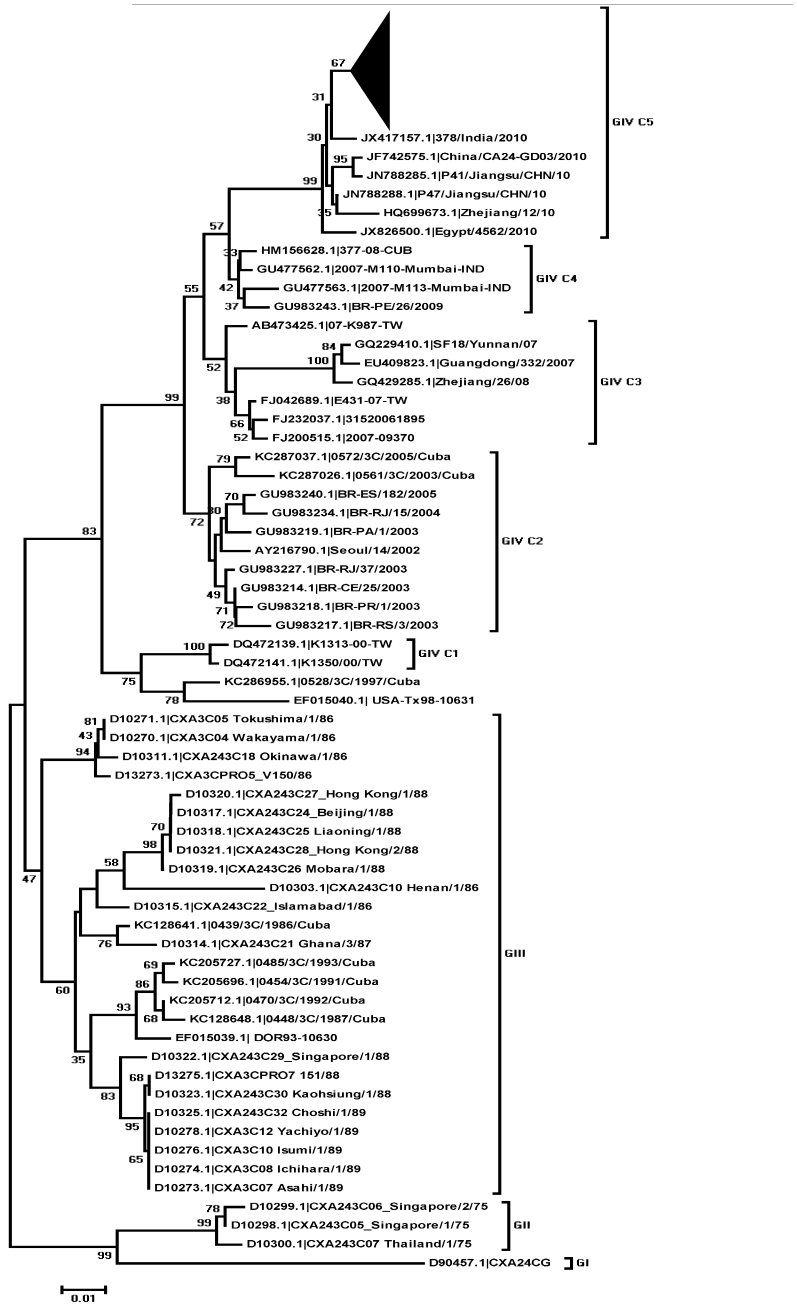

Figure 10 Phylogenetic analysis of the full-length 3C nucleotide sequences of CV-A24.

Strains identified in this study are shown as one cluster located on the top of the tree (black arrowhead).

Viral conjunctivitis occurs throughout the year, but increases during the rainy season. In addition to redness of the conjunctiva, symptoms may include swelling, irritation, pus and mucus discharge. Outbreaks are often associated with close contact in the community setting, such as schools, prisons and swimming pools. The majority of patients from this study presented mild symptoms and were prescribed eye drops for redness relief. Although no vaccines and antivirals are available to prevent or treat conjunctivitis, AHC generally self-resolves and requires no further treatment.

Rapid dissemination of infectious conjunctivitis often implicates adenovirus or enterovirus in the outbreak (71, 81, 82). CV-A24 was predominantly associated with conjunctivitis in the current study. The first CV-A24 variant was isolated in Singapore in 1970 (73). Its limited circulation in India and Southeast Asia prior to 1985 was followed by eventual worldwide spread (83). In 1992, the variant was identified in Thailand, and shown to be the major cause (76.8%) of AHC via assessment of the neutralizing antibody (84). Until now, no reports of CV-A24 identification in Thailand using molecular methods have been documented.

Outbreak reports of AHC in Thailand compiled by the Ministry of Public Health from 2003 to 2012 demonstrate a cyclical and seasonal pattern of "pink eye", especially during the rainy season between July and October every two to three years. The morbidity rates of 842.58 (2002), 417.53 (2006) and 342.57 (2009) per 100000 during the rainy months is a sharp contrast to the mean morbidity rate of <200 per 100000 during the rest of the year. Limited data currently available for October suggest a decline coinciding with the end of the rainy season. So far, the calculated infection rate has been 630.76 per 100000 in the overall population, with no reported mortality.

In decreasing order, the incidence of the 2014 conjunctivitis outbreak in Thailand occurred in the northeast, north, south and central regions. The highest rates were in the northeast provinces of Amnat Charoen (1756.18 cases per 100000), Buri Ram (1499.63 cases per 100000), Prachin Buri (1498.09 cases per 100000), Ubon Ratchathani (1299.96 cases per 100000) and Maha Sarakham (1264.84 cases per 100000) (85). Notably, the infection rate increased dramatically for individuals >6 years of age. This observation coincides with the compulsory schooling of children beginning in the first grade, which places them in the community setting where the

risk of disease exposure is high. The semester break in the month of October for most schools in the country, combined with the tapering of the rainy season, is expected to affect the number of new cases of viral conjunctivitis for the remainder of 2014.

Previous studies have identified CV-A24 variants via phylogenetic analyses of the VP1 capsid, 3C protease, and RNA polymerase regions (86). Both VP1 and 3C tree data showed that all clinical isolates from this study group together, but are separate from other previously characterized strains in the database. All strains identified in this study clustered into genotype IV, which is further subdivided into five different clusters (C1-C5). Strains from China (2007-2008) formed GIV-C3, while those from India (2007) and Brazil (2009) belonged to GIV-C4. The clinical isolates identified in this study clustered into genotype GIV-C5 and subclusters A and B (97.4– 98.3% identity), similar to strains involved in the outbreaks of AHC in Taiwan, China, India, and Egypt (18, 19, 72, 81, 87).

The extensive outbreak of AHC in Thailand this year may be attributed to the initial lack of known etiologic factors, inadequate hygiene, and increased viral exposure in community settings. CV-A24 variants may also evolve in terms of virulence, which would facilitate rapid spread of the virus in many countries. Further studies on the molecular pathogenesis of new variants of CV-A24 combined with increased vigilance in control, thorough implementation, response guidelines and prevention may help to reduce the incidence of AHC in the future.

Part 2: High prevalence of coxsackievirus A2 in children with herpangina in Thailand in 2015

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Part 2: High prevalence of coxsackievirus A2 in children with herpangina in Thailand in 2015.

Human enteroviruses are commonly associated with a wide spectrum of acute and chronic diseases affecting the gastrointestinal tract (88). Infection can be asymptomatic or manifests in fever, multiple oral ulcers, diarrhea, vomiting, and vesicular rash on the hands, feet and mouth. In some cases, infection can lead to acute flaccid paralysis, severe complications of the nervous system, myocarditis, and pulmonary edema. Herpangina, a primarily pharyngeal infection in children caused by human enterovirus of the *Picornaviridae* family, is characterized by multiple oral ulcers predominantly on the soft palate and the posterior of the oral cavity. Although symptoms often spontaneously resolve within 1-2 weeks, infection contributes significantly to childhood morbidity in the Asia-Pacific region and elsewhere around the world (89).

Enterovirus 71 and several coxsackievirus (CV) serotypes (CV-A2, -A4, and -A10) are frequently implicated in herpangina and hand-foot-mouth disease. Their predominance and prevalence varied with geographical locations, seasonality, and population susceptibility (90-92). Determinants of clinical manifestation and disease severity have been linked to specific serotypes and co-infection status, which can sometimes lead to death (93). Surveillance of outbreaks is therefore important in determining the viral etiology and transmission among young children who are most at risk.

Materials and Methods

In developing countries within Southeast Asia, there is a lack of priority and resource in identifying the viral cause of herpangina. To survey and identify the enterovirus associated with this disease, we screened and characterized herpangina-associated CV species from 295 throat swabs obtained from children (53.2% male and 46.8% female) with characteristic herpangina symptoms from Khon Kaen province in northeast Thailand (n = 88) and Bangkok (n=207) between January and December 2015. This research was approved by the Ethics Committee of the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB number 286/58). All specimens were de-identified and anonymous, therefore the IRB waived

the need for consent. Inclusion criteria for herpangina symptoms include the appearance of oral ulcers in the mouth but not elsewhere on the body. Approximately 86% of the children were ≤5 years-old (mean age 3.32).

Viral RNA was extracted using the Exgene Viral DNA/RNA kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. We initial screened for enterovirus genome region spanning the conserved 5' untranslated region (5'UTR) and VP4/VP2 gene using conventional RT-PCR as previously described (94). Subsequently, partial VP1 region was amplified with degenerate primers to identify the enterovirus species (95). Nucleotide sequences were edited and analyzed with Chromas Lite (version 2.01), BioEdit (version 7.0.4.1), and BLAST (http://blast.ncbi.nlm.nih.gov/). Nucleotide sequences of the CV-A2 identified in this study were deposited in GenBank (accession numbers KX021203-KX021265). Moreover, CV-A2 strains were verified by an additional semi-nested PCR using degenerate primers CA2_F2763 (5'-TGG GAT ATA GAY ATA ATG GGG TA-3'), CA2_R3256 (5'-GCR GTG TAR TTT GGG AAA TTC TT-3'), and CA2_R3029 (5'-AAA AGT GGG RTA WCC ATC ATA GAA-3') followed by sequencing. Reconstruction of the phylogeny trees was done using the neighbor-joining method and Maximum Composite Likelihood model with MEGA v.5.0 (96). Pairwise deletions were used for missing data and the robustness of the tree was determined by bootstrapping with 1,000 pseudo-replicates.

Results

In all, 120 samples (40.7%) tested positive for human enterovirus, of which 35.8% (43/120) were CV-A2, 15.8% (19/120) were CV-A4, 10.8% (13/120) were CV-A16, and 10% (12/120) were CV-A6 (Figure 11). The remaining samples tested positive for other human enterovirus of species A, B and C. The majority of positive samples were obtained in the rainiest months (July to September). Consequently, most CV-A2 were found in June to September. Interestingly, CV-A6 comprised the majority of the virus identified in December.

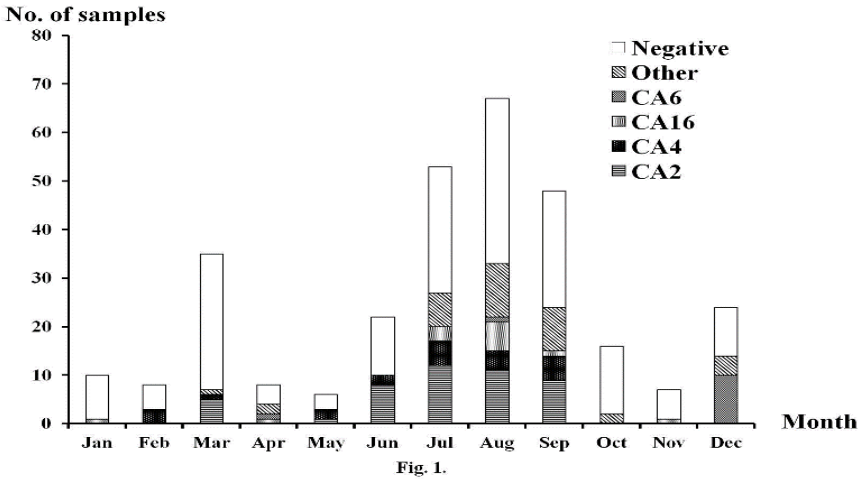

Figure 11 Distribution of herpangina samples tested positive for coxsackievirus in this study.

Since there was a predominance of CV-A2 detected in this study, we next determined the evolutionary relatedness between the CV-A2 strains and the reference strain sequences available in the GenBank database. The phylogenetic analysis of the partial VP1 nucleotide sequences showed that CV-A2 strains clustered into 3 distinct genotype subgroups. Most strains formed cluster 1 (Figure 12). The majority of the Thai CV-A2 strains in cluster 1 (n=27) were closely related to CV-A2 previously identified in Russia. An additional 11 strains were related to the strains previously isolated in Taiwan in 2012 and comprised cluster 2. Additionally, 2 Thai strains (KX021262 and KX021235) grouped with the CV-A2 previously found in China.

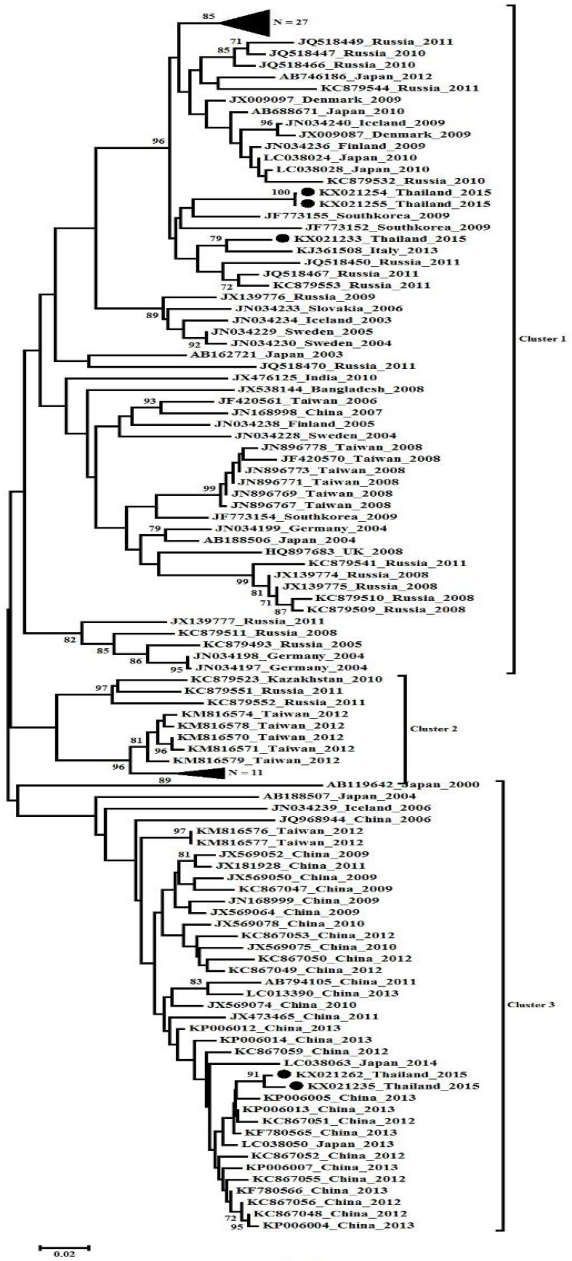

Figure 12 Phylogenetic analysis of the partial VP1 nucleotide sequences of CV-A2.

Phylogenetic trees were constructed by the neighbor-joining method implemented in MEGA v.5. Strains identified in this study are denoted as black dots or triangles (when many strains clustered together). Bootstrap resampling values >70 are indicated at the nodes. The scale bar indicates the number of substitutions per site.

Clinical presentations of herpangina and hand-foot-mouth disease overlap significantly. To examine whether herpangina is associated with distinct species of CV, this study analyzed the etiology of only clinically confirmed herpangina cases and excluded individuals with hand-foot-mouth disease. This is different from most published studies, which generally examined both diseases concurrently. Herpangina is highly contagious and the reasons for the apparent higher transmissibility in East and Southeast Asia are unclear, but factors including environmental sanitation, population density, and lifestyle may contribute to more outbreaks in the region compared to the U.S., Australia, and Europe.

Outbreaks of human enterovirus infection represent significant socio-economic burden to countries in resource-limited setting. In 2012, the unprecedented predominance of CV-A6 circulation in Thailand illustrated the ability for CV to rapidly and efficiently spread (97). Other countries in the region have continued to report outbreaks of herpangina associated with CV-A2 and CV-A4 including Taiwan in 2008, mainland China in 2009-2014, and Korea in 2009. Unlike the more severe infections caused by CV-A16 and enterovirus 71, very few or no fatalities have been associated with CV-A2 and CV-A4 infection. The viral strains in this study were very similar to those identified in previous years in East Asia probably due to continued virus co-circulations in the region.

VP1 is the determinant of viral pathogenesis and virulence, especially the antigenic BC-loop region (98). In addition to the error-prone replication mechanism of the human enterovirus, frequent recombination resulting from the exchange of structural and non-structural genes has allowed the virus to escape acquired host-immunity. The transmission of this rapidly evolving virus is thought to be mediated by the continuous interaction between spatiotemporal dispersion and natural selection process. This hypothesis is supported by studies demonstrating the rapid turnover of the CV-A4 (99) and the genetic divergence of CV-A6 (100), which has resulted in viral variants associated with novel, often more severe, clinical findings. Variations among subtypes may therefore increase viral diversity and result in a continued prevalence of CV. Examining the evolutionary rates of emerging CV variants will be useful when combined with vigilant epidemiological surveillance. Although we were unable to identify disease etiology in over half of the samples (~60%), past studies

have also reported the inability to amplify the viral nucleic acid in a significant number of samples (101). However, this is not expected to influence the diversity of the CV species identified. Frequent CV infection in many countries therefore requires good public health measures to reduce the incidence of herpangina and the socioeconomic impact of this disease.

Part 3: Human enteroviruses associated with and without diarrhea in Thailand between 2010 and 2016

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Part 3: Human enteroviruses associated with and without diarrhea in Thailand between 2010 and 2016.

Acute gastroenteritis (AGE) caused by viral infection contributes significantly to childhood morbidity and is a leading cause of death in young children (102). Despite steep declines in mortality associated with viral gastroenteritis in some countries, diarrhea in children resulting in hospitalization still contributes to significant socio-economic burden (103, 104). Viruses associated with AGE include rotavirus (RV), norovirus (NV) and adenovirus (ADV). RV infection commonly affects children <5 years of age, but has declined in many countries since the introduction of universal RV vaccination program (105). NV infection has therefore emerged as the leading cause of AGE in this age group (106, 107) with ADV infection not far behind (108, 109). Nevertheless, the etiology of a significant proportion of gastrointestinal illness remains undetermined especially in developing countries (110).

The association between human enterovirus (EV) infection and AGE is increasingly recognized (111). EV belongs to the *Picornaviridae* family and the Enterovirus genus comprising 4 EV species (A to D) and 3 rhinovirus species (A-C) (112). Collectively, they cause a broad spectrum of acute and chronic diseases especially in infants and young children (113-115). Coxsackievirus A6 and A16 cause hand-foot-and-mouth disease commonly affecting young children. Symptoms may include mild fever, oral ulcers, and vesicular rash on hands, feet, and mouth (3). Severe infection by EV such as poliovirus, EV-A71, EV-D68 can result in acute flaccid paralysis, fatal neurological and cardiac complications (89, 116). EV transmission can be direct via contact with nasal and vesicular discharge or fecal-oral route (117-119), and epidemics can demonstrate a seasonal and cyclical pattern (120, 121). Identification of the conserved 5' UTR and/or the viral capsid sequence can differentiate between different EV species and types (122).

Typical EV infection associated with mild skin and oral lesions is the hand, foot, and mouth disease (HFMD). Although HFMD is predominantly caused by members of the EV A species and does not typically result in AGE, some EV are occasionally shed in the stools of patients (123, 124). Several EV species have been

reported to cause gastroenteritis, but the molecular epidemiology of EV linked to diarrhea in children and adults have been limited (3). To determine whether certain EV species are more often associated with AGE of unknown etiology, we described a multi-year molecular surveillance of EV found in association with AGE compared to EV shed by individuals with HFMD.

Materials and methods

Study samples

The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB 491/57 and 286/58). The IRB waived the need for written informed consent because samples were de-identified and anonymous. Permission to use the samples was granted by the Director of King Chulalongkorn Memorial Hospital. Samples collected between January 2010 and December 2016 were categorized on the medical charts as infants (<2 years), pre-school children (2 to <5 years), school-age children (5 to <15 years) and individuals 15 years and older.

The first group of samples consisted of 2,692 stool specimens from individuals ages 3 days to 101 years (mean = 16.2 years; 1,465 males and 1,227 females) with AGE of unknown etiology who sought medical care at hospitals in Khon Kaen province (n = 1,406) and Bangkok (n = 1,286) (Figure 13). Inclusion criteria were symptoms of watery diarrhea (defined as ≥ 3 episodes within 24 hours) with vomiting and/or fever. These samples were subjected to screening for RV, NV, ADV, and EV.

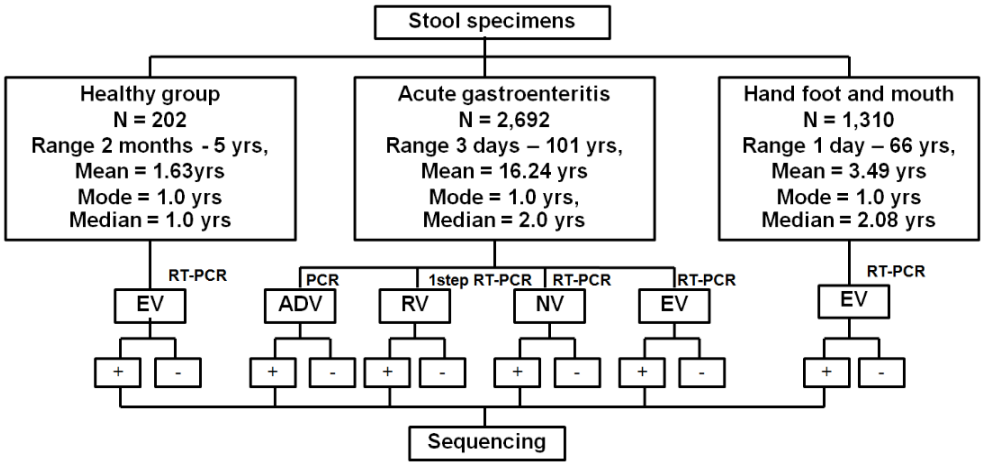

Figure 13 Schematic diagram of EV detection in stool specimens.

The presence of EV and other viral pathogens (RV, NV, and ADV) was examined in fecal samples from individuals with and without AGE. EV species identified in the AGE samples were subsequently compared to those identified in HFMD samples collected during the same period.

The second group of samples consisted of 1,310 fecal specimens from patients ages 1 day to 66 years (mean = 3.5 years; 804 males and 506 females) with no AGE symptoms, but had HFMD (defined by blister-like lesions in the buccal cavity, palms, soles, and/or buttocks) (95, 97). These samples were also obtained from Bangkok (n = 1,060) and Khon Kaen (n = 250) and were tested for EV alone.

Sample preparation

Samples were suspended in phosphate buffered saline and centrifuged at 4,000 X g for 10 minutes. Viral nucleic acid was extracted from the supernatant using RiboSpin vRD kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. The cDNA was synthesized with random hexameric primers using the Improm-II reverse transcription system (Promega, Madison, WI) according to the manufacturer's instructions.

Viral detection

Rotavirus

RV was detected by RT-PCR to amplify the conserved regions on the VP7 and VP4 genes using SuperScript III One-step RT-PCR system with Platinum Taq (Invitrogen, Carlsbad, CA) as previously described (125). The VP7 gene was amplified by Beg9 and End9 primers, while Con2 and Con3 primers were used to amplify the VP4 gene.

Norovirus

NV was detected using semi-nested PCR to identify the conserved region of the RNA-dependent RNA polymerase and VP1 gene (126). The PCR was performed using PerfectTaq MasterMix (5 PRIME, Darmstadt, Germany) according to the manufacturer's instructions. First-round PCR used forward primer JV12y and reverse primer NV2oR, while reverse primer R5591 was used in the second-round.

Adenovirus

Semi-nested PCR was used to amplify the ADV fiber gene for initial screening and hexon gene for ADV typing (77) as modified from an earlier study (127). The expected amplicon size of the hexon gene was 956 bp.

Enterovirus

Pan-EV assay using semi-nested RT-PCR to amplify the 5'UTR/VP2 was performed as previously described (94). Some HFMD samples tested positive for enterovirus have been initially reported (95, 128), but were further characterized in this study. Amplicons from EV-positive samples were agarose gel-purified and sequenced. Nucleotide sequences were analyzed using Chromas Lite (http://www.technelysium.com.au/chromas_lite.html) and Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences of EV identified from the AGE samples were deposited in the GenBank database under the accession numbers KY079137-KY079263, KR922046, KR054526-KR054554, KY774677-KY774687, and KX349962- KX349964.

Phylogenetic analysis

Nucleotide sequences of the VP4-VP2 region were aligned and subjected to phylogenetic tree reconstruction using the neighbor-joining method and maximum composite likelihood model implemented in MEGA 5.0 software (96). Pairwise deletions were utilized for the missing data, and the robustness of the tree was

determined by bootstrapping with 1,000 pseudo-replicates. Bootstrap values >70% were considered significant.

Statistical analysis

Statistical analysis was performed using IBM SPSS V21.0 package (SPSS Institute, Chicago, IL). Chi-square was used to measure differences of EV infection between age groups. The *p* value < 0.05 was considered statistically significant.

Results

Overall, AGE samples were predominantly from children less than 5 years old (males:females = 1.2:1) (Table 5). Between 2010 and 2016, samples tested positive for RV (22.7%, 611/2,692), NV (11.4%, 306/2,692) and ADV (9.3%, 249/2,692). RV constituted the major virus found in association with diarrhea between 2010 and 2014 (Figure 14A). The prevalent RV genotypes were G3P[8] (46.2%) and G1P[8] (38.1%) (Figure 15A). RV-positive samples were most often found in young children <5 years of age, while NV was more commonly found in older children and adults (Figure 14B). The majority of the NV genotype identified was GII.4 (52.6%) (Figure 15B). In this study, both NV and ADV were major enteric viruses found between 2015 and 2016. Genotype F41 comprised most of the ADV found in the samples (30.5%), followed by C2 (18.1%) and C1 (17.3%) (Figure 15C).

Table 5 Characteristics of the cohorts with (AGE) or without (HFMD) diarrhea in this study.

Characteristic		AGE (N=2,692) N (%)	HFMD (N=1,310) N (%)
Gender			
	Male	1,465 (54.4)	673 (51.4)
	Female	1,227 (45.6)	507 (38.7)
	N/I	0 (0)	130 (9.9)
Age (years)			
	< 2	1,214 (45.1)	432 (33.0)
	2 to < 5	370 (13.7)	468 (35.7)
	5 to < 15	157 (5.8)	150 (11.5)
	≥ 15	772 (28.7)	28 (2.1)
	N/I	179 (6.6)	232 (17.7)
Total EV-positive (N=168) in each age group			
	< 2	112 (66.7)	274 (33.5)
	2 to < 5	30 (17.9)	313 (38.3)
	5 to < 15	6 (3.6)	65 (8.0)
	≥ 15	11 (6.5)	13 (1.6)
	N/I	9 (5.4)	152 (18.6)
	Total	168 (100)	817 (100)

N/I = No information on gender or age.

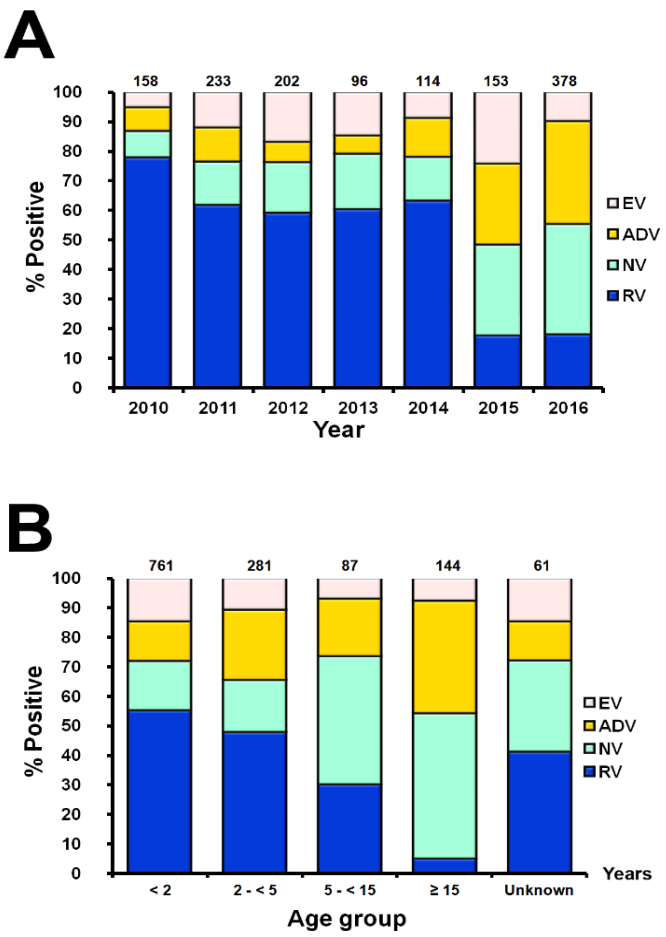

Figure 14 Enteric viruses found in AGE samples from 2010 to 2016.

Proportion of EV, ADV, NV, and RV identified by year (A) and by age group (B). The number of virus-positive samples are indicated above the bar graphs. Colors are blue for RV, green for NV, yellow for ADV, and pink, EV.

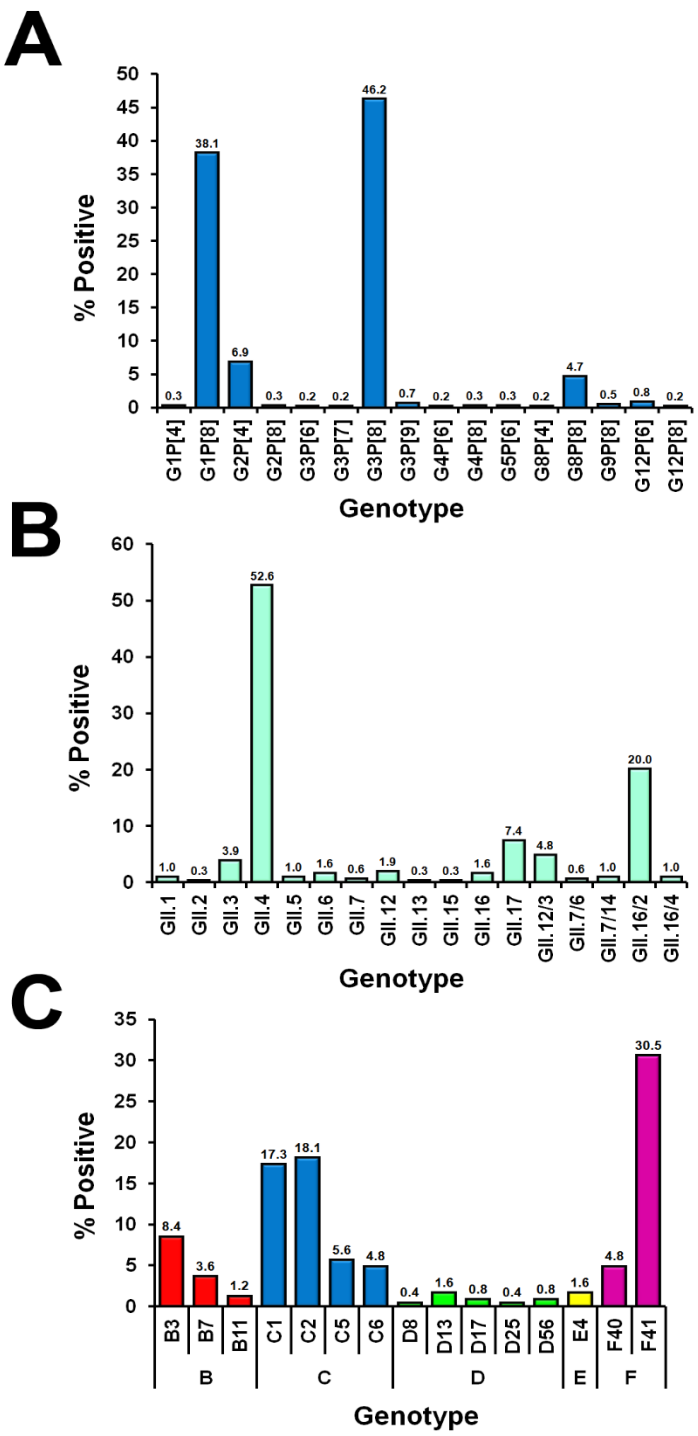

Figure 15 AGE samples tested positive for diverse genotypes of RV, NV, and ADV.

The genotype distribution of (A) RV based on the VP7 and VP4 genes, (B) NV based on the RdRp/VP1 region, and (C) ADV based on the hexon gene.

Interestingly, EV was identified either alone or in the presence of other viruses in 6.2% (168/2,692) of the AGE samples (patient mean age = 4.2 years) (Table 6). In these EV-positive AGE samples, EV was the only virus detected (54.8%, 92/168). Other samples were co-infected with RV (19.6%, 33/168), NV (11.9%, 20/168), ADV (5.4%, 9/168), or with two other viruses (8.3%, 14/168). Meanwhile, EV was detected in 62.4% (817/1,310) of all samples from HFMD, a disease not typically associated with acute diarrhea. Among EV-positive samples in the AGE and HFMD groups, children <5 years of age comprised 84.6% (142/168) and 71.8% (587/817), respectively ($p < 0.01$).

Table 6 Identification of EV alone or in the presence of other viruses in the AGE samples.

	Virus, N (%)							
	-	**RV**	**NV**	**ADV**	**RV/NV**	**RV/ADV**	**NV/ADV**	**Total**
EV-positive								
EV-A	5	0	2	2	0	0	0	9 (5.4)
EV-B	38	12	6	3	1	3	0	63 (37.5)
EV-C	22	5	6	1	1	4	1	40 (23.8)
EV-D	0	0	1	0	0	0	0	1 (0.6)
HRV-A	10	8	3	2	1	0	0	24 (14.3)
HRV-B	6	2	1	0	0	0	0	9 (5.4)
HRV-C	11	6	1	1	0	3	0	22 (13.1)
Total	92 (54.8)	33 (19.6)	20 (11.9)	9 (5.4)	3 (1.8)	10 (6.0)	1 (0.6)	168 (100)

EV, enterovirus; RV, rotavirus; NV, norovirus, ADV, adenovirus, HRV, human rhinovirus.

To further analyze EV in the AGE samples, we performed sequence and phylogenetic analysis. Four human EV species (A-D) and three human rhinovirus species (A-C) were identified (Figure 16). There were 5.4% (9/168) EV-A, 37.5% (63/168) EV-B, 23.8% (40/168) EV-C, and 0.6% (1/168) EV-D (Figure 21A). EV-A comprised genotypes CV-A4, CV-A5, CV-A8, and CV-A10. EV-B species demonstrated the most diversity (18 genotypes), most of which were CV-A9 and

echovirus E6 (8/63 for each) (Figure 21B). Of the 9 types of EV-C identified, 26 were Sabin vaccine strains of poliovirus (8 type 1, 10 type 2, and 8 type 3) (Figure 17). one fecal sample derived from a 3-year-old child tested positive for EV-D68 of clade B2 (Figure 18-20). Finally, rhinovirus was detected in 32.7% (55/168) of the samples (24 species A, 9 species B, and 22 species C), 27 of which were not co-infected with any RV, NV or ADV.

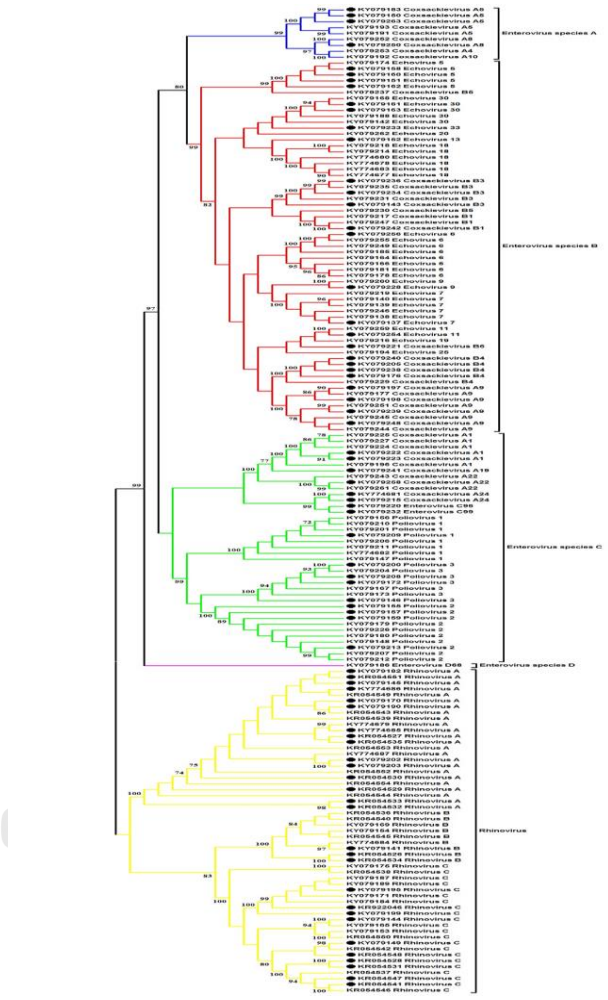

Figure 16 Cladogram analysis of the nucleotide sequences of the VP4-VP2 region from EV-positive AGE samples.

Phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA (version 5). Bootstrap resampling values >70 are indicated at the nodes. The scale bar indicates the number of substitutions per site. Black dots denote EV obtained from samples with multiple viruses. Blue, EV-A; red, EV-B; green, EV-C; purple, EV-D; yellow, rhinovirus.

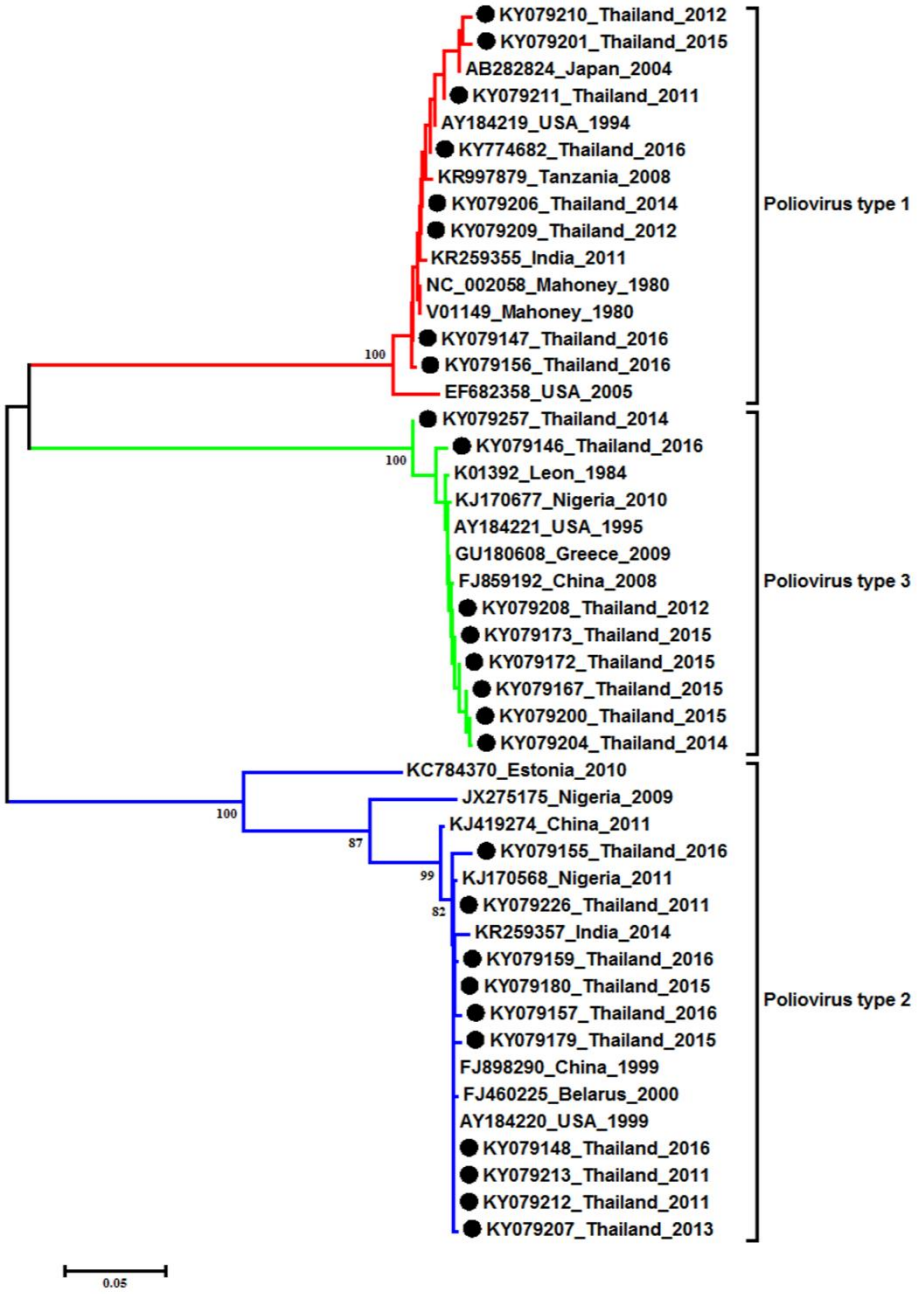

Figure 17 Phylogenetic analysis of the partial sequence (539 bp) of the 5'UTR/VP2 region of poliovirus vaccine strains found in AGE samples in this study.

Black dots denote poliovirus co-identified with multiple viruses in the sample.

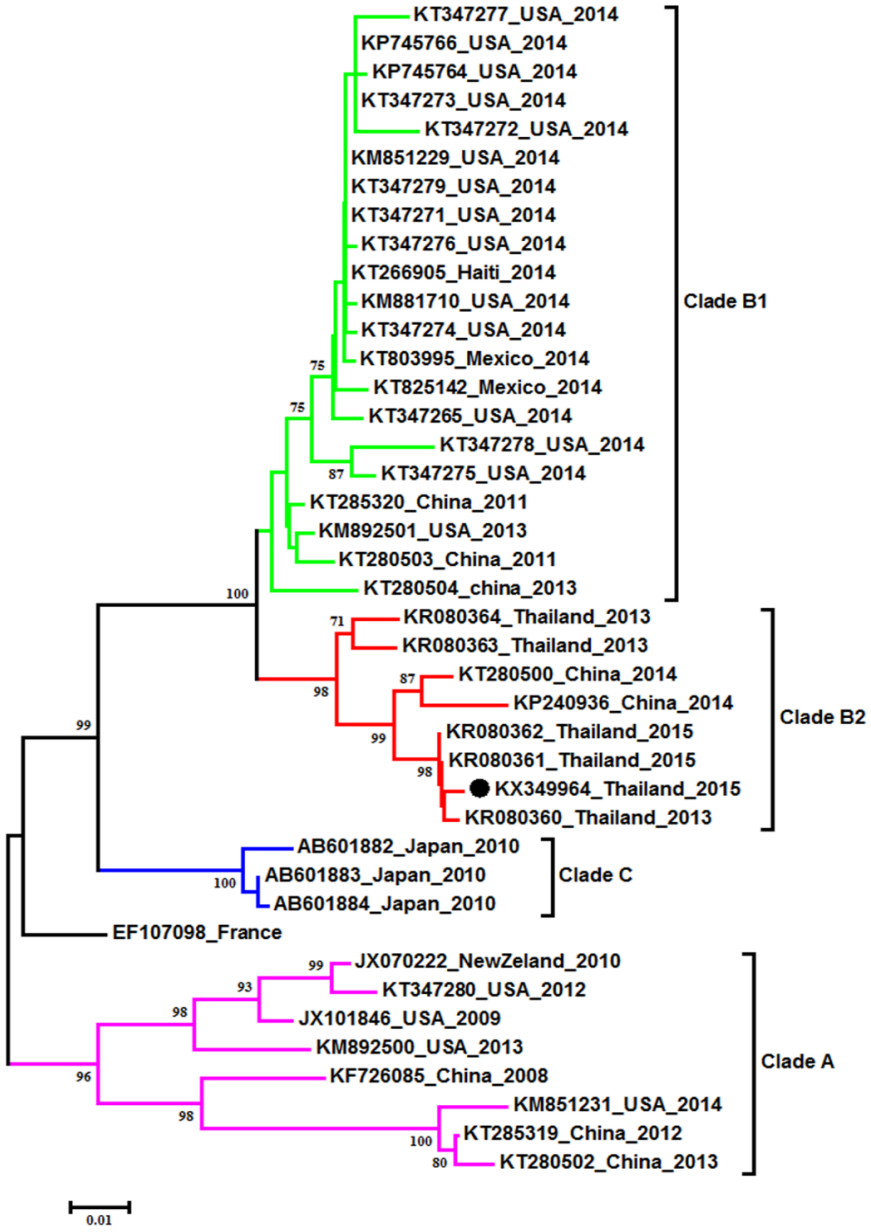

Figure 18 Phylogenetic analysis of the partial sequence (539 bp) of the 5'UTR/VP2 region of EV-D68.

Black dot indicates the virus identified in this study.

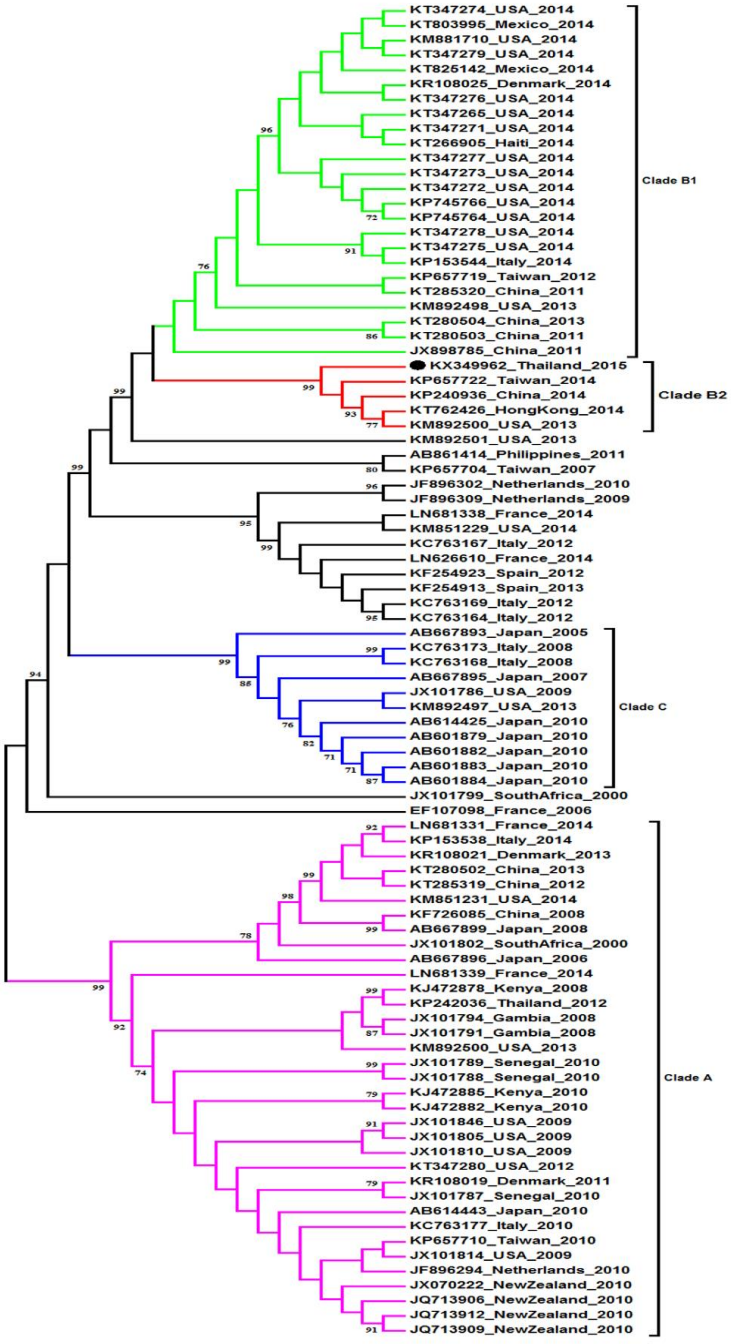

Figure 19 Cladogram analysis of the partial sequence (824 bp) of the VP1 region of EV-D68.

Black dot indicates the virus identified in this study.

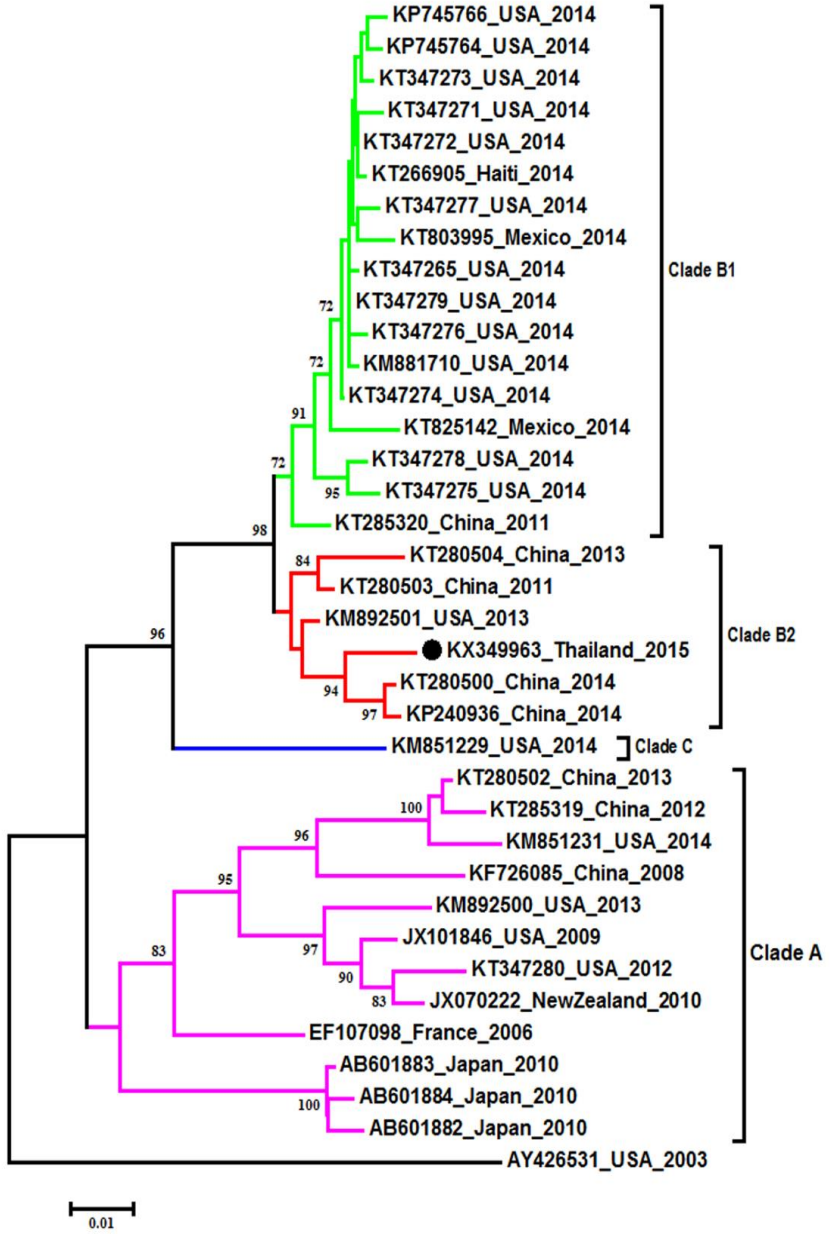

Figure 20 Phylogenetic analysis of the partial sequence (764 bp) of the 3D region of EV-D68.

Black dot indicates the virus identified in this study.

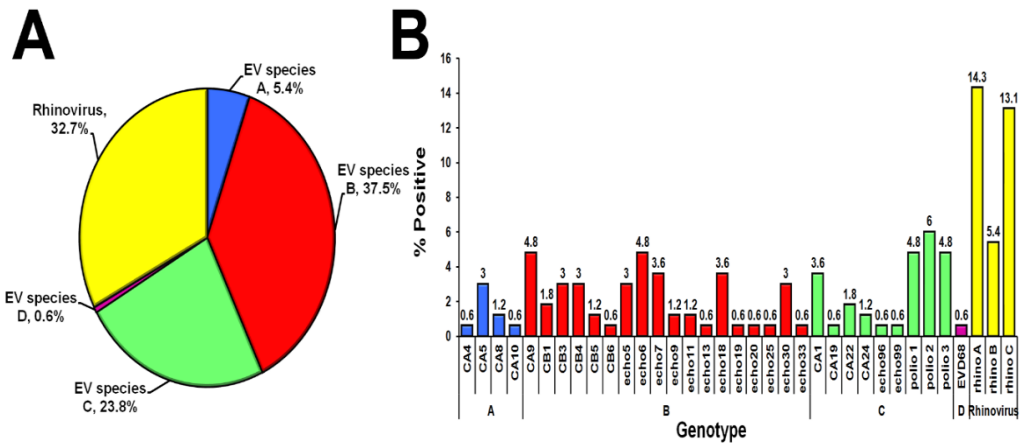

Figure 21 Distribution of EV species and types found in 168 EV-positive AGE samples.

(A) Pie chart of EV-A to -D and rhinovirus found in the fecal samples of AGE patients. (B) Genotypes of EV and their percentages (denoted by numbers above the bar graphs). Blue, EV-A; red, EV-B; green, EV-C; purple, EV-D; yellow, rhinovirus.

In contrast, analysis of the EV identified in HFMD samples revealed that an overwhelming majority (92.6%, 757/817) belonged to species A (Figure 22A). Eight EV-A identified were CV-A2, CV-A4, CV-A5, CV-A6, CV-A8, CV-A10, CV-A16, and EV-A71, most prevalent of which was CV-A6 (54.8%, 448/817) (Figure 22B). Interestingly, CV-A5, CV-A9, echovirus E18 and rhinovirus A were some of the viruses found in multiples samples from both AGE and HFMD cohorts.

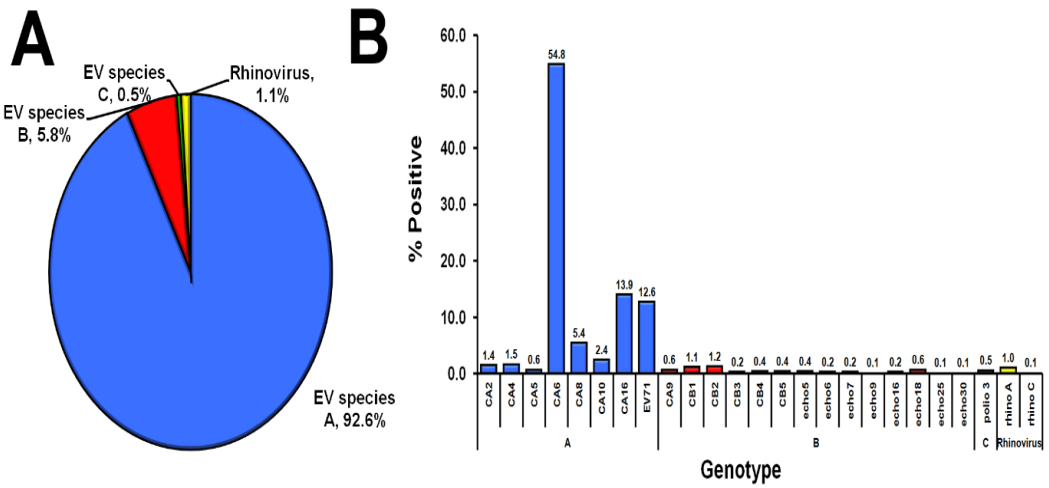

Figure 22 Distribution of EV species and types found in 817 EV-positive HFMD samples.

(A) Pie chart of EV-A to -C and rhinovirus found in the fecal samples of HFMD patients. (B) Genotypes of EV and their percentages (denoted by numbers above the bar graphs). Blue, EV-A; red, EV-B; green, EV-C; yellow, rhinovirus.

Viral gastroenteritis is a significant problem especially among children in under-resourced and developing regions. In addition to affecting the quality of life, AGE imposes a substantial medical and socio-economic burden. Infections caused by RV, NV, and ADV remain significantly underdiagnosed and are responsible for a substantial incidence of diarrhea as was shown in this study and by others (77, 108, 125, 126, 129-132). However, many EVs are also increasingly recognized as being associated with a proportion of persistent diarrhea and are often examined when stools are negative for commonly implicated enteric viruses (115, 118, 129, 133).

In this study, we investigated the molecular epidemiology of viruses typically associated with diarrhea in Thailand. The distribution of the viral etiology of AGE varied by year with RV as the leading cause of diarrhea especially in children below 5 years of age. The gradual increase in RV vaccination in Thailand has contributed to the decline of RV-related AGE and the emergence of NV GII.4 as the leading cause of AGE as was also seen elsewhere (107, 134). We were particularly interested in the co-detection of EV, especially in samples where RV, NV, or ADV were not detected. Between 2010 and 2016, multiple EV species and genotypes were detected in the

AGE samples (6.2%) including EV-B, EV-C, and all 3 species of rhinovirus. In approximately half of these samples (54.8%, 92/168), EV was the only virus present. This represents 3.4% (92/2,692) of all AGE samples, a relatively minor component if compared to RV infection. Comparison of our results to the limited published studies in developing countries with similar tropical climate showed that this rate is lower than the 12.3% of RV-negative, NV-negative, and ADV-negative fecal specimens from children with AGE in northern Ghana (135) and 9.8% in Vietnam (133). It is also lower than the prevalence of non-polio EV in RV-negative and NV-negative AGE in western India (14.1%) (115). The lower prevalence of EV-associated AGE observed in this study compared to others may in part be due to the population examined since approximately half of our cohort was from a major urban area of Bangkok. Although we also relied on PCR-based assays, sensitivities and specificities among studies vary. Additionally, viral burdens are expected to be different among different developing countries due to living conditions, diet, and cultural practices.

CV-A6, CV-A16, and EV-A71 were the three most commonly identified EV associated with HFMD. This finding is consistent with our previous reports (95, 97). Our study also revealed several interesting observations. For example, the finding of all 3 poliovirus types in 15.5% (26/168) of the AGE samples in this study was not unexpected because children can sometimes experience diarrhea as a result of poliovirus vaccination (136). One AGE sample from a child with fecal occult blood but no neurological manifestation tested positive for EV-D68. Although EV-D68 causes respiratory infection, detection in stool has been reported (137). Similarly, all three rhinovirus species were present in the AGE samples despite the absence of other enteric viruses in agreement with a number of other studies (129, 138). Why nearly half (45.2%) of AGE samples with identifiable enteric virus also had EV is quite puzzling, but the availability of clinical information regarding the severity of diarrhea should enable examination of possible additive effect EV may have on AGE given the diverse genotypes of EV-B and rhinoviruses found in the samples. Finally, the overlapping presence of CV-A5, CV-A9, CV-B1, echovirus E18 and rhinovirus A in both AGE and HFMD samples will require further studies. Of interest are CV-A9,

CV-B1, and echovirus E18, which can cause viral meningitis (139, 140). In addition, CV-A9 and CV-B1 have also been linked to childhood diabetes (141, 142).

There were several limitations in this study. Although it would have been ideal to compare EV found in AGE samples with fecal specimens from age-matched healthy controls who showed no AGE symptoms, a preliminary investigation we performed on 200 fecal specimens from healthy children ≤5 years of age did not show any detectable EV (Figure 13, unpublished data). We were unable to exclude the possibility that some AGE episodes were caused by bacteria or other less common enteric viruses such as astrovirus, sapovirus, and bocavirus. Although we amplified viral nucleic acid directly from clinical samples, we did not perform confirmation by isolating the viruses using cell culture as was done in some studies. However, we were able to identify one or more viruses in the fecal specimens and verified each with nucleotide sequencing. Future studies involving prospective sequential sampling of stools during healthy and AGE episodes from birth to adulthood may better clarify the burden of EV in diarrheal disease. In summary, the data from this study suggest an association between several EV genotypes and a proportion of AGE cases in Thailand, which underscores the diversity of clinical manifestations afforded by EV.

Part 4: Prevalence of poliovirus vaccine strains in Thai: Implications of the oral to inactived poliovirus vaccine transition

(Submitted in: Biomed Environ Sci)

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Part 4: Prevalence of poliovirus vaccine strains in Thai: Implications of the oral to inactived poliovirus vaccine transition.

Prior to the development of the poliovirus (PV) vaccine, polio was a devastating childhood disease. Although mild symptoms were characterized by vomiting, headache, myalgia, paralytic poliomyelitis caused debilitating morbidity. PV infects the central nervous system and replicates in the motor neurons of the spinal cord, brainstem, or motor cortex (143-145). Humans are the primary natural hosts in which transmission is via fecal-oral route. Some infected individuals, especially those who are immunocompromised, may shed PV for weeks to months (146).

The introduction of the Salk inactivated polio vaccine (IPV) in 1955 was followed by the more highly effective Sabin live attenuated trivalent oral polio vaccine (tOPV) in 1961, which included all three attenuated PV strains (PV genotype 1 to 3). The tOPV has proven to be inexpensive to produce, easily administered orally, and stable during storage and transportation. Since 1978, the World Health Organization (WHO) expansion of tOPV in the vaccine immunization programs has resulted in a drastic decrease in the incidence of poliomyelitis worldwide. As a result of global immunization efforts, only Pakistan, Afghanistan, and Nigeria remained endemic for PV (147-150).

Although tOPV can efficiently eradicate wild PV, it is not a perfect vaccine. Occasional shedding of viral revertants in some vaccinees reintroduces poliovirus into the environment (151). Moreover, underlying immune deficiency in some vaccinees can result in poliomyelitis after vaccination (152). Such vaccine-associated paralytic poliomyelitis (VAPP) is estimated at approximately 2.9-4.7 annual cases per million births globally, representing one poliomyelitis per 1-2.5 million OPV dose or susceptible OPV recipients. Therefore, worldwide incidence of polio including those attributed to the vaccine are still reported in Africa, Eastern Mediterranean, and Western Pacific as of 2016 (147-150).

In 1988, the WHO launched the Global Polio Eradication Initiative to eliminate wild PV and vaccine-derived poliovirus (VDPV). Strategies included 1) detecting transmission, 2) monitoring the impact of extensive use of OPV immunization, 3) surveillance of acute flaccid paralysis (AFP) cases possibly

attributed to infection from VDPV, and 4) minimizing the risk of VDPV. Although the Global Commission for the Certification of Poliomyelitis Eradication (GCC) has already certified the elimination of wild PV genotype 2 worldwide on September 2015 (22, 153) and wild PV genotype 3 has not been detected since November 2012, but wild PV genotype 1 currently remains endemic in several countries (154). Presently, >80% of the world's populations live in certified polio-free regions, which includes North America, Australia, and Europe.

To eventually eliminate VDPV from the environment, the launch of the "Polio Eradication and Endgame Strategic Plan 2013–2018" aimed to withdraw all OPVs while retaining high immunization coverage. This is possible with the availability of an improved IPV, which has enhanced potency, increased effectiveness, and no serious systemic adverse reactions compared to the original IPV. Its administration provides close to 100% seroconversion in all settings (155). IPV is now used exclusively in many higher-income countries in their national immunization programs, which has effectively eliminated wild and VAPP. This new campaign recommends the administration of at least one IPV dose in all countries (155-157). There are major barriers, however, in IPV introduction in low and middle-income countries due to cost, availability, and distribution logistics.

In anticipation for the change in polio vaccination, infants in Thailand began receiving IPV at month 4 in December 2015 under the existing vaccination regimen of tOPV at month 2, 4, 6, 18, and 48–60. From May 2016 onward, all tOPV stock in the country was withdrawn (158) and its use was replaced by the bivalent OPV (bOPV) devoid of PV genotype 2. Overall, polio immunization coverage in Thailand is reportedly 99% (159). Additionally, the use of bOPV is expected to be discontinued by 2020 whereby all infants would only be immunized with IPV. Given such changes in the vaccine policy, we sought to identify PV in stool samples from patients with suspected enterovirus infection in the past seven years, which encompassed this transition period. This cohort includes stool samples were obtained for enteric virus testing and therefore served as convenient samples for PV screening. PV strains identified in this study were genetically characterized. These results are expected to assist in evaluating vaccine coverage and PV surveillance in Thailand, which are crucial towards regional PV elimination.

Materials and Methods

Clinical samples

This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB 002/60). The IRB waived the need for written informed consent because samples were de-identified and anonymous. Stool samples were conveniently available were screened for the presence of PV. A total of 6,817 samples from patients of all ages from Bangkok (n = 4,310) and Khon Kaen province (n = 2,507) were obtained between January 2010 and April 2018.

Enterovirus and PV identification

Stool samples were diluted in phosphate buffer saline to a 10% suspension and centrifuged. Viral nucleic acid was extracted from the supernatant using a viral nucleic extraction kit (GeneAll, Seoul, Korea). Initial screening for enterovirus was performed using pan-enterovirus assay and one-step real-time reverse-transcription polymerase chain reaction (RT-PCR) (SensiFAST, Bioline Reagent, London, UK) to identify EV-A71, CV-A6, and CV-A16 as previously described (160). Pan-enterovirus positive samples were subjected to complementary DNA (cDNA) synthesis using Improm-II Reverse Transcription system (Promega, Madison, WI), amplified with enterovirus primer sets 5'UTR/VP2 region (94), and sequenced. PV-positive samples were subjected to complete VP1 gene amplification using published primers (161). Nucleotide sequences obtained from this study were deposited in GenBank database under the accession numbers MF593155-MF593178.

Phylogenetic analysis

VP1 nucleotide sequences were analyzed using Basic Local Alignment Search Tool (BLAST) on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), edited using Chromas Lite software version 2.01 (Technelysium, South Brisbane, Australia), and assembled using SeqMan II (DNA STAR, Madison, WI). The phylogenetic tree was constructed using the maximum likelihood and optimal nucleotide substitution models as identified in ModelTest and implemented in the MEGA software package (96) with bootstrap value of 1,000 replicates. The complete VP1 sequences available in the NCBI database served as references. Additional phylogenetic analysis using ClustalW (162) and BEAST software (163) in combination with BEAGLE package was

performed to estimate the sequence change rates (164, 165). Phylogenetic inferences utilized the maximum-likelihood method and the nucleotide substitution model (JModelTest was the best-fit model) in conjunction with the uncorrelated lognormal molecular clock model and prior clock rate (166). A Bayesian coalescent method with general time-reversible (GTR) model was used to infer the rates of evolution of each PV genotype. The Bayesian Markov Chain Monte Carlo framework analyses for 100 million generations took into account regular samples until convergence was reached. Sampling was performed every 10,000, discarding 10% of the chain as burn-in. All other parameters were optimized during the burn-in period. Convergence of the Maximum Clade Credibility (MCC) trees output was assessed using Tracer software (167) in which effective sample size (ESS) values >200 were accepted. All estimation parameters were shown as mean and 95% highest posterior density (HPD). Nucleotide substitution rates and estimates for time to a most recent common ancestor (tMRCA) were calculated. An MCC tree was constructed with TreeAnnotator (163) to represent the best posterior distribution and visualized in Figtree software (http://tree.bio.ed.ac.uk/software/figtree/).

Analysis of selective pressure on PV

The individual site-specific selection pressure on VP1 was measured using the likelihood-based fixed effects likelihood (FEL) and single likelihood ancestor counting (SLAC) methods contained in the HYPHY package of Datamonkey (168). The significance level for the positively selected site was accepted at <0.1 (two-tailed binomial distribution).

Results

Detection of PV in stool samples

During this study period, 20.3% (1,382/6,817) specimens tested positive for non-polio enterovirus. Analysis of the 5'UTR/VP2 region revealed 0.45% (31/6,817) of samples were PV (Table 7). Slightly more PV genotypes 2 and 3 (35% each, 11/31) were identified compared to genotype 1 (29%, 9/31). Most were identified from stool samples belonging to children younger than 18 months of age, all of whom were born after the universal polio vaccination. Two PV-positive samples (1 each of PV genotype 2 and 3) were from adults older than 30 years of age in the AGE group whose vaccination histories were not available. Although polio vaccination in

Thailand transitioned from tOPV to bOPV in April 2016, PV genotype 2 remained detectable in stool samples in May 2016 (Figure 23). For each of these years, the detection of different PV genotypes did not appear to cluster.

Table 7 Demographic data of individuals whose samples were PV-positive.

		Total cases	Genotype		
			Poliovirus 1	Poliovirus 2	Poliovirus 3
Age (months)					
	0 < 2	245	-	1	3
	2 - < 4	118	3	5	2
	4 - < 6	188	1	1	2
	6 - < 18	1581	3	3	2
	≥ 18	4685	2	1	2
Province					
	Bangkok	4310	6	7	9
	Khon Kaen	2507	3	4	2
Sex					
	Male	4168	6	9	4
	Female	2649	3	2	7
Year					
	2010	312	1	-	-
	2011	351	1	3	-
	2012	1162	2	-	1
	2013	264	-	1	-
	2014	260	1	-	3
	2015	595	1	2	4
	2016	1062	3	5	3
	2017	1583	-	-	-
	2018	1228	-	-	-
Total		6817	9	11	11

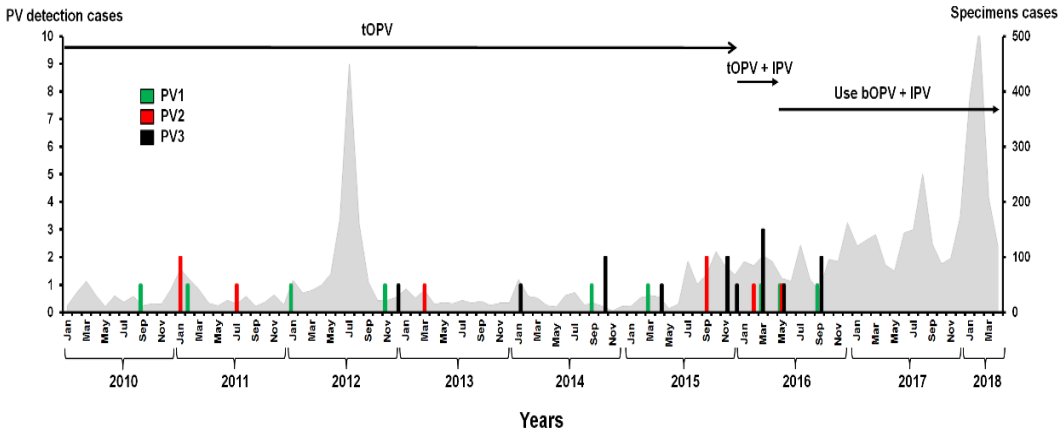

Figure 23 The distribution of PV identified between 2010 and 2018.
Left scale represents the number of PV-positive samples, while the right scale represents the total number of samples screened.

Phylogenetic and sequence analysis of PV

The phylogenetic analysis of 24 complete VP1 sequences from which amplification was successful showed three clusters of PV lineage consistent with 3 PV genotypes (Figure 24). It also supported the initial PV genotype assignments by the 5'UTR/VP2 region in which all sequences were closely related to their respective Sabin vaccine strains. No nucleotide deletions or insertions were observed among the PV identified in this study, while most nucleotide substitutions in the coding region were synonymous.

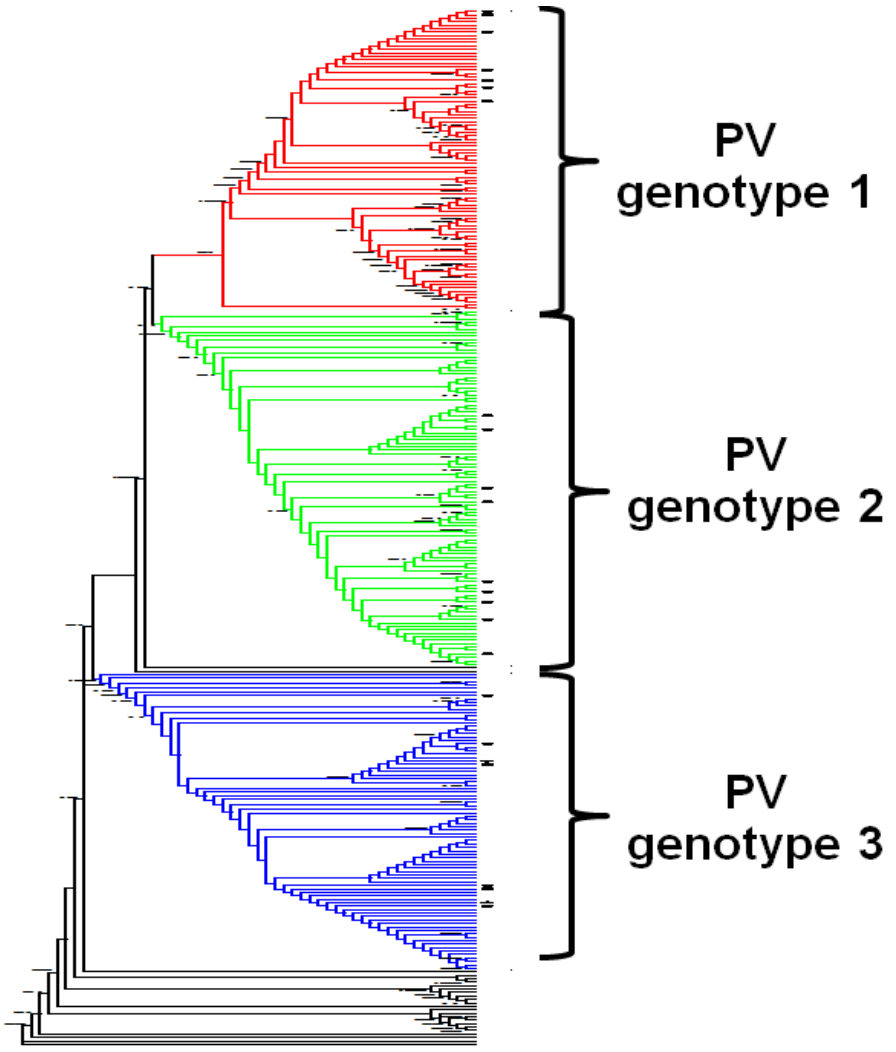

Figure 24 BEAST analysis of each PV genotype to cluster three lineage based on VP1 region.

To evaluate the adaptive molecular evolution of PV, we estimated the ratio of non-synonymous to synonymous substitutions (dN/dS) within the VP1 region. The overall dN/dS value was 0.056, while dN/dS values were 0.047 (genotype 1), 0.066 (genotype 2), and 0.036 (genotype 3). Given that PV genome replication introduces 10^{-4} substitutions/nucleotide on average (169), we performed molecular clock calculations in order to estimate the rate of mutations/synonymous site/year in the VP1 gene. Our results also showed the evolution rates of PV capsid region at $3.7x10^{-4}$. Thus, there were no evidence of positive selection, suggesting that nucleotide

changes identified in this study were below the thresholds for possible emergence of VDPV.

Official epidemiological surveillance of PV has reported the presence of poliomyelitis in Thailand since 1952 (Figure 25). Incidence of AFP possibly linked to PV infection in the country was also monitored beginning in 1992. Although tOPV vaccination in selected Thai provinces began in 1977, implementation nationwide did not occur until 1982 (Figure 26). Since then, national disease surveillance combined with improved vaccine coverage resulted in a dramatic decrease in the incidence of poliomyelitis in Thailand (147, 165, 170-173). The last reported wild PV in Thailand occurred in Loei province in April 1997 (identified from stool sample of a 10 year-old child who had received only one tOPV dose) and the last vaccine-derived poliomyelitis was reported in 2003 (PV genotype 2 identified from an 18-month old boy who completed polio vaccination). As per WHO recommendation, the Thai Ministry of Public Health has implemented the administration of IPV in combination with tOPV or bOPV for childhood vaccination beginning in December 2015.

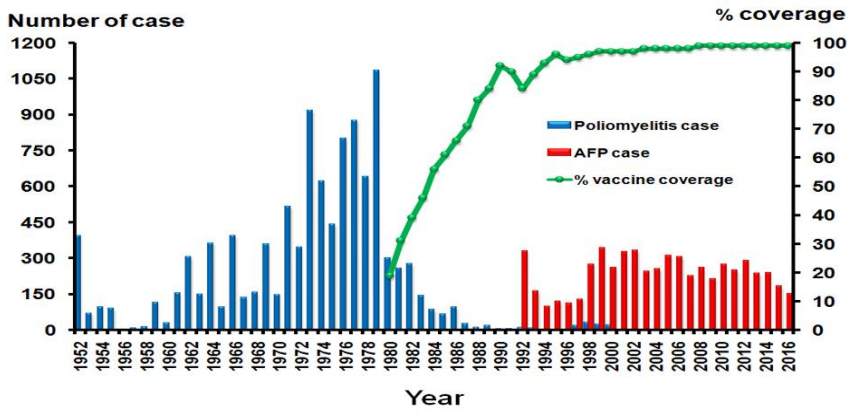

Figure 25 The annual incidence of poliomyelitis and AFP relative to polio vaccine coverage between 1952 and 2017 in Thailand.

Blue bars represent poliomyelitis cases due to natural PV infection, while red bars represent AFP cases (left scale). Polio vaccine coverage is denoted by the dotted green line (right scale)

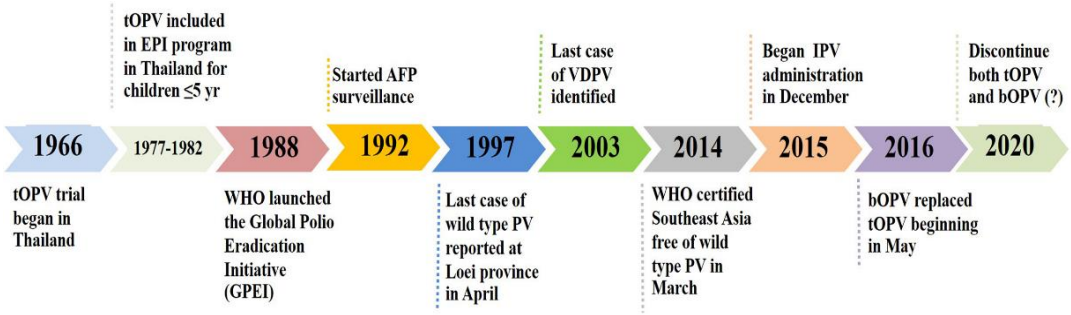

Figure 26 Timeline for PV vaccination in Thailand.

Although tOPV has contributed to a significant decrease in infection by wild PV due to its many advantages, there are several reasons to discontinue tOPV in favor of bOPV. Phenotypic changes in the attenuated PV strains in the tOPV due to their genetic instability could emerge (174) and the resulting variants can cause poliomyelitis (175). Studies on PV in feces and in sewage have suggested that VDPV replication in populations with low polio vaccine coverage can result in reversions of the attenuating mutations (176). PV genotype 2 has been associated with VAPP cases (174, 177, 178). Given the currently low incidence of polio around the world, the WHO Strategic Advisory Group of Experts on Immunization (SAGE) has determined that the risks of tOPV outweighed the benefits and therefore promoted the use of bOPV as an alternative.

The near elimination of all PV around the world has also made the use of IPV an attractive option. The inactivated IPV also contains all 3 PV genotypes, which provides protection from genotype 2 lacking in bOPV. Even outbreaks attributed to VDPV genotype 1 are reported (148, 152, 179, 180). An increasing number of countries are using IPV for routine vaccinations (181, 182) because it does not cause PV shedding in feces (183) nor contribute to VAPP (184). For these reasons, IPV has a prominent role in the ongoing efforts towards an eventual global PV eradication.

In this study, the majority of PV-positive samples were from patients born after the universal polio vaccination. PV was detected in 0.45%, which was lower than rates previously reported (185). The reason for this may be attributed to the screening of such convenient stool samples. Prevalence of poliovirus detected in other studies also reflected different cohort characteristics and geographical regions. All

three PV genotypes found here were closely related to the vaccine strains, most of which were identified in children who had received polio vaccine. Interestingly, two PV-positive samples were from adult. Due to the lack of clinical data, we could only assume that these adults had exposure to PV possibly from households with recently vaccinated children or from other environmental exposure. Moreover, PV has not been detected since 2017, possibly due to the fact that presently almost all children received IPV.

As with any live attenuated virus in vaccines, the genetic basis of Sabin strain attenuated phenotype relies on few substitutions in their genome (186). Three key substitutions (A481G, U2909C, and U398C) on VP1 have been reported in the loss of attenuation (187), but these mutations were not found in any of the strains found in this study. Therefore, it is unlikely that the PV strains identified have the potential to cause VAPP.

PV vaccine strains of all 3 genotypes were identified in fecal samples while tOPV was in use. The withdrawal of tOPV beginning in 2016 coincided with the beginning of bOPV and IPV vaccination combination, which will eliminate the circulation of PV genotype 2. Continued surveillance of PV infection from yearly incidence of poliomyelitis and AFP will provide crucial assessment of VDPV.

Part 5: Molecular characterization of human enterovirus with aseptic meningoencephalitis in childhood

(Under revised before submission)

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Part 5: Molecular characterization of human enterovirus with aseptic meningoencephalitis in childhood.

Patients with viral meningitis can be lethargic and impaired by a headache while motor or sensory deficits, altered mental status and disturbed consciousness may show in encephalitis patients (188). It often occurs in children and adolescents. World Health Organization (WHO) indicated that this disease is responsible around 12% of deaths/year worldwide in children (26). Although, bacterial and viral meningitis often present same clinical signs and symptoms. The rate of bacterial meningitis was greatly reduced after widespread use of Haemophilus influenza type b (Hib) conjugate vaccine (189, 190). Thus, viral meningitis becomes significant. Although the majority of patients continue to have unknown etiologies, some pathogen requires urgent therapy for cure and survival (191). Many etiologic viruses can cause meningoencephalitis such as Enterovirus, Herpes simplex virus, West Nile virus, Varicella zoster virus and Cytomegalovirus (192); the incidence rate has been estimated between 0.26 and 17 cases per 100000 population depend on the age or vaccination status. It's usually present with fever, headache, sepsis, nausea, vomiting and neck stiffness but self-limiting. Infants and young children represent the most susceptible population.

Human enteroviruses (EV) is the leading causative agents in children accounting for up to 80-90% of cases, and many genotypes circulate worldwide (193-195). EV meningitis is self-limited with a low rate of complications or sequelae while encephalitis is less common but more severe manifestations. EV is more likely to have a fatal outcome especially if treatment is delayed. The neurological complications can occur directly from EV infection to CNS (196, 197). The most outbreaks belong to EV B species (Coxsackie B viruses and various echoviruses) which reported worldwide in the varying size of epidemics (27, 139, 198, 199). Cerebrospinal fluid (CSF) is mainly diagnostic based to analyze with EV infection. Although EV presents the low viral load in the CSF, several studies recommend screening blood specimens in combination while detection in the stool or respiratory secretions is less value in the diagnosis of CNS infection (200). In the other hand, a

higher viral load was not correlated with more severity (201). There are no specific beneficially therapies proven.

The viral isolation from CSF and identification by virus neutralization assay with type-specific antisera is highly specific but time-consuming. Present, polymerase chain reaction (PCR) assay at 5' untranslated region (5'UTR) and part of P1 region has a classical method which more sensitive and highly specific for the diagnosis. It helpful for patient management by avoids unnecessary antibiotic treatment and decrease hospitalization period (199).

EV has been found recombination frequently among members of the same species. Most cases have been reported in encoding nonstructural protein gene. The rate of their recombination is correlated with the endemicity and emergence cycle. Therefore, EV typing and phylogenetic analyses are equally essential to identify emerging, mutation, recombination and their transmission. Herd immunity against each EV type determines their epidemic patterns. Statistical phylogenetic methods have been developed to address and apply to investigate virus migration.

The information of EV distribution in meningitis and encephalitis in Thailand is still lacking. This study aimed to survey the crucial data and characterize the EV strains circulating in aseptic meningoencephalitis patients among childhood and explore the genotype correlation with other countries based on sequences analysis.

Materials and Methods

Study samples and Specimens' collection

This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB 002/60). The Director of King Chulalongkorn Memorial Hospital has authorized the use of these stored samples. In this study, anonymous of 269 childhood patients who had clinically suspected an EV infection and hospitalized in Bangkok, Thailand in 2016 were enrolled. Demographic and clinical data of all cases were collected. There were 20 neonates, 81 infants, 126 preschools and 42 childhoods. Various clinical specimens (stool, throat swab, CSF, etc.) were collected individually by clinicians from each case. A bacterial cause was excluded by application of conventional culture and gram staining from the routine laboratory.

Viral identification

The viral nucleic acids were extracted by using RiboSpin vRD kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. The one-step real-time reverse-transcription PCR (real-time RT-PCR) was performed for EV screening (160). It consisted of two sets for screening pan-enterovirus and characterized EV A species (Enterovirus A71, Coxsackie A16, and Coxsackie A6).

The pan-enterovirus and negative results were selected to perform RT-PCR screening with conserve region at 5'UTR/VP2 by semi-nested PCR (94) and sequencing. Each case was transcribed to cDNA by using Improm-II^{TM} Reverse Transcription system (Promega, Madison, WI) and subjected to viral screening. The Basic Local Alignment Search Tool (BLAST) analysis was performed to identify EV genotype from sequencing results.

Extracted clinical data which suspicion of aseptic viral meningitis or encephalitis based on physician judgment, hospitalized with neurological dysfunction and high fever symptoms from retrospective medical record review. Then, performed EV characterization by amplified the complete VP1 region and partial 3D region (100) for analyzing recombination event. The positive amplicons were subjected to sequencing and constructed a phylogenetic tree of predominant genotype by using MEGA5 software (96).

Phylogeographic analysis

The correlated VP1 sequences data which deposited in GenBank with known time and geographical of isolation were included with predominant genotype sequences in this study. Sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The JModelTest (166) was performed to identify the best fitting nucleotide substitution model for multiple alignment sequences. A molecular clock was performed in BEAST software (202), using an uncorrelated lognormal relaxed molecular clock and Bayesian skyline tree prior. The multiple Monte Carlo Markov Chain (MCMC) runs of 100 million chains; the 10% were discarded as burn-in. The Figtree software (203) was used for visualizing the phylogenetic tree. Transmission pattern of circulation clades was analyzed by the SPREAD v1.0.6 software (Spatial Phylogenetic Reconstruction of Evolutionary Dynamics) (204), the latitude and longitude data were plotted together with sequences

data and visualized with Google Earth Engine (http://www.google.com/earth/download/ge/) to reveal the phylogeographic.

Selective pressure analysis

The HYPHY package on the Datamonkey website (168) was performed to analyse the selective pressure in VP1 region. The selective pressure was measured using the likelihood-based fixed effects likelihood (FEL) and single likelihood ancestor counting (SLAC) methods. The significance level for the positively selected site was accepted at <0.1 (two-tailed binomial distribution).

Virus cultivation

RD cells were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) 1 ml, supplemented with 10% fetal bovine serum (FBS) in 96-well plates and grown to approximately 80% confluence. The cells were infected with 10 µl of samples which have a complete VP1 sequence and incubate in 37°C for followed cytopathic effect (CPE).

Viral isolation and Whole genome Next-generation sequencing (WG-NGS)

The supernatant of these CPE positive was collected for viral RNA isolation by E.Z.N.A. Viral Extraction Kit (Omega Bio-tek, Norcross, GA). The QuantiNova™ SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) was used with 10 µM of ENRI primer (205) and 2 µl of vRNA in 20 µl of reaction to confirm EV infection.

NanoDrop (Wilmington, DE) was utilized to quantify and determines genomic quality. Purified amplicons were end-repaired, adaptor-ligated and cleaned up. PCR amplified DNA library obtained from each sample with different index primers and purified with AmPure XP beads (Beckman Coulter, CA). The different indexes were pooled together. Deep sequencing was performed using MiSeq v2 reagent kit (Illumina, CA) and carried out on MiSeq platform (Illumina, CA) according to the standard protocol. FASTQ data were processed and analyzed by using CLC genomic workbench version 8 (http://www.clcbio.com/). Low-quality reads (Q-score < 30) and adaptors sequences were excluded, while low-quality regions of sequences were trimmed.

Results

In this study, the positive detection of EV A species (EV-A71, CA16, and CA6) by real-time RT-PCR was 24.2% (65/269), pan-enterovirus 22.3% (60/269) and negative 53.3% (144/269). Among of pan-enterovirus and negative result, 28.4% (58/204) was positive by semi-nested PCR. It consists of EV A species 33, EV B species 11, EV C species 2 and Rhinovirus 12. Interestingly, all of positive for EV B species were had symptoms related to meningitis or encephalitis, and echovirus 18 was the most predominant 72.2% (8/11) while finding coxsackievirus B5, echovirus 5 and echovirus 6 individually 9.1% (1/11). Complete VP1 gene sequences of echovirus 18 were obtained and deposited in GenBank database as accession number MH427204 – MH427216. The phylogenetic tree of those was constructed with all available complete VP1 sequences in GenBank database. The same sequences or collected in the same period were excluded. The results as in figure 27A and 27B showed that echovirus 18 was clustering in three sub-genotype (A-C). The echovirus 18 which isolated from Thailand was a cluster in sub-genotype C and closely related to the previous finding in other countries.

Also, sequence results of 3D region in all samples revealed the different genotype with VP1. It confirmed that recombination event occurs.

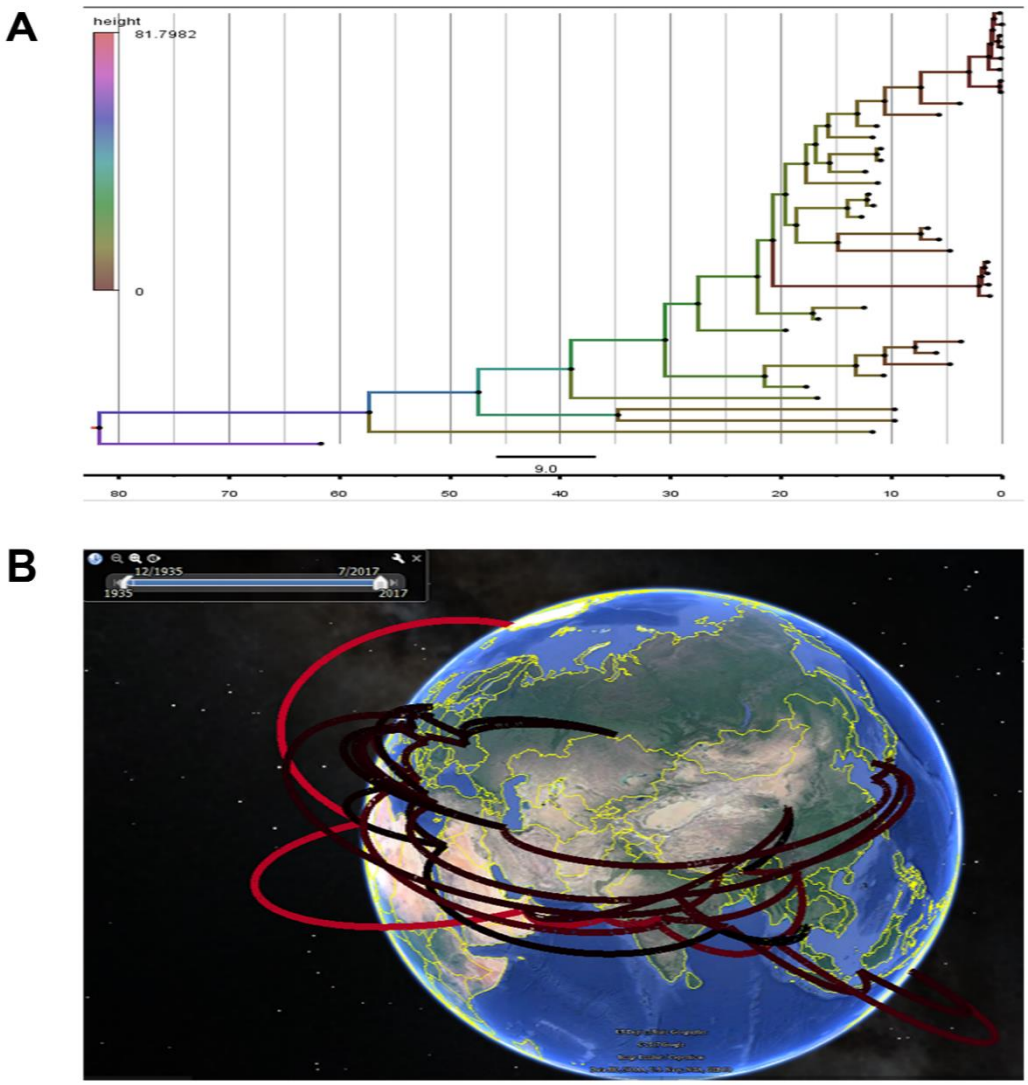

Figure 27 Phylogenetic and Phylogeographic analysis in meningitis cases.
A.) Phylogenetic analysis B.) phylogeographic analysis based on the VP1 region. It constructed by BEAST software using the GTR substitution model.

The selective pressure result was revealed in the ratio of non-synonymous to synonymous substitutions (dN/dS) values which found 0.035 and no positive selective pressure. The molecular clock calculations to estimate the rate of mutations/synonymous site/year in the VP1 gene, showed evolution rates of VP1 as $1.33x10^{-3}$ nucleotide substitution/site/year.

The official surveillance data of meningitis and encephalitis cases have reported from Bureau of Epidemiology, Ministry of Public Health, Thailand between

2007 and 2017. It was revealed as descript in Figure 28 and 29 (206). It showed that it usually found in infants and unknown specific caused agent.

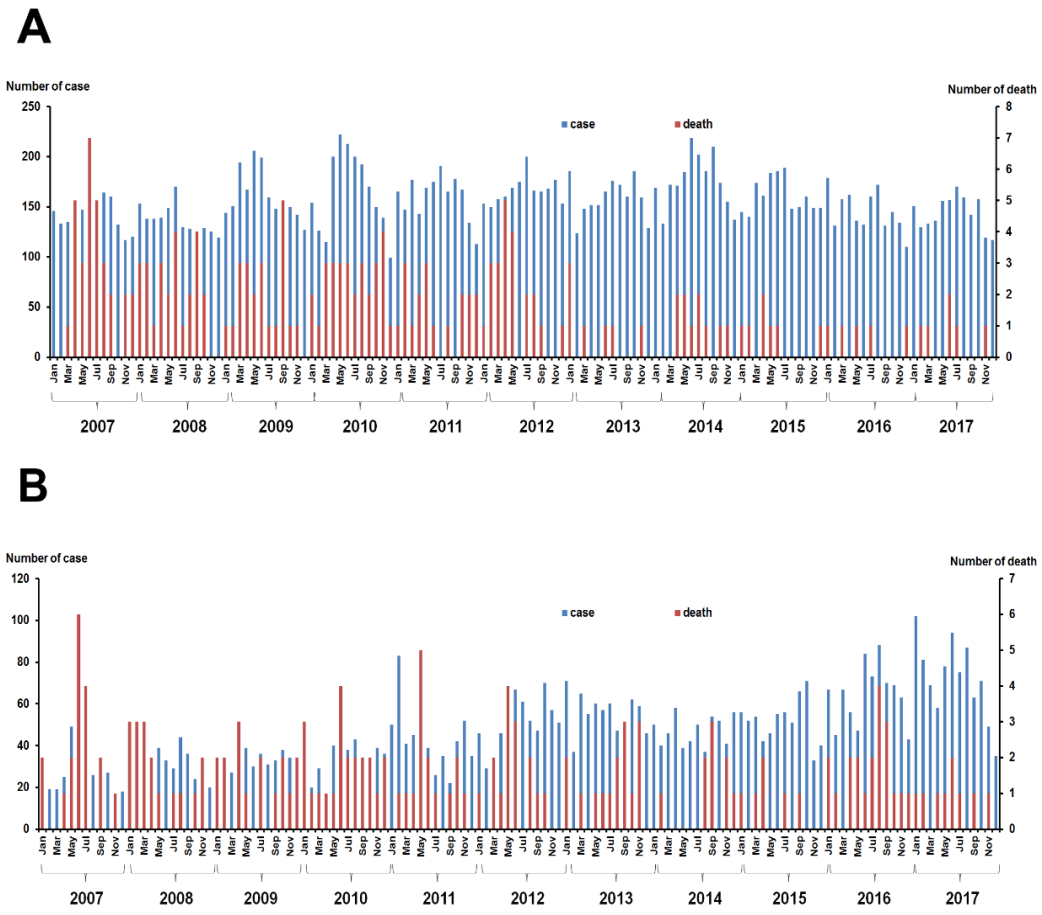

Figure 28 Meningitis and Encephalitis reported cases in Thailand from Bureau of Epidemiology, Ministry of Public Health, Thailand between 2007 and 2016. (A) Meningitis cases, (B) Encephalitis cases classified by year. Primary Y axis represented number meningitis of case, Secondary Y axis represented number of death cases.

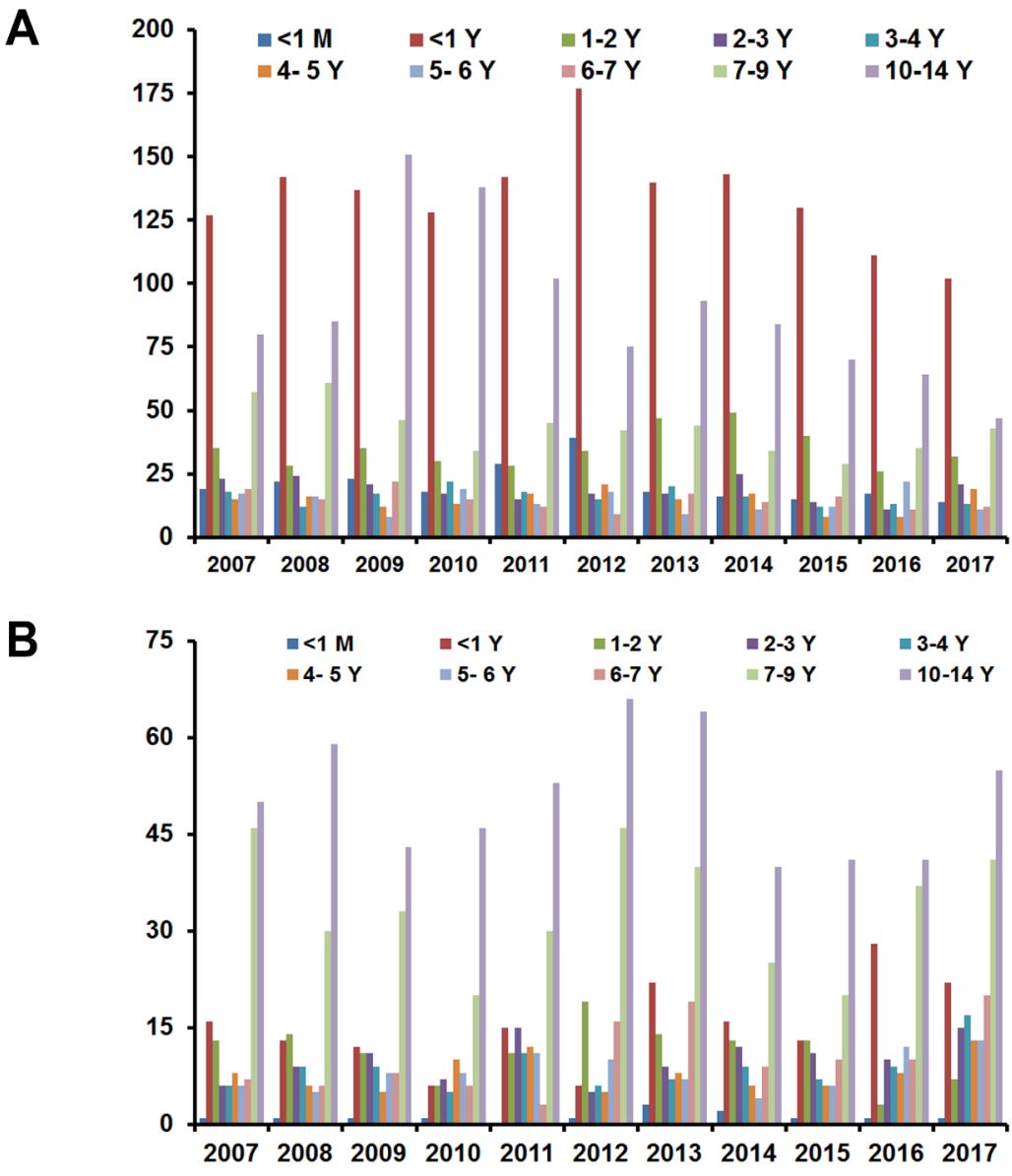

Figure 29 Meningitis and Encephalitis reported cases in Thailand from Bureau of Epidemiology, Ministry of Public Health, Thailand between 2007 and 2016. (A) Meningitis cases, (B) Encephalitis cases classified by age group

Clinical manifestations and diseases caused by EV is strongly influenced by age that leads to large numbers of hospital admissions each year. Seasonality could explain the broad range and their genotypes involved. EV and their variants cause a significant infection globally and carry significant morbidity particularly in vulnerable populations. An epidemiological investigation may provide relevant information for

monitoring and control the emerging in neurological disease (198). Meningitis and Encephalitis are the most common neurological disease reported by the CNS. Although many viruses can cause these disease that varies by distribution and occurrence in different geographical region (207) many of EV represents leading pathogens which can infect different compartments of the CNS by crossing the blood-brain barrier or retrograde axonal transport. The disease occasionally found in children under 15-year-olds. They are also most likely to present with fever, respiratory symptoms, and leukocytosis. Older ages rarely have complicated meningitis and recover without sequelae (197, 208). The strong evidence finding in this study was a predominance of the EV B species as previous studies (139, 209). Therefore, it becomes a significant agent in the diagnosis of CNS disease and associated complications such as myocarditis and hepatitis, particularly in neonates. Epidemiological surveillance can help to understand the disease association and changing pattern of infection. Thus, accurate genotyping and phylogenetic analysis need to identify emerging variants and their transmission.

Even though the aseptic meningoencephalitis surveillance data is very limited, but many EV genotypes had been reported. Their cause a range of similar manifestation but there are differences in epidemiological and geographic area (210). The high epidemic frequency of more than one EV genotype was observed as previous reports from Europe, America and Asia (211-218). The present study revealed that EV was responsible for cases of meningitis throughout the year in infant and childhood populations, but the immune status of patients was not thoroughly investigated.

Echovirus 18 belongs to EV species B which have frequently been reported outbreaks of aseptic meningitis in many countries worldwide. In the past, the spread of echovirus 18 infections were analyzed by virus isolation by serological methods. Until now, molecular data obtained with echovirus 18 outbreaks are mostly restricted to East Asia and North America (219-224). Their clinical manifestations vary from asymptomatic to aseptic meningitis and death. Their serotype may overlap with hand foot mouth disease (225). The prototype strain (Metcalf, AF317694) was isolated from infant diarrhea patient in the sporadic case in 1955 (226) while complete nucleotide sequence was determined by Andersson *et al*. (227). The first outbreak of

echovirus 18 aseptic meningitis was reported from North Carolina in 1972 (228). All strains could be divided into three subgenotypes (A-C). The phylogenetic tree on the VP1 showed that all of echovirus 18 which found in this study was a cluster in subgenotype C1 as a previous outbreak in France and not found subgenotype C2. In another hand, a 3D region in all samples was positive for echovirus 9 that confirmed the recombination event has occurred. Although, the positive selective pressure was not found in this study, it should continue study.

Their infection in endemic or epidemic features arose from the co-circulation of lineages of one predominant genogroup. It showed that geographic distance was not a barrier to EV transmission worldwide but the epidemiological consequences were also determined by herd immunity against the imported virus lineages. Thus, the phylogeographic study will help to show a large array of virus migrations events across between different countries. This pattern suggests frequent and complex of EV lineages movements worldwide which may include different transmission features and geographic distributions and closely related virus migrations. However, phylogeographic patterns were influenced by missing data, sequence datasets. The limitation of this study was a retrospective and single center of an admitted child with these diseases and did not follow up to collect their specimens. In the other hand, EV in meningitis patients in Europe mostly reported as echovirus 30 (139, 200, 229, 230) but in this study did not find. It is possible that this EV may not transport worldwide or limited area.

In conclusion, it is important to continue surveillance of aseptic meningitis and encephalitis agents to effective therapy and prevents unnecessary use of antibiotics and/or antiviral medication. This study revealed that echovirus 18 which found in meningitis patients in Thailand were closely related with previously reported in other countries. It supported that this virus has circulated worldwide and may drive their evolution. Therefore, characteristic clinical, pathological, molecular finding can be associated with certain genotypes of EV. Further investigations with large populations for EV surveillance are needed to substantiate epidemiologically. It can promote to evaluate the association of their genome-related clinical information.

CHAPTER IV
DISCUSSION AND CONCLUSION

Various species and genotypes of EV has been recognized as etiological agents for many diseases which impact to public health although most infections are asymptomatic. The association between EV genotype and clinical manifestations have not been conclusively established. Their replication often significantly disturb host homeostasis but the rate of induction of severe disease is low. For example, PV infections, about 10% of which were lethal while most cases resolved with some residual paralysis or resolved completely (231). The extent of viral disease and its outcome are related to the impact that a specific virus genotype has upon the host. Although, EV do not have virulence genes but have evolved specific genomic regions which play key roles cause disease. The best evidence support that the 5'UTR and the capsid protein determining virulence phenotypes, the IRES employed in translational initiation may differentially affect virus replication depending on cell type which EV is replicating (232, 233). Moreover, the selection of a stable, virulent, quasispecies population during replication within host is influenced by various factors. One factor is the high rate of nucleotide misincorporation without editing function in RdRp which poor fidelity introduces one misincorporation per replication event (234, 235). Another factor is recombination that occurs among related species between two viral genomes permits (41, 236, 237). Finally, factor must consider the genome of the infecting genotype which close to virulent genome. For the host, many factors such as age and gender plays a great role in this effort.

In summary, the ability of EV to induce disease rests upon the viral genetics and host environment (238). Viral genetics are important to the process but are modulated by diverse host factors. They can be variable upon transmission between outbred individuals. Entry into the new host effects new pressure upon any specific viral genome which effect to the result of permitting disease or not and determining how efficient the next cycle of infection to the next host becomes.

Epidemiology and genetic variation of enteroviruses in various clinical manifestation

The successful of the epidemiological data of EV in various symptoms are depended on many factors such as frequency of detection, population size, appropriate specimens and reliable methods. Molecular techniques become suitable method to identify EV. The knowledge of the viral genome sequence is determinants for biologic properties that correlates of viral phenotypes could potentially be obtained directly by PCR. Regarding to these method, sequence analysis of a target fragment of highly conserved 5'UTR and partial capsid coding sequences are reasonable. Therefore, PCR approach in this study could be utilized as a broad and sensitive diagnosis implement for elucidating aspects of the epidemiology.

In Thailand, although these epidemic status of EV in many diseases are still often underdiagnosed and EV typing data is not currently collected. To solve these problem, this study has retrospectively analyzed the epidemiology of comprehensive population-based of many diseases caused by EV infection by using RT-PCR amplification and molecular genotyping. In the present study, we investigated the EV genotypes distribution in many disease. We enrolled 119, 295, 1310, 2692, 6817 and 269 suspected cases of AHC, HA, HFMD, AGE, surveillance PV and meningoencephalitis, respectively. Our finding results showed the significant of EV genotype association with each disease as following; EV-A were the most common pathogens causing HA and HFMD; EV-B were mostly detected in meningoencephalitis; EV-C were detected in AHC while several species were detected in AGE. The limitation in this study is some of specimens were not sufficient for repeated test and/or incomplete demographic data.

According to AHC cases in this study, the result showed that only CV-A24 caused this disease and did not found recombination event when considered VP1 and 3C regions. In tropical countries, epidemics of viral conjunctivitis mainly occur during hot and rainy season. The mostly attributed to EV and HAdV in some reported. EV is responsible for a large range of infections with two main genotypes EV-70 and CV-A24. However, several epidemics in the last decade in Asia and other continents caused by CV-A24 and its variants which revealed high level of sequence similarity. Based on the 3C region, can classified into genotype I-IV. Moreover, subgenotypes of GIV have been identified as cluster C1-C5 which revealed three waves emergence of

GIV-C2, C3 and C5. Phenomena multiple lineages co-circulating simultaneously and recombination events, which common in EV, was not appear in CV-A24 (239).

For HA cases, various genotypes of EV associated. The previous reported revealed that the predominant genotype differ in many regions and years which may be distinct clusters related to their geographic origins, such as CV-A2 in Taipei 2008 (240), CV-A5 in Korea 2009 (241), CV-A6 and CV-A10 in France 2010 (242), in this study CV-A2 is predominant in 2015.

For HFMD cases, average incidence in Thailand around 20 cases per 100,000 population during 2007-2012 and 3-fold higher in 2012. This study revealed consistently results with previous reported that EV-A species are majority pathogens in HFMD cases and EV-A71 and CV-A16 were the major caused in worldwide. However, CV-A6 become more dominant causative agents significantly in Europe, Asia and USA since 2008 (243). CV-A6 is becoming an increasingly crucial agent as it was the only pathogen documented differences in symptomatology from HFMD disease. In addition, the widespread of the new recombinant forms of CV-A6 have become in Europe and other geographical regions. The most frequently detected recombinant form A, displayed decades-long circulation and was the ancestor of five distinct recombinant group E, F, H, K and J that have emerged in the past 5-10 years. This typical pattern for recombinant form was its rapid emergence, variable penetrance into the sampled population and relatively rapid extinction, within years rather than decades.

For AGE, one of the common diseases in infants and children. Although lower percentage of EV was detected when compare with other enteric viruses (rotavirus, norovirus, adenovirus, etc.) but it still detected significantly especially in pediatric patients. A few studies have suggested that some genotypes are potentially to be associated with diarrhea. In this study, several of EV-B, C species and HRV are found in AGE (244-246).

For poliovirus, the use of OPV in mass vaccinations has resulted in dramatically reductions poliomyelitis cases. However, major flaw of OPV is genetic unstable that makes it particularly susceptible to evolve into circulating vaccine-derived polioviruses via recombination between PV and other closely related EV which every 2-3 million doses of OPV administered in poorly immunized

communities. To reduce VAPP cases among vaccine recipients, IPV was recommended by replacing the first two vaccine doses of the immunization schedule.

For meningitis cases, although one of the most frequently genotype in many regions of Europe which caused many large outbreak is echovirus 30 but co-circulation of several EV-B species are reported (139, 209, 211).

Genetic diversity of enteroviruses

Genetic diversity is ultimately the result of mutation and recombination. The average variation exhibited by a specific virus type is depend on diverse characteristics. The increasing of genetic diversity often leads to phenotypic variation which is problematic for clinical therapy. For mutation, normally, RNA viruses have mutation rates between 10^{-3} to 10^{-5} per base per generation while DNA viruses have mutation rates between 10^{-6} to 10^{-8} (247). For recombination, it is a relatively common phenomenon in RNA viruses. These mechanisms have a high impact factor on EV epidemiology and evolution. EV-B has been shown undergo much more frequent recombination events than found for EV-A and EV-C. They exist as highly dynamic global gene pools. This events in non-structural regions were tightly correlated with nucleotide divergence within the VP1 region. Moreover, the variability and complexity in recombination frequencies between types is influenced by their different epidemiology, geographical expansion or by different compatibility restrictions concentrating on chances for generate sustainable recombinant virus. So, the fitness in non-structural region use for define the recombination events before the emergence of a novel EV lineage. The phylogenetic discordancy has been qualified to recombination events. For example, EV several types which co-circulate in an outbreak can easily undergo recombination, leading to circulation of new strains with similar non-capsid region. The non-structural genes are arbitrarily shuffled between genotypes every few years, so observation on both capsid and non-capsid regions could be more effective to detect with sufficient statistical support (248). The half-lives of recombination forms in circulation may vary in different genotypes. The example in some strain such as E6, E9, E11 and E30 were estimated at 0.87, 1.3, 9.8 and 3.1 years, respectively. In the same way, EV-A such as CV-A2, CV-A4 and CV-A10 were calculated to 3.5 years (249, 250).

For rates of molecular evolution, the relationship between genetic divergence and time of isolation are important, which determining evolution under positive selection. Based on Bayesian MCMC methods, the evolution rate of many EVs were observed based on VP1 region such as EV-A71 genogroup B ($4.2x10^{-3}$ s/s/y), EV-A71 genogroup C ($3.4x10^{-3}$ s/s/y), CV-A16 ($9.1x10^{-3}$ s/s/y), CV-A2 ($8.3x10^{-3}$ s/s/y), CV-A4 ($5.5x10^{-3}$ s/s/y), CV-A6 ($8.1x10^{-3}$ s/s/y), CV-A10 ($14.1x10^{-3}$ s/s/y), CV-A21 ($3.1x10^{-3}$ s/s/y), CV-B5 ($4.2x10^{-3}$ s/s/y), E-6 ($11.2x10^{-3}$ s/s/y), E-9 ($5.8x10^{-3}$ s/s/y), E-11 ($4.8x10^{-3}$ s/s/y), E-30 ($8.8x10^{-3}$ s/s/y), CV-A24 ($1.2x10^{-3}$ s/s/y) and WPV ($3x10^{-2}$ s/s/y) (249-253). In this study, evolution analysis was tested in poliovirus surveillance and meningitis cases. Both tested did not found positive selective pressure, evolution rates are $3.7x10^{-4}$ and $1.33x10^{-3}$ s/s/y, respectively. All results based on sequence data sets retrieve from database to analyze, so it possible under-estimate. However, substitution rates estimated from large data sets should still be reliable indicators of the average speed of evolution.

In conclusion, this study provided evidence for the molecular epidemiological and evolution data of EV caused for AHC, Herpangina, HFMD, meningitis and poliovirus surveillance in Thailand which gaining a long term better understanding of the nature of EV and their clinical specifically associated outcomes in the community. Furthermore, it supported changing serotyping to molecular typing for ongoing surveillance. The need for continuous epidemiological and frequent recombination of EV strains surveillance each disease together with development of diagnostic methods is important for recognizing, predicting and distinguishing the potential emergence of new EV variants and transmission patterns.

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APPENDIX

APPENDIX A

Table S1: Sequences information in our study

Region	Code	Human enterovirus genotype	Accession number
VP1	CU_CJ1	Coxsackievirus A24	KP122020
VP1	CU_CJ2	Coxsackievirus A24	KP122021
VP1	CU_CJ4	Coxsackievirus A24	KP122022
VP1	CU_CJ5	Coxsackievirus A24	KP122023
VP1	CU_CJ6	Coxsackievirus A24	KP122024
VP1	CU_CJ8	Coxsackievirus A24	KP122025
VP1	CU_CJ9	Coxsackievirus A24	KP122026
VP1	CU_CJ11	Coxsackievirus A24	KP122027
VP1	CU_CJ12	Coxsackievirus A24	KP122028
VP1	CU_CJ13	Coxsackievirus A24	KP122029
VP1	CU_CJ14	Coxsackievirus A24	KP122030
VP1	CU_CJ17	Coxsackievirus A24	KP122031
VP1	CU_CJ18	Coxsackievirus A24	KP122032
VP1	CU_CJ20	Coxsackievirus A24	KP122033
VP1	CU_CJ21	Coxsackievirus A24	KP122034
VP1	CU_CJ22	Coxsackievirus A24	KP122035
VP1	CU_CJ23	Coxsackievirus A24	KP122036
VP1	CU_CJ24	Coxsackievirus A24	KP122037
VP1	CU_CJ25	Coxsackievirus A24	KP122038
VP1	CU_CJ26	Coxsackievirus A24	KP122039
VP1	CU_CJ27	Coxsackievirus A24	KP122040
VP1	CU_CJ29	Coxsackievirus A24	KP122041
VP1	CU_CJ30	Coxsackievirus A24	KP122042
VP1	CU_CJ34	Coxsackievirus A24	KP122043
VP1	CU_CJ35	Coxsackievirus A24	KP122044
VP1	CU_CJ37	Coxsackievirus A24	KP122045
VP1	CU_CJ38	Coxsackievirus A24	KP122046
VP1	CU_CJ39	Coxsackievirus A24	KP122047
VP1	CU_CJ40	Coxsackievirus A24	KP122048
VP1	CU_CJ41	Coxsackievirus A24	KP122049
VP1	CU_CJ42	Coxsackievirus A24	KP122050
VP1	CU_CJ45	Coxsackievirus A24	KP122051
VP1	CU_CJ46	Coxsackievirus A24	KP122052
VP1	CU_CJ47	Coxsackievirus A24	KP122053
VP1	CU_CJ48	Coxsackievirus A24	KP122054

Region	Code	Human enterovirus genotype	Accession number
VP1	CU_CJ51	Coxsackievirus A24	KP122055
VP1	CU_CJ52	Coxsackievirus A24	KP122056
VP1	CU_CJ56	Coxsackievirus A24	KP122057
VP1	CU_CJ58	Coxsackievirus A24	KP122058
VP1	CU_CJ64	Coxsackievirus A24	KP122059
VP1	CU_CJ66	Coxsackievirus A24	KP122060
VP1	CU_CJ69	Coxsackievirus A24	KP122061
VP1	CU_CJ71	Coxsackievirus A24	KP122062
VP1	CU_CJ74	Coxsackievirus A24	KP122063
VP1	CU_CJ81	Coxsackievirus A24	KP122064
VP1	CU_CJ83	Coxsackievirus A24	KP122065
VP1	CU_CJ85	Coxsackievirus A24	KP122066
VP1	CU_CJ87	Coxsackievirus A24	KP122067
VP1	CU_CJ88	Coxsackievirus A24	KP122068
VP1	CU_CJ90	Coxsackievirus A24	KP122069
VP1	CU_CJ92	Coxsackievirus A24	KP122070
VP1	CU_CJ93	Coxsackievirus A24	KP122071
VP1	CU_CJ94	Coxsackievirus A24	KP122072
VP1	CU_CJ95	Coxsackievirus A24	KP122073
VP1	CU_CJ96	Coxsackievirus A24	KP122074
VP1	CU_CJ97	Coxsackievirus A24	KP122075
VP1	CU_CJ99	Coxsackievirus A24	KP122076
VP1	CU_CJ100	Coxsackievirus A24	KP122077
VP1	CU_CJ101	Coxsackievirus A24	KP122078
VP1	CU_CJ102	Coxsackievirus A24	KP122079
VP1	CU_CJ104	Coxsackievirus A24	KP122080
VP1	CU_CJ106	Coxsackievirus A24	KP122081
VP1	CU_CJ107	Coxsackievirus A24	KP122082
VP1	CU_CJ108	Coxsackievirus A24	KP122083
VP1	CU_CJ109	Coxsackievirus A24	KP122084
VP1	CU_CJ112	Coxsackievirus A24	KP122085
VP1	CU_CJ114	Coxsackievirus A24	KP122086
VP1	CU_CJ115	Coxsackievirus A24	KP122087
VP1	CU_CJ117	Coxsackievirus A24	KP122088
VP1	CU_CJ118	Coxsackievirus A24	KP122089
VP1	CU_CJ119	Coxsackievirus A24	KP122090
VP1	CU_CJ36	Coxsackievirus A24	KP137044
VP1	CU_CJ91	Coxsackievirus A24	KP137045
VP1	CU_CJ105	Coxsackievirus A24	KP137046
3C	CU_CJ2	Coxsackievirus A24	KP122091
3C	CU_CJ4	Coxsackievirus A24	KP122092
3C	CU_CJ5	Coxsackievirus A24	KP122093
3C	CU_CJ6	Coxsackievirus A24	KP122094

Region	Code	Human enterovirus genotype	Accession number
3C	CU_CJ8	Coxsackievirus A24	KP122095
3C	CU_CJ9	Coxsackievirus A24	KP122096
3C	CU_CJ11	Coxsackievirus A24	KP122097
3C	CU_CJ12	Coxsackievirus A24	KP122098
3C	CU_CJ13	Coxsackievirus A24	KP122099
3C	CU_CJ17	Coxsackievirus A24	KP122100
3C	CU_CJ18	Coxsackievirus A24	KP122101
3C	CU_CJ20	Coxsackievirus A24	KP122102
3C	CU_CJ21	Coxsackievirus A24	KP122103
3C	CU_CJ22	Coxsackievirus A24	KP122104
3C	CU_CJ23	Coxsackievirus A24	KP122105
3C	CU_CJ24	Coxsackievirus A24	KP122106
3C	CU_CJ25	Coxsackievirus A24	KP122107
3C	CU_CJ26	Coxsackievirus A24	KP122108
3C	CU_CJ27	Coxsackievirus A24	KP122109
3C	CU_CJ29	Coxsackievirus A24	KP122110
3C	CU_CJ30	Coxsackievirus A24	KP122111
3C	CU_CJ34	Coxsackievirus A24	KP122112
3C	CU_CJ35	Coxsackievirus A24	KP122113
3C	CU_CJ36	Coxsackievirus A24	KP122114
3C	CU_CJ37	Coxsackievirus A24	KP122115
3C	CU_CJ38	Coxsackievirus A24	KP122116
3C	CU_CJ39	Coxsackievirus A24	KP122117
3C	CU_CJ40	Coxsackievirus A24	KP122118
3C	CU_CJ41	Coxsackievirus A24	KP122119
3C	CU_CJ42	Coxsackievirus A24	KP122120
3C	CU_CJ45	Coxsackievirus A24	KP122121
3C	CU_CJ46	Coxsackievirus A24	KP122122
3C	CU_CJ47	Coxsackievirus A24	KP122123
3C	CU_CJ48	Coxsackievirus A24	KP122124
3C	CU_CJ51	Coxsackievirus A24	KP122125
3C	CU_CJ52	Coxsackievirus A24	KP122126
3C	CU_CJ56	Coxsackievirus A24	KP122127
3C	CU_CJ58	Coxsackievirus A24	KP122128
3C	CU_CJ60	Coxsackievirus A24	KP122129
3C	CU_CJ62	Coxsackievirus A24	KP122130
3C	CU_CJ64	Coxsackievirus A24	KP122131
3C	CU_CJ66	Coxsackievirus A24	KP122132
3C	CU_CJ71	Coxsackievirus A24	KP122133
3C	CU_CJ72	Coxsackievirus A24	KP122134
3C	CU_CJ74	Coxsackievirus A24	KP122135
3C	CU_CJ75	Coxsackievirus A24	KP122136
3C	CU_CJ81	Coxsackievirus A24	KP122137

Region	Code	Human enterovirus genotype	Accession number
3C	CU_CJ83	Coxsackievirus A24	KP122138
3C	CU_CJ85	Coxsackievirus A24	KP122139
3C	CU_CJ86	Coxsackievirus A24	KP122140
3C	CU_CJ87	Coxsackievirus A24	KP122141
3C	CU_CJ88	Coxsackievirus A24	KP122142
3C	CU_CJ90	Coxsackievirus A24	KP122143
3C	CU_CJ92	Coxsackievirus A24	KP122144
3C	CU_CJ93	Coxsackievirus A24	KP122145
3C	CU_CJ94	Coxsackievirus A24	KP122146
3C	CU_CJ95	Coxsackievirus A24	KP122147
3C	CU_CJ96	Coxsackievirus A24	KP122148
3C	CU_CJ97	Coxsackievirus A24	KP122149
3C	CU_CJ99	Coxsackievirus A24	KP122150
3C	CU_CJ100	Coxsackievirus A24	KP122151
3C	CU_CJ101	Coxsackievirus A24	KP122152
3C	CU_CJ102	Coxsackievirus A24	KP122153
3C	CU_CJ106	Coxsackievirus A24	KP122154
3C	CU_CJ107	Coxsackievirus A24	KP122155
3C	CU_CJ108	Coxsackievirus A24	KP122156
3C	CU_CJ109	Coxsackievirus A24	KP122157
3C	CU_CJ112	Coxsackievirus A24	KP122158
3C	CU_CJ114	Coxsackievirus A24	KP122159
3C	CU_CJ117	Coxsackievirus A24	KP122160
3C	CU_CJ118	Coxsackievirus A24	KP122161
3C	CU_CJ119	Coxsackievirus A24	KP122162
3C	CU_CJ14	Coxsackievirus A24	KP137042
3C	CU_CJ69	Coxsackievirus A24	KP137043
VP1	Coxsackievirus_A2_A1677	Cosackievirus A2	KX021224
VP1	Coxsackievirus_A2_A1678	Cosackievirus A2	KX021225
VP1	Coxsackievirus_A2_A1693	Cosackievirus A2	KX021226
VP1	Coxsackievirus_A2_A1706	Cosackievirus A2	KX021227
VP1	Coxsackievirus_A2_A1727	Cosackievirus A2	KX021228
VP1	Coxsackievirus_A2_A1733	Cosackievirus A2	KX021229
VP1	Coxsackievirus_A2_A1736	Cosackievirus A2	KX021230
VP1	Coxsackievirus_A2_A1739	Cosackievirus A2	KX021231
VP1	Coxsackievirus_A2_A1741	Cosackievirus A2	KX021232
VP1	Coxsackievirus_A2_A1742	Cosackievirus A2	KX021233
VP1	Coxsackievirus_A2_A1743	Cosackievirus A2	KX021234
VP1	Coxsackievirus_A2_A1747	Cosackievirus A2	KX021235
VP1	Coxsackievirus_A2_A1756	Cosackievirus A2	KX021236
VP1	Coxsackievirus_A2_A1757	Cosackievirus A2	KX021237
VP1	Coxsackievirus_A2_A1772	Cosackievirus A2	KX021238
VP1	Coxsackievirus_A2_A1790	Cosackievirus A2	KX021239

Region	Code	Human enterovirus genotype	Accession number
VP1	Coxsackievirus_A2_A1792	Cosackievirus A2	KX021240
VP1	Coxsackievirus_A2_A1795	Cosackievirus A2	KX021241
VP1	Coxsackievirus_A2_A1798	Cosackievirus A2	KX021242
VP1	Coxsackievirus_A2_A1799	Cosackievirus A2	KX021243
VP1	Coxsackievirus_A2_A1818	Cosackievirus A2	KX021244
VP1	Coxsackievirus_A2_A1826	Cosackievirus A2	KX021245
VP1	Coxsackievirus_A2_A1842	Cosackievirus A2	KX021246
VP1	Coxsackievirus_A2_A1852	Cosackievirus A2	KX021247
VP1	Coxsackievirus_A2_A1863	Cosackievirus A2	KX021248
VP1	Coxsackievirus_A2_A1877	Cosackievirus A2	KX021249
VP1	Coxsackievirus_A2_A1878	Cosackievirus A2	KX021250
VP1	Coxsackievirus_A2_A1902	Cosackievirus A2	KX021251
VP1	Coxsackievirus_A2_A1904	Cosackievirus A2	KX021252
VP1	Coxsackievirus_A2_A1907	Cosackievirus A2	KX021253
VP1	Coxsackievirus_A2_A1912	Cosackievirus A2	KX021254
VP1	Coxsackievirus_A2_A1930	Cosackievirus A2	KX021255
VP1	Coxsackievirus_A2_A1931	Cosackievirus A2	KX021256
VP1	Coxsackievirus_A2_A1945	Cosackievirus A2	KX021257
VP1	Coxsackievirus_A2_A1946	Cosackievirus A2	KX021258
VP1	Coxsackievirus_A2_A1953	Cosackievirus A2	KX021259
VP1	Coxsackievirus_A2_A1962	Cosackievirus A2	KX021260
VP1	Coxsackievirus_A2_A1969	Cosackievirus A2	KX021261
VP1	Coxsackievirus_A2_A1970	Cosackievirus A2	KX021262
VP1	Coxsackievirus_A2_A1972	Cosackievirus A2	KX021263
VP1	Coxsackievirus_A2_A1990	Cosackievirus A2	KX021264
VP1	Coxsackievirus_A2_A2008	Cosackievirus A2	KX021265
5’UTR	B1324	Echovirus E7	KY079137
5’UTR	B1290	Echovirus E7	KY079138
5’UTR	B1337	Echovirus E7	KY079139
5’UTR	B1333	Echovirus E7	KY079140
5’UTR	B2966	Rhinovirus B69	KY079141
5’UTR	B2947	Echovirus 30	KY079142
5’UTR	B2939	Coxsackievirus B3	KY079143
5’UTR	B2891	Rhinovirus C	KY079144
5’UTR	B2839	Rhinovirus A	KY079145
5’UTR	B2837	Poliovirus 3	KY079146
5’UTR	B2834	Poliovirus 1	KY079147
5’UTR	B2831	Poliovirus 2	KY079148
5’UTR	B2830	Rhinovirus C	KY079149
5’UTR	B2773	Coxsackievirus A5	KY079150
5’UTR	B2717	Echovirus E5	KY079151
5’UTR	B2682	Echovirus E13	KY079152
5’UTR	B2681	Rhinovirus C	KY079153

Region	Code	Human enterovirus genotype	Accession number
5’UTR	B2679	Rhinovirus B	KY079154
5’UTR	B2644	Poliovirus 2	KY079155
5’UTR	B2627	Poliovirus 1	KY079156
5’UTR	B2614	Poliovirus 2	KY079157
5’UTR	B2606	Echovirus E5	KY079158
5’UTR	B2615	Poliovirus 2	KY079159
5’UTR	B2610	Echovirus E5	KY079160
5’UTR	B2593	Echovirus E30	KY079161
5’UTR	B2575	Echovirus E5	KY079162
5’UTR	B2592	Echovirus E30	KY079163
5’UTR	B2565	Echovirus E6	KY079164
5’UTR	B2516	Rhinovirus C	KY079165
5’UTR	B2500	Echovirus E6	KY079166
5’UTR	B2434	Poliovirus 3	KY079167
5’UTR	B2405	Echovirus E30	KY079168
5’UTR	B2399	Rhinovirus B	KY079169
5’UTR	B2398	Rhinovirus A	KY079170
5’UTR	B2376	Rhinovirus C	KY079171
5’UTR	B2366	Poliovirus 3	KY079172
5’UTR	B2338	Poliovirus 3	KY079173
5’UTR	B2318	Echovirus E5	KY079174
5’UTR	B2301	Rhinovirus C	KY079175
5’UTR	B2257	Coxsackievirus B4	KY079176
5’UTR	B2215	Coxsackievirus A9	KY079177
5’UTR	B2202	Echovirus E6	KY079178
5’UTR	B2180	Poliovirus 2	KY079179
5’UTR	B2176	Poliovirus 2	KY079180
5’UTR	B2175	Echovirus E6	KY079181
5’UTR	B2167	Rhinovirus A	KY079182
5’UTR	B2165	Coxsackievirus A5	KY079183
5’UTR	B2136	Rhinovirus C	KY079184
5’UTR	B2113	Echovirus E6	KY079185
5’UTR	B2109	Enterovirus D68	KY079186
5’UTR	B2106	Rhinovirus C	KY079187
5’UTR	B2104	Echovirus E30	KY079188
5’UTR	B2097	Rhinovirus C	KY079189
5’UTR	B2074	Rhinovirus A98	KY079190
5’UTR	B2096	Coxsackievirus A5	KY079191
5’UTR	B2083	Coxsackievirus A10	KY079192
5’UTR	B2082	Coxsackievirus A5	KY079193
5’UTR	B2078	Echovirus E25	KY079194
5’UTR	B2073	Rhinovirus C	KY079195
5’UTR	B2070	Coxsackievirus A1	KY079196

Region	Code	Human enterovirus genotype	Accession number
5’UTR	B2067	Coxsackievirus A9	KY079197
5’UTR	B2065	Coxsackievirus A9	KY079198
5’UTR	B2058	Rhinovirus C	KY079199
5’UTR	B2048	Poliovirus 3	KY079200
5’UTR	B2039	Poliovirus 1	KY079201
5’UTR	B2038	Rhinovirus A81	KY079202
5’UTR	B2035	Rhinovirus A81	KY079203
5’UTR	B1997	Poliovirus 3	KY079204
5’UTR	B1995	Coxsackievirus B4	KY079205
5’UTR	B1987	Poliovirus 1	KY079206
5’UTR	B1747	Poliovirus 2	KY079207
5’UTR	B1680	Poliovirus 3	KY079208
5’UTR	B1669	Poliovirus 1	KY079209
5’UTR	B1323	Poliovirus 1	KY079210
5’UTR	B1105	Poliovirus 1	KY079211
5’UTR	B1037	Poliovirus 2	KY079212
5’UTR	B1016	Poliovirus 2	KY079213
5’UTR	B860	Echovirus E18	KY079214
5’UTR	B930	Coxsackievirus A24	KY079215
5’UTR	B906	Echovirus E19	KY079216
5’UTR	B894	Coxsackievirus B1	KY079217
5’UTR	B816	Echovirus E18	KY079218
5’UTR	B1283	Echovirus E7	KY079219
5’UTR	B1264	Echovirus EC96	KY079220
5’UTR	B1254	Coxsackievirus B6	KY079221
5’UTR	B1215	Coxsackievirus A1	KY079222
5’UTR	B1207	Coxsackievirus A1	KY079223
5’UTR	B1205	Coxsackievirus A1	KY079224
5’UTR	B1203	Coxsackievirus A1	KY079225
5’UTR	B1196	Poliovirus 2	KY079226
5’UTR	B1193	Coxsackievirus A1	KY079227
5’UTR	B1188	Echovirus E9	KY079228
5’UTR	B1172	Coxsackievirus B4	KY079229
5’UTR	B1169	Coxsackievirus B5	KY079230
5’UTR	B1160	Coxsackievirus B3	KY079231
5’UTR	B1126	Echovirus EC99	KY079232
5’UTR	B1096	Echovirus E33	KY079233
5’UTR	B1061	Coxsackievirus B3	KY079234
5’UTR	B1054	Coxsackievirus B3	KY079235
5’UTR	B1004	Coxsackievirus B3	KY079236
5’UTR	B1667	Coxsackievirus B5	KY079237
5’UTR	B1611	Coxsackievirus B4	KY079238
5’UTR	B1607	Coxsackievirus A9	KY079239

Region	Code	Human enterovirus genotype	Accession number
5’UTR	B1602	Coxsackievirus B4	KY079240
5’UTR	B1600	Coxsackievirus A19	KY079241
5’UTR	B1544	Coxsackievirus B1	KY079242
5’UTR	B1982	Coxsackievirus A22	KY079243
5’UTR	B1557	Coxsackievirus A9	KY079244
5’UTR	B1448	Coxsackievirus A9	KY079245
5’UTR	B1437	Echovirus E7	KY079246
5’UTR	B1542	Coxsackievirus B1	KY079247
5’UTR	B1540	Coxsackievirus A9	KY079248
5’UTR	B1517	Echovirus E6	KY079249
5’UTR	B1516	Coxsackievirus A8	KY079250
5’UTR	B1499	Coxsackievirus A9	KY079251
5’UTR	B1496	Coxsackievirus A8	KY079252
5’UTR	B1467	Coxsackievirus A4	KY079253
5’UTR	B1930	Echovirus E11	KY079254
5’UTR	B1929	Echovirus E6	KY079255
5’UTR	B1925	Echovirus E6	KY079256
5’UTR	B1864	Poliovirus 3	KY079257
5’UTR	B1846	Coxsackievirus A22	KY079258
5’UTR	B1830	Echovirus E11	KY079259
5’UTR	B1825	Echovirus E9	KY079260
5’UTR	B1817	Coxsackievirus A22	KY079261
5’UTR	B1812	Echovirus E20	KY079262
5’UTR	B1792	Coxsackievirus A5	KY079263
5’UTR	B1838	Rhinovirus C	KR922046
5’UTR	B1497	Rhinovirus C	KR054542
5’UTR	B1784	Rhinovirus C	KR054550
5’UTR	B1748	Rhinovirus C	KR054548
5’UTR	B934	Rhinovirus C	KR054528
5’UTR	B1013	Rhinovirus C	KR054531
5’UTR	B1746	Rhinovirus C	KR054547
5’UTR	B1346	Rhinovirus C	KR054537
5’UTR	B1447	Rhinovirus C	KR054541
5’UTR	B1681	Rhinovirus C	KR054546
5’UTR	B1365	Rhinovirus C	KR054538
5’UTR	B1008	Rhinovirus A	KR054530
5’UTR	B1533	Rhinovirus A	KR054544
5’UTR	B1953	Rhinovirus A	KR054554
5’UTR	B977	Rhinovirus A	KR054529
5’UTR	B1080	Rhinovirus A	KR054533
5’UTR	B1055	Rhinovirus A	KR054532
5’UTR	B1768	Rhinovirus A	KR054549
5’UTR	B1515	Rhinovirus A	KR054543

Region	Code	Human enterovirus genotype	Accession number
5’UTR	B1938	Rhinovirus A	KR054553
5’UTR	B929	Rhinovirus A	KR054527
5’UTR	B1811	Rhinovirus A	KR054551
5’UTR	B1816	Rhinovirus A	KR054552
5’UTR	B1380	Rhinovirus A	KR054539
5’UTR	B1266	Rhinovirus A	KR054535
5’UTR	B801	Rhinovirus B	KR054526
5’UTR	B1084	Rhinovirus B	KR054534
5’UTR	B1672	Rhinovirus B	KR054545
5’UTR	B1331	Rhinovirus B	KR054536
5’UTR	B1400	Rhinovirus B	KR054540
5’UTR	B2983	Echovirus E18	KY774677
5’UTR	B3016	Echovirus E18	KY774678
5’UTR	B3035	Rhinovirus A	KY774679
5’UTR	B3036	Echovirus E18	KY774680
5’UTR	B3043	Coxsackievirus A24	KY774681
5’UTR	B3050	Poliovirus 1	KY774682
5’UTR	B3117	Echovirus E18	KY774683
5’UTR	B3150	Rhinovirus B	KY774684
5’UTR	B3256	Rhinovirus A	KY774685
5’UTR	B3259	Rhinovirus A	KY774686
5’UTR	B3370	Rhinovirus A	KY774687
VP1	A93	Poliovirus 1	MF593155
VP1	A1572	Poliovirus 3	MF593156
VP1	A2128	Poliovirus 2	MF593157
VP1	A2325	Poliovirus 3	MF593158
VP1	A2327	Poliovirus 3	MF593159
VP1	B1016	Poliovirus 2	MF593160
VP1	B1037	Poliovirus 2	MF593161
VP1	B1105	Poliovirus 1	MF593162
VP1	B1196	Poliovirus 2	MF593163
VP1	B1323	Poliovirus 1	MF593164
VP1	B1680	Poliovirus 3	MF593165
VP1	B1747	Poliovirus 2	MF593166
VP1	B1864	Poliovirus 3	MF593167
VP1	B1987	Poliovirus 1	MF593168
VP1	B2039	Poliovirus 1	MF593169
VP1	B2048	Poliovirus 3	MF593170
VP1	B2176	Poliovirus 2	MF593171
VP1	B2180	Poliovirus 2	MF593172
VP1	B2338	Poliovirus 3	MF593173
VP1	B2614	Poliovirus 2	MF593174
VP1	B2831	Poliovirus 2	MF593175

Region	Code	Human enterovirus genotype	Accession number
VP1	B2834	Poliovirus 1	MF593176
VP1	B2837	Poliovirus 3	MF593177
VP1	B3050	Poliovirus 1	MF593178
VP1	A2242	Echovirus 18	MH427204
VP1	A2262	Echovirus 18	MH427205
VP1	A2269	Echovirus 18	MH427206
VP1	A2281	Echovirus 18	MH427207
VP1	A2283	Echovirus 18	MH427208
VP1	A2326	Echovirus 18	MH427209
VP1	B2983	Echovirus 18	MH427210
VP1	B3016	Echovirus 18	MH427211
3Dpol	A2281	Echovirus 9	MH427212
3Dpol	A2269	Echovirus 9	MH427213
3Dpol	A2242	Echovirus 9	MH427214
3Dpol	A2326	Echovirus 9	MH427215
3Dpol	B3016	Echovirus 9	MH427216

APPENDIX B

Table S2: Sequence primers for detect other viruses in this study

Primer's name	Sequence (5' – 3')	Region	Virus
Beg9	GGC TTT AAA AGA GAG AAT TTC CGT CTG G	VP7	Rotavirus (125)
End9	GGT CAC ATC ATA CAA TTC TAA TCT AAG		
Con3	TGG CTT CGC CAT TTL ATA GAC A	VP4	
Con2	ATT TCG GAC CAT TTT ATA ACC		
F4895	GAT TTA GGT GAC ACT ATA GYD STT YTC HTT YTA YGG KGA YGA TGA	VP1	Norovirus (126)
R5591	AWT CGG GCA RGA GAT YGC GAT C		
R5393	GCC TGY ACA AAR TTA TTS ATT ATC CA		
ADV_FO	AYG CYA MCT TYT TYC CCA TGG C	Fiber	Adenovirus (77)
ADV_R	AAR CCC TGR TAN CCD ATR TTG TA		
ADV-F1	TYT TYC CCA TGG CNC ACA ACA C		
ADV_F2	TTY CCC ATG GCN CAC AAC AC	Hexon	
ADV_R2	GYY TCR ATG AYG CCG CGG TG		

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