ระบาดวิทยา การพัฒนาการตรวจวินิจฉัยระดับโมเลกุลของเชื้อฮิวแมนเอนเทอโรไวรัส และการเกิด
รีคอมบิเนชันและวิวัฒนาการของเชื้อฮิวแมนคอกแซกกีไวรัส เอ6

นางสาวจิรัชญา พื้นผา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Epidemiology, development of molecular diagnostic of human enterovirus and

recombination and evolutionary dynamics of human coxsackievirus A6

Miss Jiratchaya Peunpa

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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By	Miss Jiratchaya Peunpa
Field of Study	Medical Science
Thesis Advisor	Professor Yong Poovorawan, M.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

_____ Dean of the Faculty of Medicine

(Professor Suttipong Wacharasindhu, M.D.)

THESIS COMMITTEE

_____ Chairman

(Associate Professor Padet Siriyasatien, M.D.)

_____ Thesis Advisor

(Professor Yong Poovorawan, M.D.)

_____ Examiner

(Professor Duangporn Werawatganon, M.D.)

_____ Examiner

(Assistant Professor Sunchai Payungporn)

_____ External Examiner

(Associate Professor Teeraporn Chinchai)

จิรัชญา พื้นผา : ระบาดวิทยา การพัฒนาการตรวจวินิจฉัยระดับโมเลกุลของเชื้อฮิวแมนเอนเทอโรไวรัส และการเกิดรีคอมบิเนชัน และวิวัฒนาการของเชื้อฮิวแมนคอกแซกกีไวรัส เอ6 (Epidemiology, development of molecular diagnostic of human enterovirus and recombination and evolutionary dynamics of human coxsackievirus A6) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ. ยง ภู่วรวรรณ, 117 หน้า.

โรคมือ เท้า ปาก มีสาเหตุเกิดจากเชื้อไวรัสในกลุ่มเอนเทอโรไวรัส สปีชีย์เอ ซึ่งมีอยู่มากกว่า 20 สายพันธุ์ ที่เป็นปัญหาใหญ่ทาง เศรษฐกิจ สังคม ในประเทศไทยและทั่วโลก หนึ่งในไวรัสที่มีบทบาทสำคัญต่อการเกิดโรคคือ เชื้อเอนเทอโรไวรัส 71 สามารถก่อให้เกิดโรคแทรก ซ้อนทางระบบประสาทและสมองที่รุนแรงจนเป็นเหตุให้เสียชีวิตได้ ปัจจุบันยังไม่มีวัคซีนและยารักษาที่มีประสิทธิภาพ งานวิจัยนี้มีวัตถุประสงค์ เพื่อศึกษาระบาดวิทยาและการเปลี่ยนแปลงสายพันธุ์ไวรัส มือ เท้า ปาก ที่เกิดการระบาดในประเทศไทยในปี พ.ศ. 2555 เปรียบเทียบกับการ ระบาดในอดีตที่ผ่านมา โดยทำการตรวจวิเคราะห์ตัวอย่างจากกลุ่มผู้ป่วยโรคมือ เท้า ปาก, โรคเฮอร์แปงไจนา และผู้ป่วยติดเชื้อในระบบ ทางเดินหายใจ ทำการตรวจหาเชื้อฮิวแมนเอนเทอโรไวรัส โดยอาศัยวิธีการ semi-nested RT-PCR ผลการวิจัยพบว่าสามารถตรวจพบเชื้อฮิว แมนเอนเทอโรไวรัสทั้งสิ้น 17 สายพันธุ์ (คอกซากีไวรัส เอ 4, เอ 5, เอ 6, เอ 8, เอ 9, เอ 10, เอ 12, เอ 16, เอ 21, บี 1, บี 2, บี 4, บี 5, เอคโคไวรัส 7, 16, 25 และเอนเทอโรไวรัส 71) โดยเชื้อคอกซากีไวรัส เอ 6 เป็นสายพันธุ์ที่พบมากที่สุดในกลุ่มผู้ป่วยโรคมือ เท้า ปากและผู้ป่วยติดเชื้อใน ระบบทางเดินหายใจ คิดเป็นร้อยละ 33.5 และร้อยละ 1.5 ตามลำดับ เชื้อคอกซากีไวรัส เอ 8 พบมากที่สุดในกลุ่มผู้ป่วยโรคเฮอร์แปงไจนา คิด เป็นร้อยละ 19.3 ลักษณะการระบาดของเชื้อเอนเทอโรไวรัสในช่วงที่มีศึกษานี้พบว่ามีการกระจายตัวของเชื้อเอนเทอโรไวรัสตลอดทั้งปี และพบ มากที่สุดในเดือนกรกฎาคม ในกลุ่มผู้ป่วยทั้งสามกลุ่มมีอัตราการติดเชื้อเอนเทอโรไวรัสมากที่สุดในเด็กอายุมากกว่าหรือเท่ากับ 1 ปี จากนั้นได้ ทำการพัฒนาการตรวจวินิจฉัยระดับโมเลกุลของเชื้อฮิวแมนเอนเทอโรไวรัสโดยเฉพาะสายพันธุ์ที่พบในประเทศไทย ด้วยเทคนิค multiplex real-time RT-PCR ผลการศึกษาพบว่า ในการทดสอบความจำเพาะและความไวของเทคนิค multiplex real-time RT-PCR พบว่า ไม่เกิด cross reactivity ต่อเชื้อไวรัสชนิดอื่นที่ก่อโรคทั้งในระบบทางเดินอาหารและทางเดินหายใจ แสดงให้เห็นว่าไพรเมอร์และโพรบในงานวิจัยนี้มี ความจำเพาะสูงต่อเชื้อเป้าหมาย และผลความไวของเทคนิคต่อเชื้อเป้าหมาย พบว่า สามารถตรวจจับได้ในปริมาณ 10 copies/µl เมื่อ เปรียบเทียบประสิทธิภาพของเทคนิค multiplex real-time RT-PCR กับ conventional RT-PCR ในการตรวจหาเชื้อเอนเทอโรไวรัสจำนวน 1,313 ตัวอย่าง จากกลุ่มผู้ป่วยโรคมือ เท้า ปาก, โรคเฮอร์แปงไจนา ผลการวิเคราะห์พบว่าเทคนิค multiplex real-time RT-PCR มีความไวและ ความจำเพาะในการตรวจจับเชื้อฮิวแมนเอนเทอโรไวรัสมากกว่าเทคนิค conventional RT-PCR ดังนั้น เทคนิค multiplex real-time RT-PCR ในงานวิจัยนี้มีประโยชน์สูงทั้งในด้านความเร็วในการตรวจวินิจฉัยของโรค มีความจำเพาะและมีความไวต่อเชื้อฮิวแมนเอนเทอโรไวรัส ซึ่งเป็นวิธี ที่เหมาะสมที่สุดเพื่อที่จะนำมาใช้ในการควบคุมและป้องกันการระบาดของโรคมือเท้าปาก นอกจากนี้ ในช่วงระยะเวลาใกล้เคียงกันหลายๆ ประเทศก็ได้มีการรายงานอุบัติการณ์การเพิ่มขึ้นของการติดเชื้อคอกซากีไวรัส เอ 6 ที่เกี่ยวข้องกับการเกิดโรคมือ เท้า ปาก ซึ่งเริ่มมีรายงานการ ระบาดใหญ่ครั้งแรกที่ประเทศฟินแลนด์ในปี พ.ศ. 2551 พบว่าการติดเชื้อไวรัสสายพันธุ์นี้ มีแนวโน้มที่ผู้ป่วยจะแสดงอาการรุนแรงมากกว่าการ ติดเชื้อฮิวแมนเอนเทอโรไวรัสสายพันธุ์อื่นๆ งานวิจัยนี้ทำการศึกษาวิวัฒนาการและการเกิดรีคอมบิเนชันของเชื้อคอกซากีไวรัส เอ 6 โดยเก็บ ตัวอย่างจาก 5 ประเทศ ได้แก่ ไทย สวีเดน เดนมาร์ค สเปน และ เยอรมัน ผลการวิจัยพบว่า อัตราของการเปลี่ยนแปลงทางวิวัฒนาการของเชื้อ ไวรัสนี้เท่ากับ 8.1×10^{-3} จำนวนนิวคลีโอไทด์ที่ถูกแทนที่ต่อหนึ่งตำแหน่งต่อปี ในการเกิดรีคอมบิเนชันของเชื้อไวรัสนี้ที่ช่วงระยะเวลาต่างๆ พบว่าเกี่ยวข้องกับการแทนที่ยีนที่ไม่ใช่ยีนโครงสร้าง เมื่อคำนวณระยะเวลาที่ครึ่งหนึ่งของเชื้อไวรัสนี้จะเกิดรีคอมบิเนชัน มีค่าเท่ากับ 3.1 ปี สรุปได้ว่าการเกิดความหลากหลายทางพันธุกรรมของเชื้อไวรัสนี้ใช้เวลาที่สั้นกว่าเชื้อเอนเทอโรไวรัส 71 นอกจากนี้ จุดที่เกิดการรวมของจีโนไทป์ (recombination breakpoint) ของเชื้อไวรัสนี้พบว่าเกิดขึ้นที่บริเวณยีน 2A-2C และ 5´ untranslated region กล่าวโดยสรุปงานวิจัยนี้แสดงให้ เห็นถึงการเกิดสายพันธุ์ใหม่ของเชื้อคอกซากีไวรัส เอ 6 และทำให้เกิดการระบาดของโรคมือ เท้า ปากทั่วโลก

สาขาวิชา วิทยาศาสตร์การแพทย์	ลายมือชื่อนิสิต	_____
ปีการศึกษา 2558	ลายมือชื่อ อ.ที่ปรึกษาหลัก	_____

v

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KEYWORDS: EPIDEMIOLOGY/ RECOMBINATION/ EVOLUTIONARY DYNAMICS/ HUMAN ENTEROVIRUS/ HUMAN COXSACKIEVIRUS A6

JIRATCHAYA PEUNPA: Epidemiology, development of molecular diagnostic of human enterovirus and recombination and evolutionary dynamics of human coxsackievirus A6. ADVISOR: PROF. YONG POOVORAWAN, M.D. , 117 pp.

Hand, foot, and mouth disease (HFMD) is a common infectious disease caused by several genotypes of human enterovirus species A and represent a significant global public health concern. Enterovirus 71 (EV71) infections cause a deadly neurological disease for which no specific anti-viral drugs or vaccines has received regulatory approval. Published reports have described a dramatically increased incidence of coxsackievirus A6 (CV-A6) infections associated with HFMD since their occurrence in 2008 in Finland. The aim of this study was to evaluate the burden of human enteroviruses associated to HFMD and herpangina in patients and establish epidemiological profiles of these viruses in Thailand in 2012. Detection and genotype determination of enteroviruses were accomplished by reverse transcription-polymerase chain reaction and sequencing of the VP1 region. Enterovirus-positive samples were differentiated into 17 genotypes (CV-A 4, A5, A6, A8, A9, A10, A12, A16, A21, B1, B2, B4, B5, echovirus 7, 16, 25 and EV71). The result showed CV-A6 (33.5%), followed by CV-A 16 (9.4%) and EV71 (8.8%) as the most frequent genotypes in HFMD, CV-A 8 (19.3%) in herpangina and CAV6 (1.5%) in influenza like illness. Enterovirus infections were most prevalent during July with 34.4% in HFMD, 39.8% in herpangina and 1.6% in ILI. The higher enterovirus infection associated with HFMD and herpangina occurred in infants over one year-old. This represents the first report describing the circulation of multiple enteroviruses in Thailand. Furthermore, multiplex real-time PCR using TaqMan probes for broad detection of EVs and differentiation of EV71, CV-A6 and CV-A16 were developed and validated. The multiplex real-time PCR system which was run in two separate tubes was capable of screening and specific detection of the three selected enteroviruses, without cross-reactions with the other examined RNA viruses. The detection limit of the assays was 10 copies/µl for EV71, CV-A6, CV-A16 and panenterovirus. The overall diagnostic sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the multiplex rRT-PCR assay were greater than those of conventional RT-PCRs. The multiplex rRT-PCR assays provide the highly sensitive detection and rapid simultaneous typing of EV71, CV-A6 and CV-A16 thus rendering it feasible and attractive for large scale surveillance of EVs associated with HFMD outbreaks. Moreover, this study also investigated the relationship between these disease outbreaks with the evolutionary dynamics of CV-A6 and the appearance of novel recombinant forms (RFs) of the virus. Based on the analysis of the VP1 gene, the substitution rates of CV-A6 was estimated at 8.1×10^{-3} substitutions/site/year. There was an increasing likelihood between CV-A6 genome recombination and VP1 sequence divergence, with an estimated half-life of the RFs of 3.1 years. Bayesian phylogenetic analysis of the data showed that recently occurring recombination groups (RF-E, -F, -H, -J and -K) shared a common ancestor (RF-A). Recombination breakpoints were frequently observed between the 2A-2C gene and the 5´ untranslated region. This study revealed the potential for new CV-A6 variants to emerge and potentially modify disease outcomes of this major etiologic agent for HFMD affecting children worldwide.

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

Academic Year: 2015   Advisor's Signature --

ACKNOWLEDGEMENTS

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จุหาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF ABBERVIATIONS

α	Alpha
β	Beta
μ	Micro
μg	Microgram
μl	Microliter
%	Percent
℃	Degree Celsius
3´UTR	3´ untranslated region
5´UTR	5´ untranslated region
aa	Amino acid
AFP	Acute flaccid paralysis
Anx2	Annexin 2
BHQ	Black hole quencher
BLAST	Basic local alignment search tool
bp	Base pairs
BrCr	Enterovirus 71 prototype strain
CAR	Coxsackie-adenovirus receptor
CEB	Chick embryo cells
CI	Confidence intervals
CODEHOP	Consensus degenerate hybrid oligonucleotide primer
CV	Coefficients of variation
CV-A	Coxsackievirus A
CV-B	Coxsackievirus B
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CSF	Cerebrospinal fluid
Ct	Threshold cycle

DAF	The decay-accelerating factor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
E	Echovirus
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
EV	Enterovirus
FMDV	Foot-mouth disease virus
g	grams
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HA	Herpangina
hEV	Human enterovirus
hEV-A	Human enterovirus group A
hEV-B	Human enterovirus group B
hEV-C	Human enterovirus group C
hEV-D	Human enterovirus group D
HFMD	Hand, foot and mouth disease
Hr	Hour
HRVs	Human rhinoviruses
HS	Heparan sulphate
ICAM-1	Intercellular adhesion molecule-1
ICTV	International Committee on the Taxonomy of Viruses
ILI	Influenza-like illness
IRES	Internal ribosome entry site
Kb	Kilobases
LDL-R	The low-density lipoprotein receptor
M	Molar
MEGA	Molecular evolutionary genetics analysis

NCBI	The National Center for Biotechnology Information
NPV	Negative predictive value
nt	Nucleotide
NS	Non-structural protein
NTP	Nucleotide triphosphate
ORF	Open reading frame
PCR	Polymerase chain reaction
Poly(A)	Polyadenylated
PPV	Positive predictive value
PSGL-1	Human P-selectin glycoprotein ligand-1
PV	Poliovirus
PVR	Poliovirus receptor
RD	Rhabdomyosarcoma (cells)
RdRp	RNA-dependent RNA polymerase
RF	Recombination form
RNA	Ribonucleic acid
RNAse	Ribonuclease
RSV	Respiratory syncytial virus
RT	Reverse transcription
SA	Sialic acid
SCARB2	Human scavenger receptor class B member 2
ss	Single-stranded
VCAM-1	Vascular adhesion molecule-1
VP	Viral protein
VPg	Viral protein genome-linked

CHAPTER I

GENERAL INTRODUCTION

Background and rationale

Enterovirus (EV) infections are the immense health issue worldwide, affect both children and adults, and causing a wide spectrum of diseases ranging from mild (common cold) to life-threatening (poliomyelitis, hepatitis, meningitis, myocarditis and encephalitis). In particular, hand, foot, and mouth disease (HFMD) can be caused by various EV species A, most of them are coxsackievirus A4 (CV-A4), CV-A5, CV-A6, CV-A7, CV-A10, CV-A12, CV-A16 and EV-A71 (1). Although most of these virus infections are not serious in healthy individual, but HFMD caused by EV-A71 is occasionally leading to fatal neurological diseases (2). Recent repeated outbreak of EV-A71 subgenogroup C4 associated with severe encephalitis with cardiovascular collapse has occurred in Cambodia in 2012, resulting in more than 50 deaths (3).

This thesis will focus mainly on EV species A (especially EV-A71, CV-A6 and CV-A16) which are the predominant causes of HFMD and herpangina in the human population, with an incidence rate up to 500,000 - 1,000,000 cases per year (4). Therefore, epidemiological surveillance of EV infections in the population will help understand the pattern of EV transmission, and the epidemic background of circulating EVs, which could help interpret the EV trend and prevent the spread of these viruses. These informative data will provide major implications for future research on the pathogenic roles of EVs in HFMD and herpangina disease. In addition, this study has developed the multiplex real-time PCR panel allowing simultaneous detection of EV-A71, CV-A6 and CV-A16 by comparing with standard conventional diagnostic (RT)-PCR assays. This panel provides a rapid, specific and sensitive diagnostic approach and will be useful for routine diagnostics, epidemiological detection of EV infections and

preventing and controlling of the progress of disease. Moreover, the study provides clear evidence for recombination events in the founding of new CV-A6 lineages over an evolutionary time scale which remain a major etiologic agent for HFMD affecting children worldwide, comparable to that previously observed among EV species B.

Objectives

It is hypothesizes that there are other enteroviruses play an important role during the large scale outbreaks of HFMD and herpangina in Thailand during the rainy season in 2012. Although previous studies have shown that the viruses predominately associated with disease were EV-A71 and CV-A16 (5, 6). This study hypothesizes that CV-A6 may be the most frequent viral agent associated with the diseases. And there are some virus-specific factors that contribute to changes in clinical phenotype of CV-A6. Therefore the objectives of this study were to:

(i)   To evaluate the burden of human enteroviruses associated to HFMD and herpangina in patients and establish epidemiological profiles of these viruses during the study period.

(ii)   To develop a rapid, sensitive and specific assay based on one-step multiplex RT-PCR for use in Enterovirus 71, Coxsackievirus A6, Coxsackievirus A16 and panenterovirus detection.

(iii)   To investigate the evolutionary dynamics and molecular epidemiology of circulating strains of Coxsackievirus A6 from different countries (Germany, Spain, Sweden, Denmark, and Thailand) between 2013 and 2014 through analysis of structural and non-structural genome regions.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

In general, human enterovirus (EV) species A (especially EV-A71, CV-A6 and CV-A16) is one of the most common etiological agents of hand, foot, mouth disease (HFMD) and herpangina. They have been classified in the genus *Enterovirus* belonging to the family *Picornaviridae* which contains crucial pathogenic viruses such as poliovirus, human enterovirus (hEV), hepatitis A virus (HAV) and foot-and-mouth disease virus (FMDV). The Picornaviridae is one of the largest family and currently comprises 29 genera (50 species), seven of which include human pathogens (1) (Figure 1).

Enteroviruses were originally identified more than 100 immunologically distinct serotypes in the 1950s. These viruses can be classified by several parameters including the clinical manifestations observed in human infection, their pathogenesis in suckling mice, evolutionary relationships, antiviral drug susceptibility, and receptor specificity (7). By using the basis of a molecular classification system, human enteroviruse has been taxonomically categorized into 12 species: previously defined species EV-A (25 types), EV-B (63 types), EV-C (23 types), EV-D (5 types), HRV-A (80 serotypes), HRV-B (32 serotypes), and the new species HRV-C (55 types proposed based on determinant-encoded gene), and five EV species that only detect in animals (EV-E to EV-J). Although the polioviruses fall within the same genetic cluster as specie C, they are currently assigned to a distinct species in recognition of their biological uniqueness as agents of poliomyelitis. The classification of new types or strains within a species is based on the similarity of nucleotides and amino acid sequences of the capsid coding regions. A new type of EV is commonly defined by less than (<) 75% nucleotide and <85% amino acid sequence identity with known members across the VP1 (1).

Family	Genus	Specie	Disease	Associated genotypes
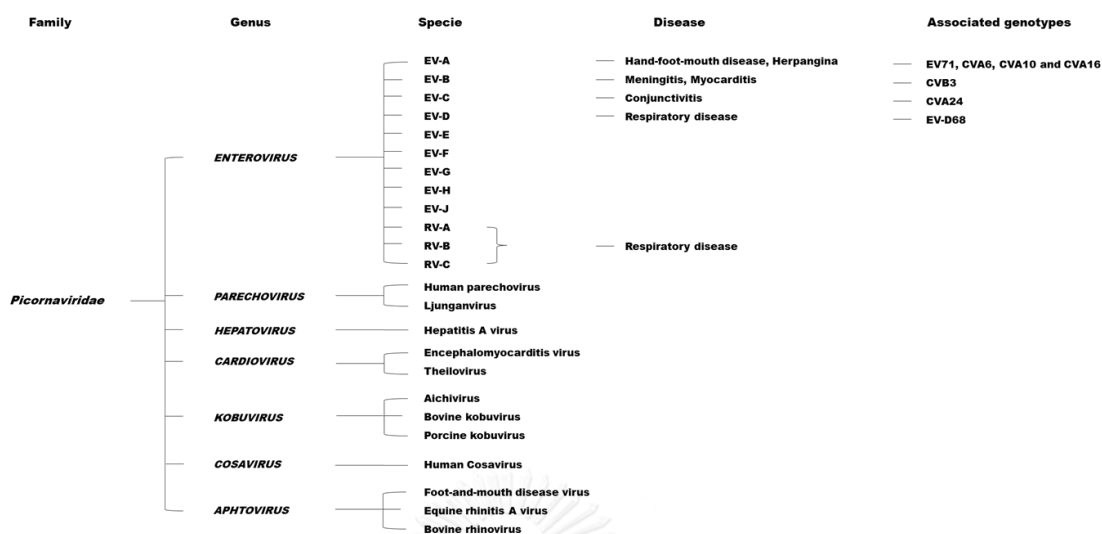
Figure 1. Members of the Picornaviridae family as classified based on Maximum likelihood tree in capsid proteins by Knowles (1) (Modified from van der Linden (8)).

2.2 Structure of Enterovirus Particles

Enterovirus capsids are encoded by the P1 region of the genome, serves multiple functions in many stages of the virus life cycle such as cell surface receptor binding, capsid internalization and destabilization, and transferring the viral genome into host cell (9). The mature virions containing 60 copies each of four viral polypeptides (VP1-VP4) and associate to form an icosahedral shell. The outer shell of the capsid is formed of parts of the three large proteins VP1, VP2 and VP3 that comprise wedge-shaped eight stranded μ-barrels. 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. Each capsid protein taking the form of four loops (BC, HI, ED and FG) connect with these eight strands. The small VP4 molecules is located completely on the inner surface of the capsids (10) (Figure 2).

Many reports have documented the particle structures of enterovirus including poliovirus (11), rhinovirus (12), CV-A21 (13), CV-B3 (14), and CV-A16 (15). Recently, (16-18) reporting the structural resolution of the mature virions of EV-A71 (subgenogroups B3, C2 and C4) revealed the capsid expansion without RNA encapsidation and the stabilization of virus by the presence of pocket factor which unlike in other enteroviruses. There is canyon surrounding the five-fold axes, which serve as the receptor binding site for enterovirus. Furthermore, the hydrophobic pocket within VP1 (the floor of canyon) has been recognized to be the directed binding site of several antiviral compounds including disoxaril (WIN 51711), WIN 54954, WIN 61209, WIN 68934, WIN 65099 and pleconaril (WIN 63843) (19).
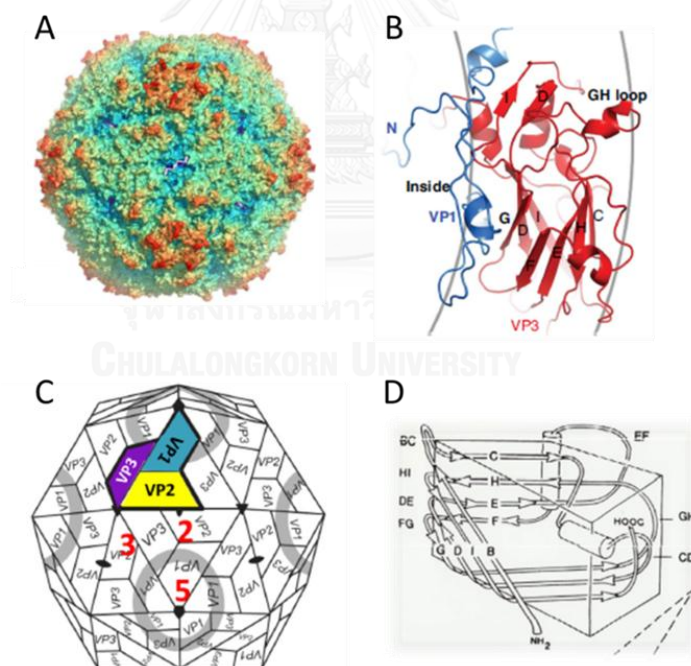
Figure 2. The 3D structure of the Enterovirus particle. (A) The crystal structure shows the surface of the 80S-like EV-A71 particle colour coded by radius. (B) The mature EV-A71 virion show structural regions in VP1 (blue) and VP3 (red) due to capsid expansion. (C and D) Icosahedral structure and schematic representation showing the positions of an enteroviral major capsid protein (VP1, VP2 and VP3), associate with eight stranded antiparallel beta sheet structures. (Modified from Jiang (20) and Ren (15))

2.3 Viral genome organization

Enterovirus is a small non-enveloped virus (30 nm) which comprises a positive single-stranded RNA genome (+ssRNA) of approximately ~7.2 to 8.4 kb long, which are encased by a highly structured icosahedral capsid. The viral genome is covalently linked to the small viral peptide VPg (22 to 25 amino acids) at the 5´-uridine of the genome (Tyr-p-U) (21). It is translated into a large polyprotein with a single open reading frame (ORF) flanked by 5´ untranslated regions (UTR) containing replication signals and the internal ribosome entry site (IRES), and 3´UTR with polyadenylated tail of variable length as shown in figure 3. The virus polyprotein is proteolytically cleaved by viral protease into structural protein P1 (viral capsid protein (VP) 1 to VP4) and nonstructural proteins P2 (2A to 2C) and P3 (3A to 3D) which have different functions important for virus survival, shown in table 1. VP1 has been recognized as an essential part of the viral antigenic determinant, and it is also utilized as binding sites of many cellular receptors and neutralization antibodies (9).
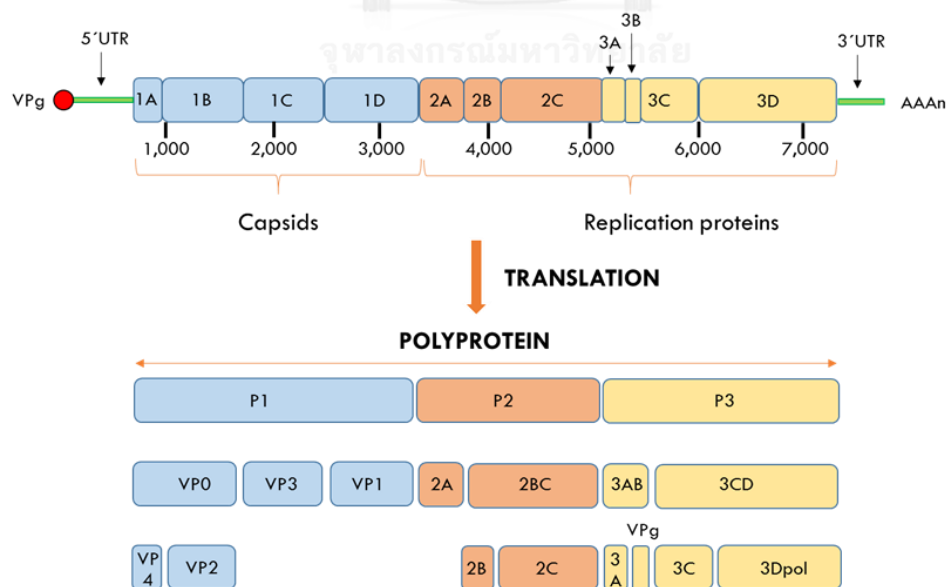
Figure 3. A Schematic representing the enteroviral genome composition and organization.

Table 1. Characteristics and functions of several genes of enterovirus.

Genomic Region	Length (bp)	Protein designation	Protein Function
P1	~200	VP4	Small capsid protein implicated in virion uncoating
	~800	VP2	Major capsid protein
	~700	VP3	Major capsid protein
	~900	VP1	Receptor binding site
P2	~400	2A	Chymotrypsin-like cysteine protease releasing capsid precursor from the nascent polypeptide, inhibit host transcription and maturate viral protein
	~300	2B	Increased membrane permeability, inhibiton of cellular secretory pathways
	~1,000	2C	Vesicle formation and NTPase
P3	~200	3A	Inhibition of intracellular transport
	~100	3B	VPg primes RNA synthesis
	~500	3C	Protease
	~1,400	3D	RNA-dependent RNA polymerase

The P2 and P3 regions are comprised of 7 viral genes which 2A is included into 2C, and 3A is included into 3D. $2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$ are encoded as viral protease, and play essential roles in viral polyprotein processing. The first cleavage reaction in all enteroviral polyproteins is catalyzed by the $2A^{pro}$. Viral proteases not only cleave viral polypeptides, but also inhibit various host machineries (22-24). Viral protein 2B contain two hydrophobic regions, which is crucial for multimerization, integrating into the membrane of the host Golgi and ER complex, producing virus-induced vesicles, and forming the viroporin complex (25). The 2C protein is a multifunctional protein comprising 329 amino acid residues. The protein 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 (26, 27). Protein 3A which is a membrane binding protein plays a role in inhibiting cellular protein secretion and mediating presentation of membrane proteins during viral infection (28, 29). The enteroviral 3B protein (VPg) is small peptides (21-23 aa), which is utilized as a primer in both positive- and negative-strand RNA synthesis (30, 31). The viral RNA-dependent RNA polymerase 3D is one of the major components of the viral RNA replication complex (32, 33).

2.4 Replication cycle

EV is transmitted predominantly via oral-fecal route, but also through contact with virus-contaminated oral secretions and vesicular fluid, surfaces and fomites as well as through direct contact of patient's respiratory droplets (34). EV can infect a wide range of different cells with the different in the viral replication capacity. Virus entry into susceptible host cells involves several processes, including viral surface attachment, receptor binding and then the virus particles are uptake into host cell through endocytic pathway (Figure 4). Subsequently, the viral RNA is released into the cytoplasm after the

virus is uncoated. After that, the ORF is translated in the cytoplasm to produce a single,

large polyprotein of approximately 2,200 amino acid residues, which may be co- and post-

translationally cleaved into P1, P2 and P3 precursors upon the synthesis of virus encoded

protease ($2A^{pro}$ and $3C^{pro}$). During replication initiation with a secondary RNA structure in

the genome as a template, $3D^{pol}$ and 3C protease uridylylates the VPg protein by

uridylylation process (VPg-pUpU) (30). After that, the newly made viral RNA is replicated

and translated prior to form new infectious virus particles which exits the host cell either

after cell lysis.
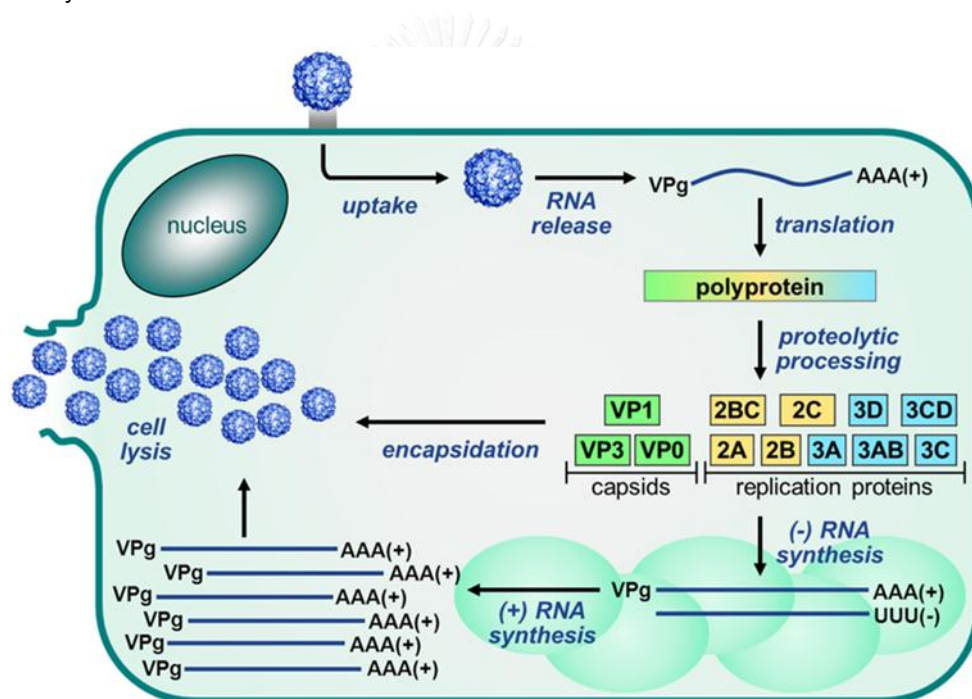
Figure 4. Overview of the Enterovirus life cycle.

From van der Linden, 2015 (8).

2.5 Enterovirus Receptors

By attaching on the surface of host cell, Enterovirus receptors play an crucial role

in the internalization and destabilization of the capsid. Several viruses in this family have

similar receptor molecules. Table 2 provides an overview of the receptors identified

among enteroviruses and their functions. However, the specific host cellular receptor for

EV remains unknown. Until recently, there are at least four different types of human cellular receptors specific to EV-A71 and CV-A16 infection such as human scavenger receptor class B member 2 (SCARB2), Human P-selectin glycoprotein ligand-1 (PSGL-1), ANX2, and Sialylated Glycans.

2.5.1 Human scavenger receptor class B member 2

The first characterized receptor is human scavenger receptor class B member 2 (SCARB2), which also known as lysosomal integral membrane protein II, CD36b like-2 or HLGP85. SCARB2 is a large family of the scavenger receptor family. They are involved in physiological function including transfer β-glucocerebrosidase from the endoplasmic reticulum to lysosomes and reorganize the endosomal/lysosomal compartment (35). SCARB2 is expressed on many cells including neurons in the central nervous system, lung pneumocytes, hepatocytes, renal tubular epithelium, splenic germinal centers and intestinal epithelium and expression can enhance efficient viral replication (36). SCARB2 is used by EV-A71 for cell attachment through the binding of α5 helix within the head region (amino acids 142-204) (37). The interaction of EV-A71 with SCARB2 induces a conformational changes which lead to the uncoating of the virion at acidic pH (below 6.0) (38). In addition, SCARB2 also functions as a receptor for Coxsackievirus A7 (CV-A7), CV-A14 and CV-A16, which are in the same species with EV-A71 in *Enterovirus* genus (39).

2.5.2 Human P-selectin glycoprotein ligand-1

Human P-selectin glycoprotein ligand-1 (PSGL-1) is a second characterized receptor for EV-A71 (40). It is a membrane protein expressed by leukocytes and involve in inflammatory responses including tethering and rolling of leukocytes due to the recruitment of cells from blood vessels (41). The expression of PSGL-1 is essentially ubiquitous, with expression found on myeloid, lymphoid, dendritic lineages and platelets.

The binding of PSGL-1 on EV-A71 was determined to be close to the five-fold vertex via an electrostatic interaction (42) (Figure 5).
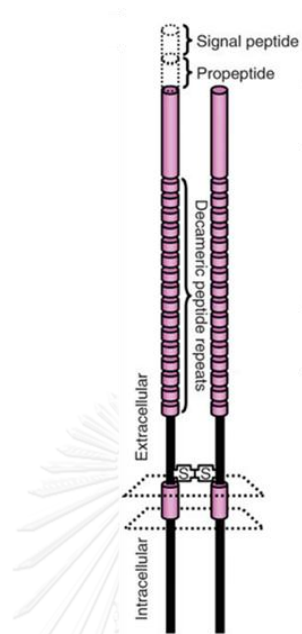
Figure 5. The structure of human P-selectin glycoprotein ligand-1 (PSGL-1).

From Nishimura Y, 2009 (40).

2.5.3 Anx2

Anx2 is the member of the annexin family, the members of which are involved in a range of functions including a profibrinolytic coreceptor for tissue plasminogen activator and plasminogen on endothelial cells (43). The Anx2 binding site on EV-A71 was mapped by yeast two-hybrid analysis to be close to VP1 amino acids 40–100, which comprise b-sheet B and the partial BC loop and not in the canyon region (44).

2.5.4 Sialic acid

The expression of sialic acid (SA)-linked glycan on intestinal epithelial cells corresponds to the known tropism of the viruses that use this molecule as a receptor (45). The direct interaction between sialylated glycans and EV-A71 is still unclear

2.6 Viral Pathogenesis

2.7 Virus entry and spread

Initial viral replication is presumed to occur in the lymphoid tissues of the tonsils and Peyer's patches and further multiplication in the regional lymph nodes. Subsequently, the virus particles begin to appear in the respiratory and alimentary tracts leading to prolong virus shedding up to 2 weeks post infection. EV-A71 can persistently present in alimentary tract up to 11 weeks after the symptoms recovered (46). Furthermore, studies in mice indicated that CNS invasion may occur at this point through retrograde axonal spread along cranial or peripheral nerves (47). Secondary site of infection is the central nervous system (CNS) and dissemination of EV-A71 could also be found in the reticulo-endothelial system (liver, spleen, bone marrow and lymph nodes), heart, lung, pancreas, skin, and mucous membrane, leading to cause mild viremia (46).

2.6.2 Tropism

Despite the genome and life cycle of EV-A71 is similar to other picornaviruses, the associated pathological conditions differ significantly. Coxsackievirus B3 (CVB3) induces myocarditis and cardiomyocyte apoptosis (48). Various factors could affect severe disease or pathogenesis, including age, viral genotype, viral proteins, the host genetic background, the host immunity and co-infection with other viruses such as adenovirus 21 (49). The host immune response may induce serious complications of EV-A71 patients. Additionally, inflammation of the CNS has been proposed as the underlying pathogenic mechanism of EV-A71-associated severe neurological complication. Recent studies suggest that EV-A71 can infect several types of immune cells, such as DCs, monocytes and Jurkat T-cell lines (50). Infected immune cells may contribute to pathogenesis by secreting cytokines and chemokines, which mediate effector cells. Moreover, EV-A71

infection causes the up-regulation of FasL expression and thus promotes the apoptosis of Jurkat cells (51).

Table 2. Enterovirus receptors and their functions (52-63).

Receptors	Function	Receptor / Virus domain binding	Viruses	Reference
1. Immunoglobulin Superfamily				
1.1 ICAM-1 (CD54)	Inflammatory responses		Major group of HRVs, CVA13, A17 ,A18, A20, A21 and A24	(52)
1.2 VCAM-1	Not reported	The amino-terminal domain, D1 / Canyon	EMCV	(53)
1.3 PVR (CD155)	The formation of adherens junctions		Poliovirus	(54)
1.4 CAR	Cell adhesion molecule in developing brain		CVB1 - B6	(55)
2. LDL-R	Serum cholesterol and vitamin homeostasis	the 2nd and 3rd domains / HI & BC surface loops	Minor group of HRVs	(56)
3. DAF (CD55)	Regulate complement activation and protect cells from autologous lysis	Several binding patterns	CVB1, B3, B5, Echoviruses and EV70	(57)
4. Integrins	Regulate cell-to-cell / cell-to-matrix contacts, tissue repair, angiogenesis and inflammation	Several binding patterns	CVA9, CVB1, B3, B5, Echoviruses	(58-60)
5. Heparan sulphate	Embryonic development, wound healing and homeostasis	(+) charged amino acid residues on surface particle interact with (-) charged sulphates	CVB3, Echovirus, HRV89, FMDV	(61-63)

ICAM-1, Intercellular adhesion molecule-1; VCAM-1, Vascular adhesion molecule-1; PVR, Poliovirus receptor; CAR, Coxsackie-adenovirus receptor; LDL-R, The low-density lipoprotein receptor; DAF, The decay-accelerating factor; HRVs, Human Rhinoviruses; EMCV, Encephalomyocarditis virus and FMDV, Foot-mouth disease virus

2.6.3 EV-A71 infection induce apoptosis

Although the pathogenesis of EV-A71 infection remains unclear, the induction of cell death by EV-A71 may be a crucial factor contributing to its pathogenesis. Host-virus interactions studies suggested that many types of virus in the *Picornaviridae* family have ability to induce apoptotic cell deaths such as poliovirus, and coxsackievirus type B3 (64, 65). EV-A71 infection results in apoptosis for a wide range of cells, including rhabdomyosarcoma (RD) cells, Jurkat cells, SK-N-MC cells, glioblastoma SF268 cells, Vero cells, the human microvascular endothelial cell line and Hela cells (51, 66, 67). Cell apoptosis is necessary to induce EV-A71 viral protein synthesis. Animal studies showed that neuronal loss and apoptosis were evident in the brainstem and spinal cord of EV-A71-infected mice (68).

Several studies indicated that the proteolytic cleavage of EV-A71 3C and 2A can trigger apoptosis by activating of caspases (66, 69). A previous study identified that SUMO-modified 3C delayed the onset of cell apoptosis, providing evidence that EV-A71-3C plays a crucial role in apoptosis induction (70). The synergistic effect of many EV-A71 proteins might contribute to the pathogenesis of disease severity. Many picornaviral proteins including VP1, VP2, VP3, 2A, and 3C are reported to interact with host cellular factor by associating with apoptosis induction (71). For example, the 2C protein is a multifunctional protein comprising 329 amino acid residues. The protein contains many conserved motifs including membrane-, RNA-, and NTP-binding sites which are crucial for host cell membrane rearrangement, genome replication and virus particle encapsulation (26, 27, 72). The 2C protein also has two important functions during viral RNA synthesis: NTPase activity and directing replication complexes to the cell membrane (73). The 2C protein can specifically bind to the $3'$-end of viral RNA itself (74). It also has specific interaction with structural protein (VP3), which implicates in the genome

encapsulation (75). Furthermore, evidence from *in vitro* studies suggested that apoptosis process could be induced in chick embryo cells (CEB) and Cos-7 cells through an activation of the cytochrome-c-caspase 9 pathway by the 2C protein of avian encephalomyelitis virus (76). Recent studies also suggest that the 2C protein of EV-A71 can interact with host protein reticulon 3 to form protein complex which is important for viral replication (77). Moreover, EV-A71-2C can function as an antagonist of the TNF-α-mediated activation of NF-κB signaling pathway, which is responsible for the secretion of numerous cytokines. Through interaction with IKKβ, EV-A71 can modulate host immune reactions via its 2C protein (78). The EV-A71-2C has also been implicated as a virulence factor and regulation of host response.

2.8 Clinical Manifestation and Outbreak

EV-A71 was first isolated from the feces of a female encephalitis patient in 1969 in California (79). However, a retrospectively analysis suggested that the virus could have emerged in the Netherlands as early as 1963 (80). Infection by EV-A71 is often asymptomatic or may manifest as a self-limiting influenza-like illness. EV-A71 has been recognized as the most common pathogen of hand, foot, and mouth disease (HFMD), which are one of the most infectious diseases frequently affecting children below 5 years of age. EV-A71 can also occasionally cause serious neurological manifestations including aseptic meningitis, acute flaccid paralysis (AFP), brainstem encephalitis as well as pulmonary edema, which may lead to mortality due to the complications (81, 82). EV-A71-associated HFMD have become a major public health concern in many countries especially in the Asia-Pacific countries including China (83), Taiwan (84), Malaysia (85), Japan (86), Singapore (87), and Thailand (6). Among the epidemic of HFMD in these countries, China has experienced many large scale outbreaks of EV-A71-associated HFMD. The largest outbreak occurred in 2008 with an estimated 25.71 cases per 100,000

populations with a high fatality rate in young children (126 death cases among 490,000 HFMD confirmed cases). Auhui province was the most affected area during the outbreak period (83). Until recently, neither effective antiviral therapy nor vaccine for EV-A71 is yet available.

2.9 Antiviral Compounds

Several antiviral compounds directed against EVs have been developed as shown in table 3.

Table 3. Examples of preventative and therapeutic approaches targeting EV infections (88-93).

Type	Therapeutic agent	Action	Effect
Capsid binders	Pleconaril (WIN 63843 or Picovir)	Bind Canyon	Broad-spectrum activity against a variety of HRV and enterovirus serotypes
	BTA798	. Bind Canyon	Treated HRV infections in asthmatic patients
	Pocapavir	Bind Canyon	Against EVs and poliovirus
Protease Inhibitor	Rupintrivir (AG7088)	3C protease inhibitor	Potent activity against all HRV serotypes in vitro whereas insignificant impact in human trial.
	AG7404	3C protease inhibitor	Analog of rupintrivir with improved oral bioavailability compared to rupintrivir
	PI4KIIIβ inhibitors	3A protease inhibitor	Inhibited effect on CVB4 replication and CVB4-induced pathology
	TTP-8307	3A protease inhibitor	Against CVB3 and inhibited viral RNA synthesis
Polymerase Inhibitor	Ribavirin	$3D^{pol}$ inhibitor	Increases the error rate of $3D^{pol}$
	GPC-N114	$3D^{pol}$ inhibitor	Interferes with productive binding of the template-primer to $3D^{pol}$

Part 1: Prevalence and characterization of enterovirus infections among pediatric patients with hand foot mouth disease, herpangina and influenza like illness in Thailand, 2012

Hand, foot, and mouth disease (HFMD) and herpangina are common causes of morbidity among children particularly, elementary school children below 10 years of age. HFMD is a febrile viral illness with oral ulceration on the anterior tonsillar pillars, soft palate, buccal mucosa and uvula and vesiculo-papular rashes over the hands, feet, elbows, knees or buttocks, while pathological hallmarks of HA are fever and oral ulcers without any progression of vesicular eruption on the skin (2, 94-96). HFMD and herpangina are generally considered as asymptomatic or self-limiting infectious diseases with patients having mild clinical complications which can be resolved completely within 5-7 days post infection. Various species and genotypes of enterovirus have been recognized as etiological agents for HFMD and herpangina including human enterovirus 71 (EV-A71), coxsackievirus A genotypes 1 (CV-A1) to CV-A10, A12, A16 and A22 and coxsackievirus B genotype 2 (CV-B2) to CV-B5 and echovirus 18 (97-100).

Recent viral etiological surveillance studies have suggested that viral profiles implicated in herpangina are less complex than HFMD; notably, members of enterovirus species A have been reported to be common viruses of herpangina. Nevertheless, among these, EV-A71 and CV-A16 are the most frequent viral agents associated with the diseases (101-103). Although different types and species of enteroviruses are somewhat different in their genetic background, the clinical manifestation and severity are identical and therefore, hamper the differentiation between specific virus infections and their clinical consequences based on the sole observation of clinical signs. Some epidemiological studies have suggested that, unlike other viruses, EV-A71-induced HFMD may cause severe life-threatening neurological and systemic complications,

accompanied by brainstem encephalitis, aseptic meningitis, encephalomyelitis, poliomyelitis-like paralysis and cardiopulmonary failure, and may consequently lead to death, resulting in the recognition of EV-A71 as the most important neurotropic virus since the prevention of poliovirus by vaccine (104, 105). Furthermore, EV-A71-caused HFMD and herpangina have largely emerged and continuously led to deaths due to complications in many Asia-Pacific countries including China (83, 106-108), Taiwan (84, 109), Malaysia (85, 110), Japan (86), Singapore (87, 95), and Vietnam (105, 111). Accordingly, understanding the risk factors that may exacerbate clinical complications and establishing effective molecular detection and enterovirus serotyping methods in clinical specimens are critical for disease surveillance and public health intervention.

Studies on clinical complications caused by specific types of enteroviruses have been reported. Among multiple enterovirus types associated with recent HFMD and herpangina outbreaks, CV-A6 has been recognized as an emerging causative virus since the epidemics in Finland and Singapore in 2008 (112-114) and its global dissemination thereafter in Taiwan in 2010 (115), Japan and Spain in 2011 (116, 117) and the United State in 2012 (118). In our previous study, we have reported on large scale outbreaks of HFMD and herpangina in Thailand during the rainy season in 2012 with approximately 40,000 suspected cases all over the country, and shown that CAV6 played an important role during the outbreak in 2012 (119). Our previous enterovirus detection assays in clinical specimens targeted the consensus sites in the 5´ UTR combined with the specific primer sets for the most common viruses EV71 and CV-A16 and the recently emerged CV-A6 (5, 6, 119). Nonetheless, as the 5´ UTR site has highly conserved sequences shared by all enterovirus members, our molecular serotyping based on 5´ UTR sequences alone could not unequivocally distinguish between the genetic types of enterovirus and accordingly, only 65.7% of all pan-enterovirus positive specimens could be assigned to

any specific type (119). In addition, the underlying reasons for these changes in clinical phenotype of CV-A6 are unknown. Therefore, cohort epidemiologic studies and pathogenic role of this virus should be concerned.

Recently, many PCR techniques mainly targeting the hypervariable capsid encoding VP1 region using a consensus degenerate hybrid oligonucleotide primer (CODEHOP) have been developed to further increase sensitivity and specificity of detection as its classification results resembled the result from seroneutralization assays (120). To encounter the nonspecific amplification problem, the CODEHOP consists of two regions Degenerate Core and Consensus clamp. The degenerate core region uses inosine to make the region highly degenerative, designed from conserved motif when amino acid sequences are aligned, allowing board target specificity while consensus clamp are non-degenerate driven the hybridization of primer to template, therefore increases the stability allowing higher annealing temperature to be use and in turn diminish nonspecific amplification. These observations prompted us to conduct a study to further our insight into the genetic diversity of enteroviruses and document their epidemiological profiles associated with HFMD and herpangina diseases in Thailand in 2012.

In the present study, we investigated the involvement of multiple enterovirus types by a molecular typing method using CODEHOP primers targeting the VP1 gene in order to establish an effective method which could be directly applied for clinical specimen testing. Parallel screening for enteroviruses in influenza-like illness (ILI) patients over the same period was also performed in order to compare the viral activities in different subjects with clinical symptoms.

Materials and Methods

Ethical considerations

The research protocol was approved by the institutional review board of the Ethical Committee of the Faculty of Medicine, Chulalongkorn University, Thailand (approval number IRB390/55). All information and patient identifiers were kept anonymous to protect patient confidentiality. The stored data included age, location of hospitals or medical centers, any recorded symptoms or clinical information, referral source, month of sample collection, and, if any, the results of other virological tests for each sample. Since the data obtained in this study were de-identified, written consent from the patients was waived. Permission for specimen utilization had been granted by the Director of King Chulalongkorn Memorial hospital.

Population study

The studied population in this research was inpatients and outpatients from different parts of Thailand (Bangkok, Khonkaen, Suphanburi, Saraburi Rayong and Chantaburi) in 2012, diagnosed as suspected cases for HFMD, herpangina and influenza-like illness (Figure 6). These clinical specimens will be subjected for routine enteroviruses and respiratory viruses diagnostic and keep at the Center of Excellent in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
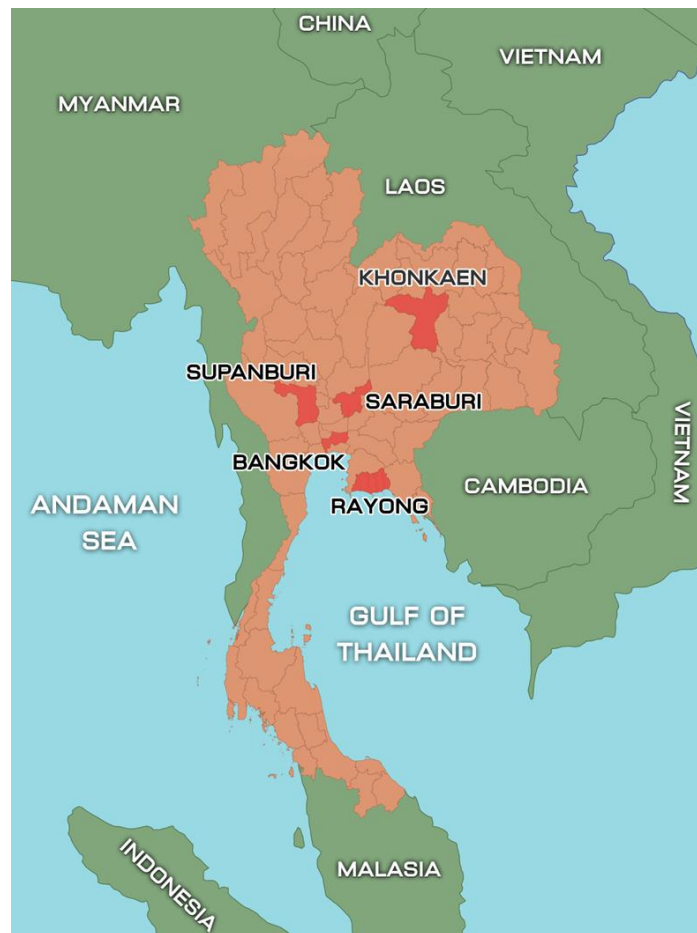
Figure 6. Location of sample collection sites during outbreak of hand, foot, and mouth disease, Thailand, 2012.

Case definition and sample collection

Herpangina

Herpangina was defined as well-characterized multiple vesicular exanthema and ulcers of the soft palate with presentation of fever, sore throat, and decreased appetite. In addition, a total of 172 clinical specimens from 166 cases were collected from herpangina cases including 133 rectal swabs, 26 throat swabs, 9 serum samples, 2 stool samples, 1 vesicle fluid, and 1 nasal swab (119).

HFMD

HFMD suspected cases were defined as having oral ulcers but chiefly on the buccal mucosa, tongue, hard and soft palate accompanied by typical vesicular rashes most commonly on the extensor surfaces of the hands, feet, knees and/ or buttock. A total of 730 clinical specimens were taken from 704 HFMD suspected patients including 578 rectal swabs, 75 stool samples, 36 throat swabs, 15 serum samples, 8 cerebrospinal fluid samples, 8 nasal swabs, 8 vesicle fluids, 1 sputum and 1 saliva (119).

Influenza like illness (ILI)

The inclusion criteria for ILI were as follows: pediatric patient aged less than 12 years with the onset of high temperature (more than 38°C) and respiratory tract symptoms such as sore throat, cough, and runny nose with difficulty to breathe. In addition, a total of 1,094 nasal and throat swab specimens had been collected from both hospitalized and non-hospitalized patients having presented with ILI symptoms and admitted to hospital between January and December 2012.

All patient samples were collected from outpatients during visit and from inpatients within 48 h of admission and were transferred in 2 ml viral transport media (VTM) modified according to recommendations by the World Health Organization to the Center of Excellence of Clinical Virology, Chulalongkorn Hospital and immediately stored at -70°C until RNA extraction.

Sample size estimation

The prevalence of enteroviruses infection in patients has been reported by several countries and years with variation considerably. As for examples, the EV prevalence values are as follows: Spain, 66%; Singapore, 62%; Korea, 43%; Hong Kong, 67%. With regard to the precise of identifying epidemic data, average values of the prevalence of

EV in patients with HFMD will be used in the experimental designs as 59.5%. The sample size required will be calculated according to the following formula:

$$n = \frac{Z\alpha^2 P(1- P)}{e^2}$$

n	=	required sample size
$Z\alpha$	=	confidence level at 95% (standard Z value of 1.96(two-tail))
P	=	Incidence proportion EV infection in patients with HFMD and herpangina estimated from the previous study
e	=	acceptable margin of error at 3% (standard value of 0.03)

Therefore, 1,029 samples will be required for the EV surveillance, respectively, in order to achieve the statistically supported results with 3% acceptable error.

Viral nucleic acid preparation

Total viral nucleic acid was extracted from 200 μl of the clinical specimens using a Viral Nucleic Acid Extraction Kit according to the manufacturer's recommendation (RBC Bioscience, Taipei, Taiwan) and the extracted solution was stored at -70°C. All experiments were performed in a Bio-safety Level 2 plus environment. RNA from individual specimens was reverse transcribed into cDNA using the ImProm-II Reverse Transcription System (Promega, Madison, WI) with random hexamer primers (First BASE Laboratories, Selangor Darul Ehsan, Malaysia).

Laboratory diagnosis for enterovirus using RT-PCR

Pan-Enterovirus detection using PCR amplification for 5′UTR

RNA from individual specimens was reverse transcribed into cDNA using the ImProm-II Reverse Transcription System (Promega, Madison, WI) with random hexamer

primers (First BASE Laboratories, Selangor Darul Ehsan, Malaysia). The primer details are listed in table 4. The PCR reaction mixture contained the following: 1 µl of cDNA, 15 µl of PerfectTaq Plus Mastermix (5Prime, Hamburg, Germany), 0.5 µM forward and reverse primers, MgCl2 and nuclease free water. The PCR for detection of enterovirus was carried out on a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: 3 min at 94°C for initial denaturation, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 60°C for 45 sec followed by 1 min at 72°C for extension, and a final extension at 72°C for 10 min. The expected 317-bp PCR products were visualized under UV light upon gel electrophoresis and staining with ethidium bromide.

Enterovirus typing using PCR amplification of VP1 region

Amplification of the VP1 encoding regions was performed using three different primer sets including specific primers for EV-A71/CV-A16, CV-A6 and CODEHOP.

To identify the type of Pan-enterovirus, samples were amplified using CODEHOP primer VP1 semi-nested PCR as previously described. Briefly, cDNA were synthesized from total RNA using four different primers (AN32-35). Enteroviruses were detected by nested-PCR with primers 222 and 224 as first-round primers and primers AN88 and AN89 for nested amplification. The CODEHOP PCR product was 350-400 bp in length.

Sequence Analysis

The amplified products from the conventional PCR reactions were purified using the HiYield Gel DNA Fragment Extraction kit (RBC Bioscience, Taipei, Taiwan). All sequences were amplified bidirectionally using the primers of the 2nd round PCR and subjected to sequencing by automated DNA cycle sequencer (First BASE Laboratories, Selangor Darul Ehsan, Malaysia). The nucleotide sequences were edited using the

Seqman program of DNASTAR Software (v5.0). Multiple sequence alignment was achieved by using the ClustalW multiple alignment programs, primer sequences were trimmed out and percent identities between pairs of sequences were calculated using the BioEdit Sequence Alignment Editor package (v7.0.9.0). To determine the genetic variation and the relationships with other reference viruses, phylogenetic trees were constructed using the neighbor-joining (NJ) method and Kimura's two-parameter distance model with 1,000 bootstrap pseudo-replicated and pair-wise deletions for missing data implemented in the MEGA software package (v5.0) (121). According to the 9th ICTV report, members of enterovirus species which share more than 75% nucleotide identity within the VP1 capsid gene, those with amino acid identities exceeding 85% would be considered as lineages of the previously defined enterovirus types (1). In this study, we maintained the criteria for type assignment originally described by ICTV and followed by some publications contributing to the discussion on phylogenetically evolving viruses.

The nucleotide sequences obtained from this study have been stored in GenBank database under accession numbers JX556422–JX556564, KF383346-KF383383 and KF661098- KF661255.

Table 4. Primers used for conventional RT-PCR assays (6, 119, 120).

Specificity	Primer name	Nucleotide sequence 5´- 3´	Strand	Target gene	Product size (bp)	Reference
panenterovirus	panEV-F1	CAA GCA CTT CTG TTT CCC CGG	Sense	5´UTR	317	(6, 119)
	panEV-R1	ATT GTC ACC ATA AGC AGC CA	Antisense	5´UTR		
	panEV-F2	AAG CAC TTC TGT TTC C	Sense	5´UTR		
	panEV-R2	CAT TCA GGG GCC GGA GGA	Antisense	5´UTR		
EV71/CAV16	EV-F2760	ATG GKT ATG YWA AYT GGG ACA T	Sense	VP1	418 (EV71),	(6, 119)
	EV71/F2788	AAC WGG TTA YGC RCA AAT GCG	Sense	VP1	306 (CAV16)	
	CA16/F2900	ACT GCA GTA CAT GTA TGT CCC	Sense	VP1		
	EV-R3206	CCT GAC RTG YTT MAT CCT CAT	Antisense	VP1		
CAV6	CA6-F2632	TGT GTG ATG AAT CGA AAC GGG GT	Sense	VP1	420	(6, 119)
	CA6-R3288	TGC AGT GTT AGT TAT TGT TTG GCT	Antisense	VP1		
	CA6-R3053	GGG TAA CCA TCA TAA AAC CAC TG	Antisense	VP1		
CAV8	CA8-F2690	GAC CAT TTC TTT TCA AGA GCA GG	Sense	VP1	440	-
	CA8-R3203	GCG CAC GTG YTT SAG GCG CAT	Antisense	VP1		
	CA8-R3127	GCG AAT GTR CCC ATC ATG TTA TT	Antisense	VP1		
Enterovirus (CODEHOP)	222	CIC CIG GIG GIA YRW ACA T	Antisense	VP1	350-400	(120)
	224	GCI ATG YTI GGI ACI CAY RT	Sense	VP3		
	AN89	CCA GCA CTG CAG CAG YNG ARA YNG G	Sense	VP1		
	AN88	TAC TGG ACC ACC TGG NGG NAY RWA CAT	Antisense	VP1		
	AN32	GTYTGCCA	Antisense	VP1		
	AN33	GAYTGCCA	Antisense	VP1		
	AN34	CCRTCRTA	Antisense	VP1		
	AN35	RCTYTGCCA	Antisense	VP1		

Results

Patient characteristics

From January 1st to December 31st, 2012, cases of HFMD (704 cases), herpangina (166 cases) and influenza-like illness (1,094 cases) were enrolled in this study. The median (range) age at the time of infection diagnosed as HFMD, herpangina and ILI were 2.0 years (from 1 month to 54 years), 2.3 years (from 3 months to 16 years) and 3.0 years (from 1 month to 12 years), respectively. Most suspected cases of HFMD (92.5%), herpangina (89.7%) and ILI (74.6%) occurred in children aged 5 years or younger. Patients with HFMD (59.9%), herpangina (53.6%) and ILI (56.5%) were male , with a M/F ratio of approximately 1.5, 1.2 and 1.3, respectively. 74.1% patients with HFMD were from urban, 25.9% from suburban areas of Thailand (Khonkaen, Suphanburi, Saraburi, Rayong and Chantaburi) (Table 5).

Table 5. Demographic characteristics of all patients.

Characteristic		HFMD (N = 704)		Herpangina (N = 166)		ILI (N = 1,094)	
		No.	%	No.	%	No.	%
Gender	Male	422	59.9	89	53.6	618	56.5
	Female	282	40.1	77	46.4	476	43.5
Area	Urban	522	74.1	91	54.8	864	79.0
	Suburb	182	25.9	75	45.2	230	21.0
Age median (yrs.)		2.0		2.25		3.0	
Age	0 – 2 yrs.	454	64.5	101	60.8	541	49.5
	3-5 yrs.	197	28.0	48	28.9	275	25.1
	6 – 12 yrs.	39	5.5	15	9.0	278	25.4
	13 – 15 yrs.	4	0.6	1	0.6	0	-
	>15 yrs.	10	1.4	1	0.6	0	-

Generally, the clinical manifestations of HFMD were fever; drooling, and refusal to eat (among young children); painful lesions in the mouth, especially on the soft palate (Figure 7, panel A); and vesicular rashes on the palms and feet (Figure 7, panels B, C). For patients affected by this outbreak, physicians from reporting sites reported anecdotally that they observed more severe skin manifestations than usual, especially on the buttocks and perianal area (Figure 7, panel D), knees, and elbows. Two cases with neurologic involvement (convulsion, altered consciousness) were caused by EV71 and were treated with intravenous immunoglobulin. No patients died.

Figure 7. Clinical manifestations in children with coxsackievirus A6 infection during outbreak of hand, foot, and mouth disease and herpangina, Thailand, 2012.
A) Lesions on the soft palate of the mouth; B) vesicular rash on the hands; C) vesicular rash on the foot; D) lesions on the buttocks and perianal area.

Temporal Distribution

The temporal distribution of enteroviruses from HFMD, herpangina and ILI patients showed that the prevalence of enteroviruses in different months was distinctly different (Figure 8). As for HFMD, EV were distributed throughout the year except for February and showed a pronounced increase in the incidence during the rainy season; 14.6% in June (103/704), 34.4% in July (242/704) and 9.9% in August (70/704). As for herpangina, EV showed seasonal variation with a peak during the rainy season. The pattern of outbreak was similar to HFMD spanning a time period of 3 months; 16.3% in June (27/166), 39.8% in July (66/166) and 6.6% in August (11/166). As for ILI, EV was detected throughout the year except for May, November and December. The prevalent months for EV were 1.0% in June (11/1094) and 1.6% in July (18/1094).
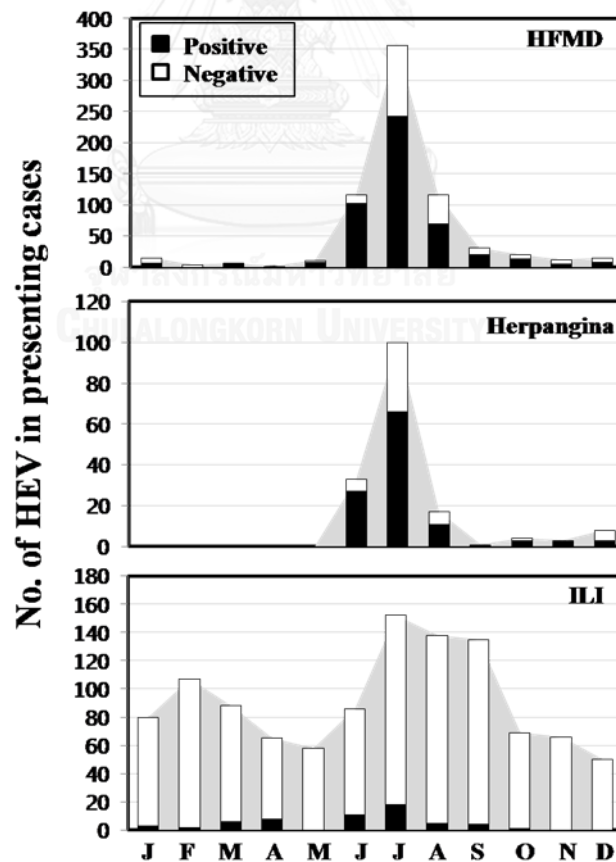
Figure 8. Seasonal distribution of hEV in Thailand 2012.

Age Distribution

       Three patient groups (HFMD, herpangina and ILI) showed a high prevalence of enterovirus infection in young children below the age of 5 years (Figure 9). As for the age of HFMD patients with enterovirus infection, extremely high infection rates were observed in 1-year-old infants (24.3%). The result showed the lowest incidence of enterovirus among the 0-6-month olds accounting for 1.6% and a peak incidence among the 7-12-month olds. Moreover, the oldest HFMD patient in this study was 36 years old. As for herpangina, the age distribution pattern was similar to HFMD. The result showed the lowest incidence of enterovirus among the 0-6-month olds accounting for 1.2% and a peak incidence among 1-year olds (18.1%). As for ILI, 1.0% of EV infections were observed in 2-year-old children. In addition, the result showed that the distribution patterns in each age group were comparable.
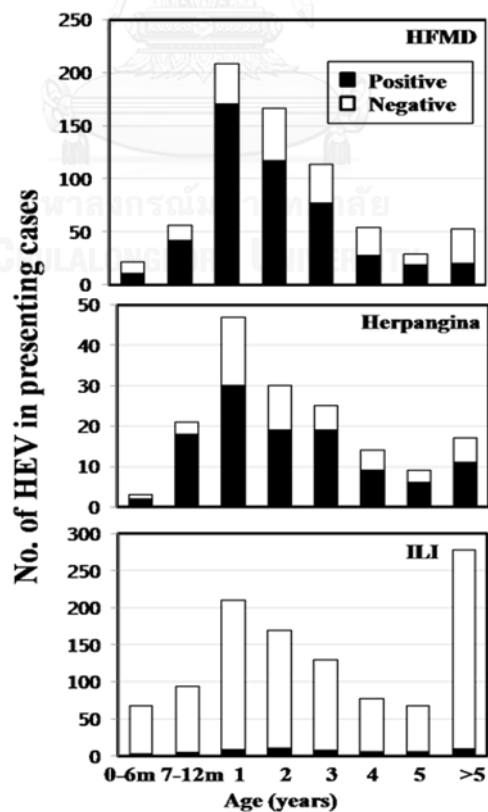
Figure 9. Age distribution of hEV infected subjects.

Enterovirus detection

Of the 704 HFMD suspected cases, 485 were virus-positive, indicating a prevalence of 68.9%. Of these, 402 samples typed positive for 15 different human enterovirus types. They are: 386 samples (54.8%) were identified as species A (CV-A4, A5, A6, A8, A10, A12, A16 and EV-A71), 15 samples (2.1%) as species B (CV-A9, CV-B1, B2, B4, B5 and Echo7), and one sample (0.1%) as species C (CV-A21). The remaining 83 samples initially tested positive (11.8%) were could not be typed. Of the 166 herpangina samples tested for hEV, 68 could be fully typed. Of those, 64 samples (94.1%) were identified as species A (CV-A4, A5, A6, A8, A10, A12 and A16), 4 samples (5.9%) as species B (CV-B2 and E25) and 46 samples (27.7%) as non-identified genotypes. hEV-species C was not detected in herpangina patients (Table 6).

Of the 1,094 influenza-like illness cases, a total of 58 samples were EV positive upon initial screening, indicating a prevalence of 5.3%. We found that 38 out of the 58 samples typed positive for 11 different human enterovirus types, including 26 samples (2.4%) identified as species A (CV-A5, A6, A8, A10 and EV-A71), 11 samples (1.0%) as species B (CV-A9, CV-B1, B2, Echo7 and Echo16), and one sample (0.09%) as species C (CV-A21). Twenty samples (1.8%) were of non-identified genotypes (Table 6). We note in table 7 that co-infection by respiratory viruses was found in 2 cases. First, a 5-year-old girl was infected by CV-A8/respiratory syncytial virus (RSV) and a 2-year-old girl was infected by CV-B2/seasonal influenza virus (H3).

The majority of enterovirus detected in three patient groups (HFMD, herpangina and ILI) belonged to human enterovirus species A. In the largest outbreak of HFMD in Thailand in 2012, CV-A6 was identified as the most prevalent virus followed by CV-A16 and EV-A71. A small outbreak of herpangina in the same year was caused in most patients

by infection with CV-A8 followed by CV-A6. Moreover, we found CV-A6 was the most predominant pathogen in ILI patients followed by CV-A8 (Figure 10).

Genotypes of human enterovirus species A present in all HFMD, herpangina and ILI groups were CV-A5, CV-A6, CV-A8 and CV-A10. Genotypes of hEV-species A detected in HFMD and herpangina were CV-A4, CV-A12 and CV-A16. Not a single herpangina patient was infected by EV-A71. Moreover, one of the herpangina patients demonstrated co-infection by CV-A8/ CV-A10 (data not show). The most common type of human enterovirus species B in HFMD and herpangina patients was CV-B2. Both CV-A9 and CV-B2 were mainly found in ILI patients. Types of hEV-species B only detected in HFMD and ILI patients were CV-A9, CV-B1 and Echo7. Furthermore, CBV4 and CBV5 were only detected in HFMD, Echo16 in ILI and Echo25 in herpangina. Moreover, CV-A21 (human enterovirus species C) was detected in both HFMD and ILI patients.

Table 6. Comparison between frequency of enterovirus genotypes in patients with hand, foot, mouth disease, herpangina and influenza-like illness, in 2012.

Virus	Influenza-like illness		Hand, foot, mouth disease		Herpangina	
	No.	%	No.	%	No.	%
Coxsackievirus A4	0	-	2	0.3	3	1.8
Coxsackievirus A5	1	0.1	2	0.3	1	0.6
Coxsackievirus A6	16	1.5	236	33.5	22	13.3
Coxsackievirus A8	6	0.5	7	1.0	32	19.3
Coxsackievirus A9	4	0.4	3	0.4	0	-
Coxsackievirus A10	1	0.1	8	1.1	3	1.8
Coxsackievirus A12	0	-	3	0.4	2	1.2
Coxsackievirus A16	0	-	66	9.4	1	0.6
Coxsackievirus A21	1	0.1	1	0.1	0	-
Coxsackievirus B1	1	0.1	2	0.3	0	-
Coxsackievirus B2	4	0.4	6	0.9	3	1.8
Coxsackievirus B4	0	-	2	0.3	0	-
Coxsackievirus B5	0	-	1	0.1	0	-
Echovirus 7	1	0.1	1	0.1	0	-
Echovirus 16	1	0.1	0	-	0	-
Echovirus 25	0	-	0	-	1	0.6
Enterovirus 71	2	0.2	62	8.8	0	-
Untypable	20	1.8	83	11.8	46	27.7
Negative	1036	94.7	219	31.1	52	31.3
Total	1094	100	704	100	166	100

Table 7. Details of patients with ILI positive for enterovirus during investigation in 2012.

Strain	Sampling Date	Gender	Age	Virus detected	Place	Accesion no.	Co-infection
Echo16_THA/B6018/2012	25/1	M	3 y	Echovirus 16	Bangkok	KF383381	-
Echo7_THA/C2541/2012	26/1	F	2 y	Echovirus 7	Khon Kaen	KF383383	-
CV-A6_THA/C2607/2012	16/2	F	7 y	Coxsackievirus A6	Khon Kaen	KF383347	-
CV-A6_THA/C2697/2012	14/3	F	3 y	Coxsackievirus A6	Khon Kaen	KF383348	-
CV-A6_THA/C2710/2012	22/3	M	10 y	Coxsackievirus A6	Khon Kaen	KF383349	-
CV-A6_THA/C2711/2012	22/3	M	10 y	Coxsackievirus A6	Khon Kaen	KF383350	-
CV-A6_THA/C2717/2012	22/3	M	5 y	Coxsackievirus A6	Khon Kaen	KF383351	-
CV-A6_THA/C2723/2012	29/3	F	4 y	Coxsackievirus A6	Khon Kaen	KF383352	-
CV-A6_THA/C2737/2012	29/3	F	2 y	Coxsackievirus A6	Khon Kaen	KF383353	-
CV-A6_THA/C2744/2012	3/4	M	7 m	Coxsackievirus A6	Khon Kaen	KF383354	-
CV-A6_THA/C2745/2012	3/4	F	1 y	Coxsackievirus A6	Khon Kaen	KF383355	-
CV-A6_THA/C2747/2012	3/4	F	3 y	Coxsackievirus A6	Khon Kaen	KF383356	-
CV-A6_THA/C2769/2012	11/4	F	10 m	Coxsackievirus A6	Khon Kaen	KF383357	-
CV-A6_THA/C2773/2012	11/4	M	5 m	Coxsackievirus A6	Khon Kaen	KF383358	-
CV-A6_THA/C2785/2012	25/4	F	13 d	Coxsackievirus A6	Khon Kaen	KF383359	-
CV-A6_THA/C2788/2012	25/4	F	2 y	Coxsackievirus A6	Khon Kaen	KF383360	-
CV-A6_THA/C2791/2012	25/4	F	1 y	Coxsackievirus A6	Khon Kaen	KF383361	-
EV-A71_THA/C2904/2012	7/6	M	1 y	Enterovirus 71	Khon Kaen	KF383376	-
CBV2_THA/C2909/2012	7/6	M	1 y	Coxsackievirus B2	Khon Kaen	KF383374	-
CAV8_THA/C2921/2012	21/6	M	1 y	Coxsackievirus A8	Khon Kaen	KF383367	-
CAV8_THA/C2928/2012	21/6	F	1 y	Coxsackievirus A8	Khon Kaen	KF383366	-
CBV2_THA/C2929/2012	21/6	M	2 y	Coxsackievirus B2	Khon Kaen	KF383373	-
CBV2_THA/C2938/2012	21/6	F	7 y	Coxsackievirus B2	Khon Kaen	KF383372	-
CAV8_THA/B6294/2012	27/6	M	8 y	Coxsackievirus A8	Bangkok	KF383362	-
CAV8_THA/B6328/2012	6/7	M	8 m	Coxsackievirus A8	Bangkok	KF383363	-
CAV5_THA/C3007/2012	10/7	M	3 y	Coxsackievirus A5	Khon Kaen	KF383380	-
CV-A6_THA/B6347/2012	14/7	M	2 y	Coxsackievirus A6	Bangkok	KF383346	-
CAV8_THA/C3021/2012	17/7	F	5 y	Coxsackievirus A8	Khon Kaen	KF383365	-
CBV1_THA/C3025/2012	17/7	F	4 y	Coxsackievirus B1	Khon Kaen	KF383382	-
CAV10_THA/B6446/2012	24/7	M	5 y	Coxsackievirus A10	Bangkok	KF383378	-
CAV8_THA/C3035/2012	24/7	F	5 y	Coxsackievirus A8	Khon Kaen	KF383364	RSV
EV-A71_THA/C3051/2012	24/7	M	2 y	Enterovirus 71	Khon Kaen	KF383377	-
CBV2_THA/B6463/2012	25/7	F	2 y	Coxsackievirus B2	Bangkok	KF383375	Seasonal influenza
CAV9_THA/C3055/2012	8/8	M	1 y	Coxsackievirus A9	Khon Kaen	KF383371	-
CAV9_THA/B6761/2012	26/8	F	3 y	Coxsackievirus A9	Bangkok	KF383368	-
CAV9_THA/B6763/2012	26/8	M	2 y	Coxsackievirus A9	Bangkok	KF383369	-
CAV9_THA/B6773/2012	28/8	M	2 y	Coxsackievirus A9	Bangkok	KF383370	-
CAV21_THA/B6982/2012	21/9	M	10 y	Coxsackievirus A21	Bangkok	KF383379	-
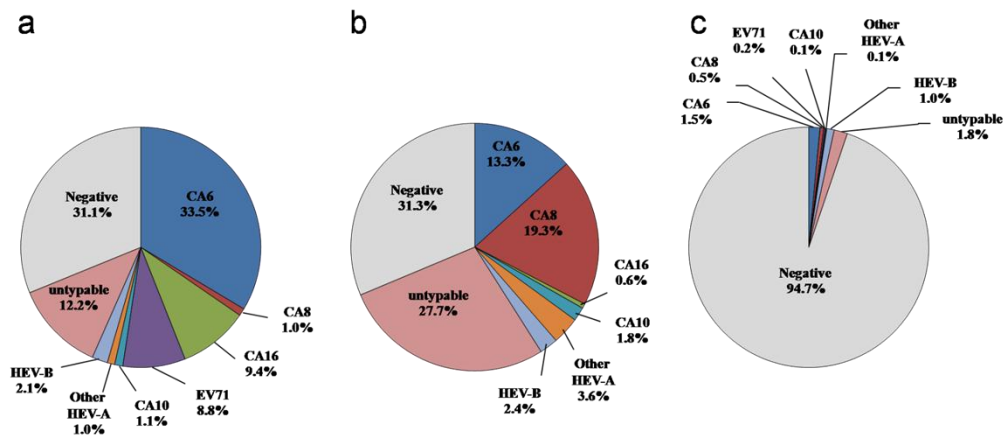
Figure 10. Distribution of human enteroviruses among hospitalized a) HFMD b) herpangina and c) influenza like illness cases in Thailand, 2012.

Phylogenetic and sequence analysis of enterovirus

Two phylogenetic trees were constructed comprising all human enterovirus species A, B and C strains from the present study along with their respective prototype strains with bootstrap values higher than 85% (Figures 11 and 12).

Phylogenetic analyses of the representative Thai CV-A6 strains are shown in figure 13a. Overall, the partial VP1 sequences were classified into ten clusters (A-J) with obvious geographical distribution patterns. All representatives CV-A6 strains identified in Thailand in 2012 segregated into cluster A and displayed a close genetic relationship with other strains from Shanghai, China 2012. They were also similar to strains from Finland and Spain in 2008 (cluster B and E) and Japan in 2011 (cluster C), which were associated with onychomadesis subsequent to HFMD (112, 113, 116, 117). The partial VP1 genes of all CV-A6 strains determined in this study exhibited 88.3% to 100% similarity. The Thai CV-A6 strains share 81.7-83.5% nucleotide identity with the prototype strain 'Gdula' and 55.7-66.7% with other members of enterovirus species A (Table 8).

The molecular epidemiology of the Thai EV-A71 strains was investigated applying a phylogenetic tree, with the representative strains selected from GenBank as known subgenotype references(A, B1-B5, C1-C5) (Figure 13b). The results have shown that all EV-A71 strains determined in this study were categorized into two genotypes, with the majority clustered with genotype B, and closely related to subgenotype B5. Interestingly, two EV-A71 strains from HFMD patients cluster with subgenotype C4a (CU562 and CU947), closely related to strains circulating in Ho Chi Minh City, Vietnam (2011) and China (2009-2010). Genetic differences within the VP1 gene of these two Thai C4a and B5 strains were 3.8% and 6.8%, respectively. Genetic differences within the VP1 gene between genotypes C4a and B5 were 14.6-16.5%. The bootstrap supported phylogenetic tree showed that EV-A71 (subgenotype B5) strains from Thailand clustered into 3 clades, namely, clades I, II and III. The results showed that most of them including two strains from ILI patients (from June to December 2012) belonged to clade I, closely related to strains circulating in Malaysia in 2008. Seven strains (the majority from January 2012) were classified into clade II, which is closely related to strains identified in Singapore in 2008. Finally, 12 strains (from June to December 2012) in clade III were closely related to strains detected in Taiwan (2007-2008).

As shown in figure 14a, the CV-A8 strains were assigned to six major clusters, denoted A, B, C, D, E, F and G based on criteria described above. The partial VP1 genes of all CV-A8 strains (n = 45) in this study exhibited 74.4% to 100% similarity. The Thai CV-A8 strains were divided into two distinct groups; the majority of which grouped with cluster A and four strains grouped with cluster C. The majority of the CV-A8 strains showed a close genetic relationship with 2007-2009 Indian strains associated with non-polio acute flaccid paralysis (122) and a minority of strains showed a close genetic relationship with the 2013 Japanese strains associated with herpangina, 2012 Chinese strains associated

with HFMD and 2008 Chinese strains associated with acute respiratory tract infection (123).

The phylogenetic relationship between CV-B2 strains was also assessed based on partial VP1 regions (Figure 14b). The nucleotide sequence identities within all Thai CV-B2 strains (n = 13) amounted to approximately 94.8% to 100% in partial VP1 regions. Comparison with the prototype strain (AF081485/Ohio strain) showed that the Thai CV-B2 strains in 2012 had less than 85% nucleotide sequence identities. CV-B2 strains were assigned to seven major clusters based on the criteria described above (A-G). All CV-B2 strains (n = 13) were grouped in cluster A, which is most closely related to strains in China (1994-2003). Cluster B comprising the strains from Russia, Bangladesh, Australia, India and United Kingdom: cluster C from USA: cluster D from China: cluster E from Denmark, Finland, Germany and France: cluster F from Denmark, Finland and Germany and cluster G from Japan. No significant clustered relationship was observed based on clinical syndromes and temporal specific distribution. In addition, the aligned amino acid positions of the BC-surface loops of CV-A6 and CV-A8 are shown in figure 15.
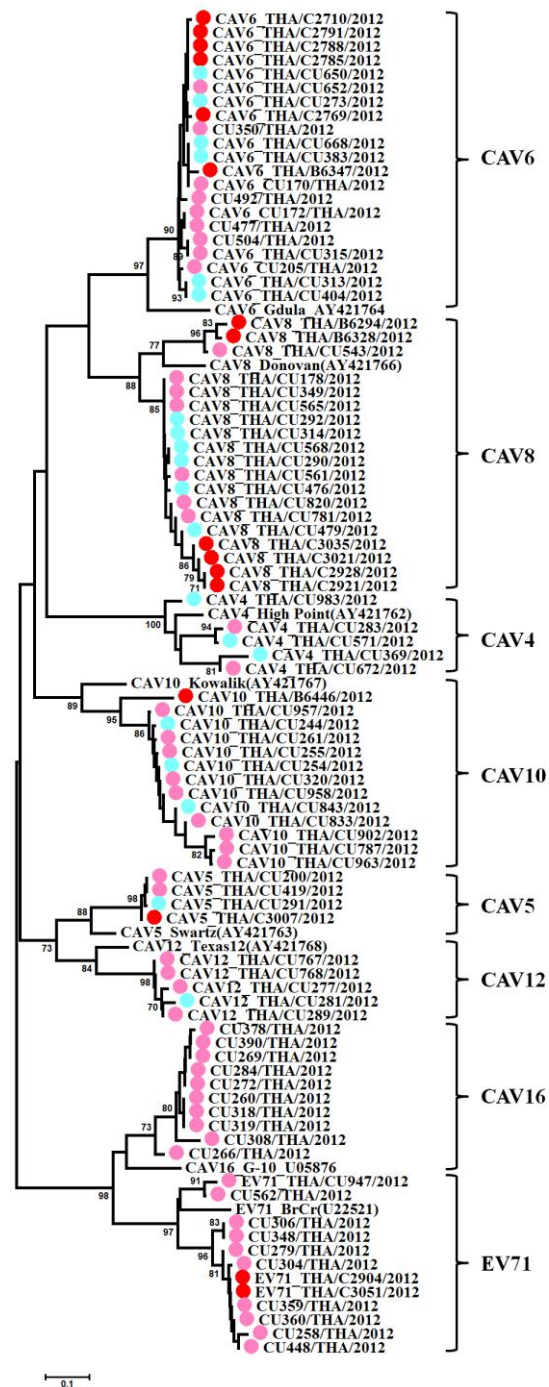
Figure 11. Phylogeny of human enterovirus specie A based on the partial VP1 region constructed by the neighbor-joining (NJ) algorithm implemented in MEGA version 5.0 using the Kimura two-parameter substitution model and 1000 bootstrap pseudo-replicates. Strains from HFMD patients are indicated in pink, herpangina patients in blue, and influenza like illness patients in red.
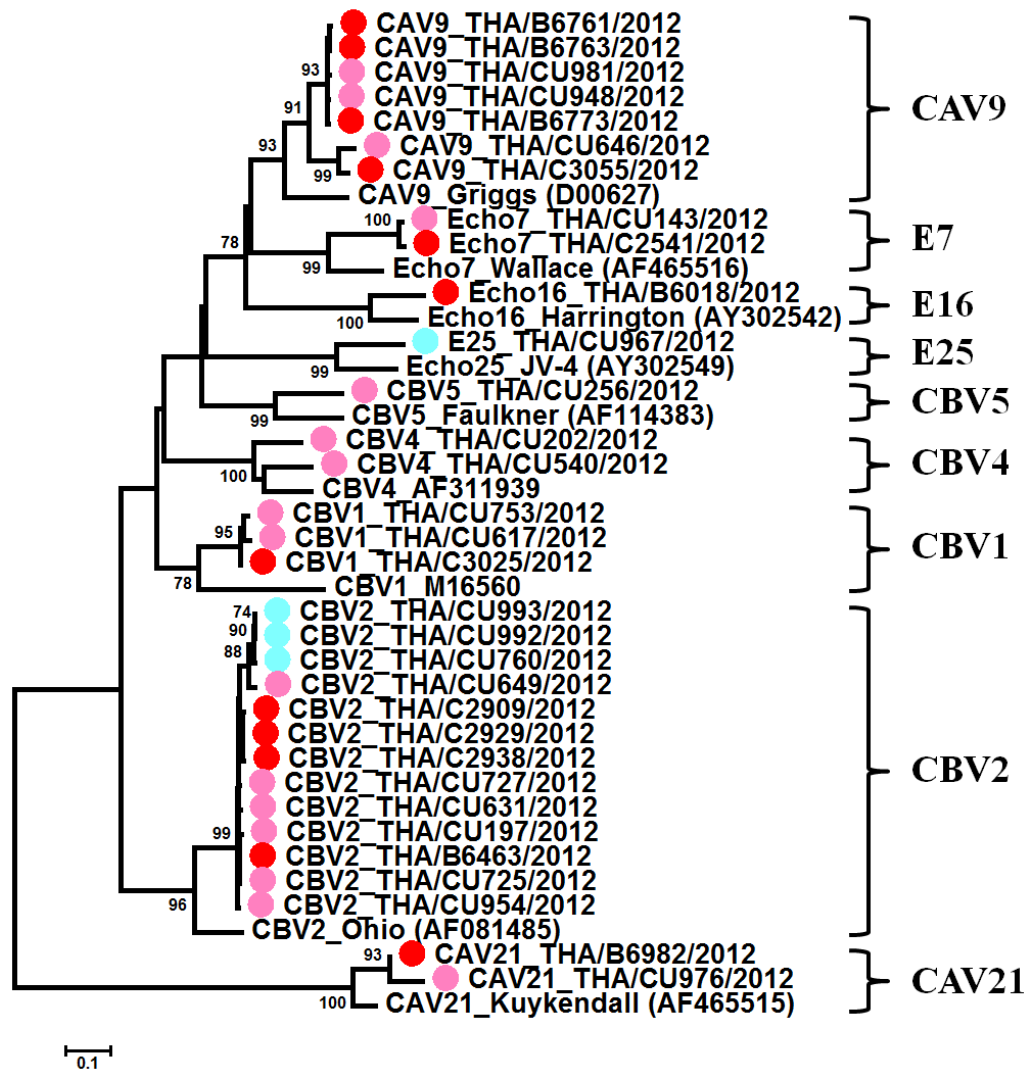
Figure 12. Phylogeny of human enterovirus specie B and C based on the partial VP1 region constructed by the neighbor-joining (NJ) algorithm implemented in MEGA version 5.0 using the Kimura two-parameter substitution model and 1000 bootstrap pseudo-replicates. Strains from HFMD patients are indicated in pink, herpangina patients in blue, and influenza like illness patients in red.

(a)
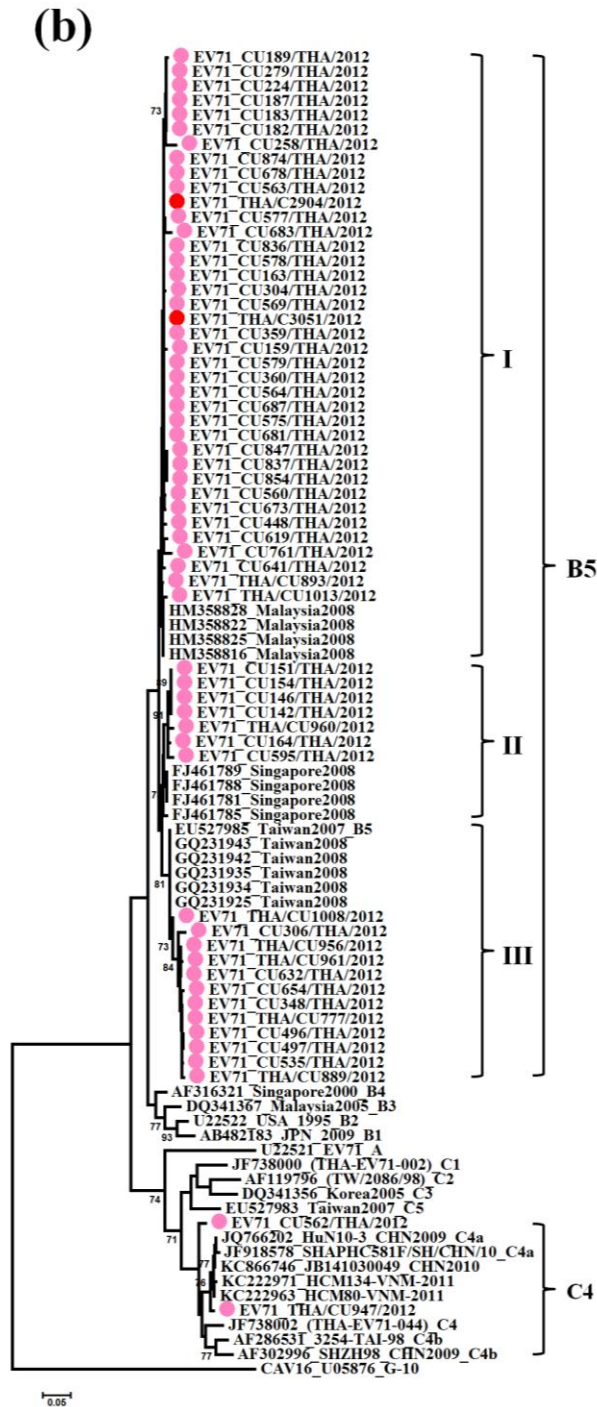
Figure 13. Phylogeny of a) CV-A6 and b) EV-A71 based on the partial VP1 region constructed by the neighbor-joining (NJ) algorithm implemented in MEGA version 5.0 using the Kimura two-parameter substitution model and 1000 bootstrap pseudo-replicates. Strains from HFMD patients are indicated in pink, herpangina patients in blue, and influenza like illness patients in red.

(a)
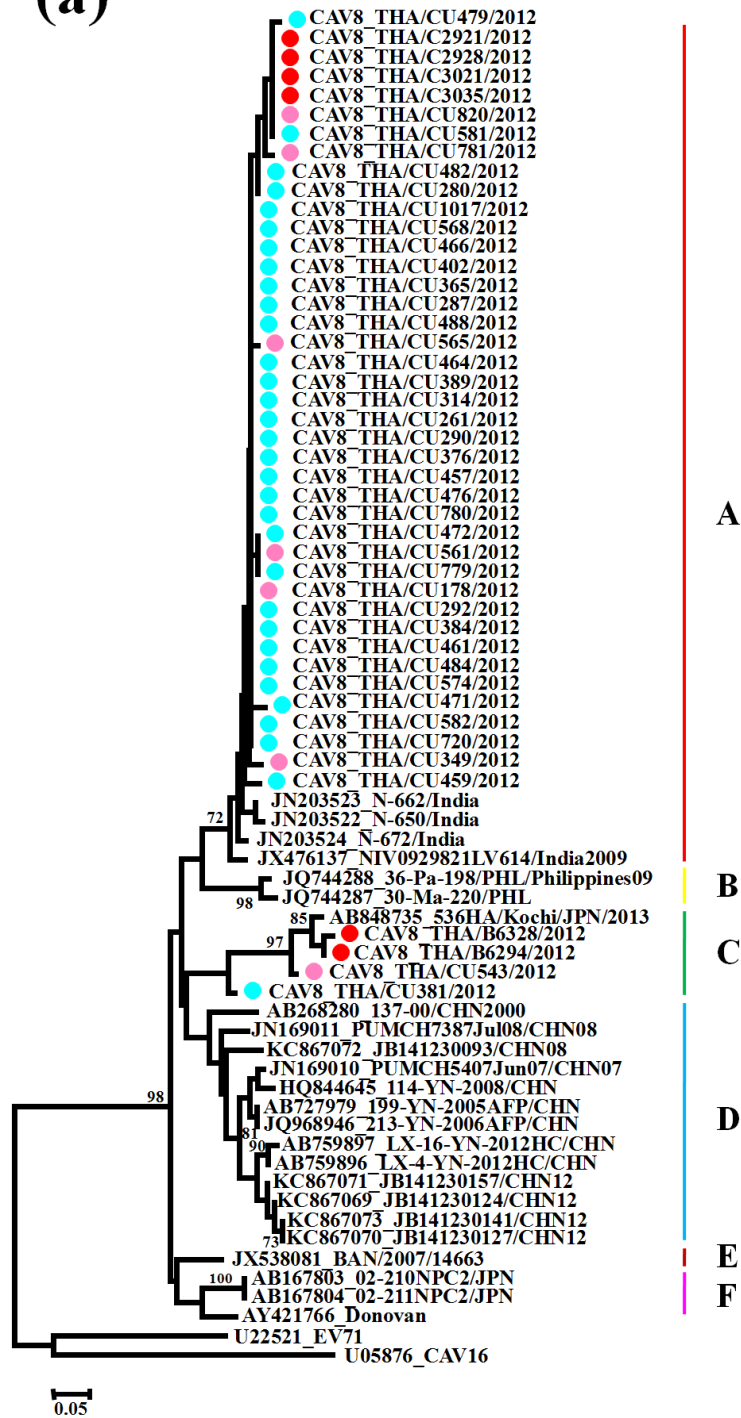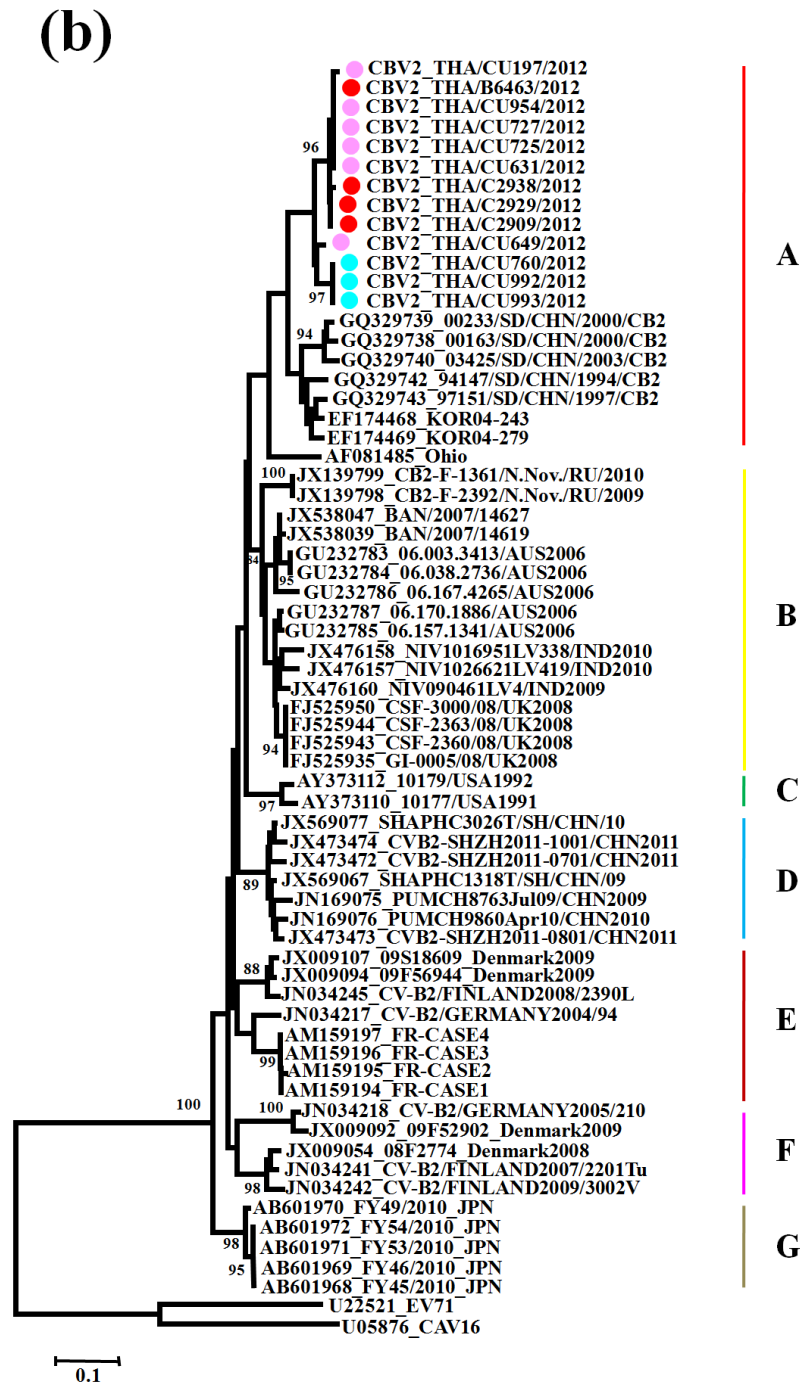
Figure 14. Phylogeny of a) CV-A8 and b) CV-B2 based on the partial VP1 region constructed by the neighbor-joining (NJ) algorithm implemented in MEGA version 5.0 using the Kimura two-parameter substitution model and 1000 bootstrap pseudo-replicates.

Strains from HFMD patients are indicated in pink, herpangina patients in blue, and influenza like illness patients in red.

Table 8. Nucleotide identity matrix obtained for the alignment of the partial VP1 region of CV-A6 strains from Thailand and reference strains of CV-A6 and other members of enterovirus species A.

Sequence	KF383346	KF383348	KF383351	KF383355	KF383358	KF383361	Gdula	Donovan	BrCr	Kowalic
KF383348	94.3									
KF383351	94.8	99.4								
KF383355	94.3	99.4	99.4							
KF383358	94.5	99.7	99.7	99.7						
KF383361	94.5	99.7	99.7	99.7	100.0					
Gdula	83.5	82.2	82.2	81.7	81.9	81.9				
Donovan	66.4	66.7	66.7	66.7	66.4	66.4	68.8			
BrCr	57.8	56.0	55.7	55.7	55.7	55.7	57.2	59.0		
Kowalic	62.8	63.6	63.9	63.9	63.9	63.9	64.1	68.5	60.6	
Swartz	65.7	66.4	66.4	65.9	66.2	66.2	65.2	62.8	56.5	67.5
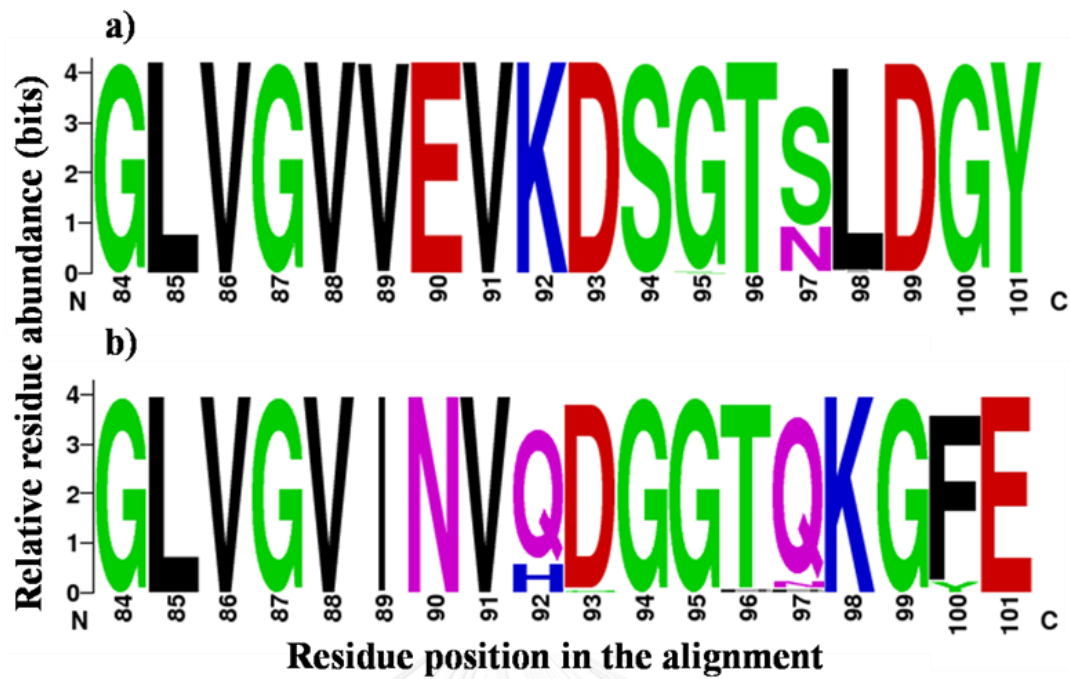
Figure 15. Genome signatures in amino acid residues at the alignment position of BC surface-exposed loop of a) CV-A6 and b) CV-A8 in Thailand in 2012.

The graphical presentation was performed using WebLogo. The height of symbol indicates the relative frequency of the corresponding amino acid at that position. Residue positions are given based on the nucleotide positions of the Gdula strain (AY421764) and the Donovan strain (AY421766).

Part 2: Development of single step multiplex real-time RT-PCR for screening (panEV) and typing (EV-A71/CV-A6/CV-A16) of Enterovirus

HFMD is one of the most infectious diseases commonly found in infant and children aged <5 years. Series of molecular surveillance study have been reported that large-outbreaks of HFMD were frequently found in Western Pacific and Southeast Asia countries with endemic findings in America and Europe countries. Since the discovery of a large outbreak of HFMD associated with CV-A6 in Finland in 2008, the virus has spread worldwide including China, Japan, Spain, Taiwan, the United State and Scotland (115-118, 124, 125). Unlike EV-A71 and CV-A16, infection with CV-A6 often causes uncommon symptoms in HFMD patients including cutaneous eruption, perioral lesions, large vesicles affecting the palms, soles, face, trunk and buttock, and also onychomadesis (nail shedding). In the past few years, HFMD has been recognized as endemic disease in Thailand and the dominant causative pathogens change every endemic season. In recent, a large CV-A6 outbreak was documented in 2012 and eclipsing EV-A71 and CV-A16 as causative agents (126). As the outbreak of CV-A6 increases, there is a requirement for early and rapid diagnostic techniques to differentiate EV-A71, CV-A16 and CV-A6, prevent the spread of these viruses and for initial treatment as soon as possible.

As these enteroviruses usually cause very similar clinical symptoms, there is a requirement for accurate diagnostic techniques to differentiate EV-A71, CV-A16 and CV-A6, prevent the spread of these viruses and for initial treatment as soon as possible. Monospecific PCR assays have been described to identify EV-A71, CV-A16 and CV-A6 of each target and are therefore expensive and resource intensive (119, 120, 127, 128). Multiplex PCR assays have a significant advantage for clinical diagnosis, as several viruses can be detected in a single reaction mixture, and also been reported for

differentiating EV-A71 and CV-A16. However, the viral targets commonly can be distinguished after electrophoresis by PCR product size or hybridization with probes after completion of the PCR reaction (129). The multiplex real-time RT-PCR (rRT-PCR) is a powerful tool used for improving the sensitivity of hEV detection and one-tube assay for multi-pathogen detection. Many assays based on multiplex rRT-PCR had been applied to the differential detection of EV-A71 and CV-A16, but none of them were targeted CV-A6 (130-133). Recently, another rRT-PCR assay was also developed for the specific detection of CV-A6 (134). However, this assay is single rRT-PCR methods that detect VP1 genes of CV-A6.

In this study, a rapid and convenient one-step multiplex real-time RT-PCR using TaqMan probes for simultaneous detection and serotypes differentiation of circulating EV in Thailand was developed and evaluated. Consequently, two separate assays were developed for screening EV and concurrently identifying their serotypes. The first assay consists of primers and probes corresponding to the viral protein gene (VP1) of EV-A71, CV-A16 and CV-A6 in order to identify the three most prominent genotypes of enterovirus capable of infecting humans. The other assay employed primers and probes target to the 5´UTR of almost all EVs specifically and GAPDH gene of host cells for screening of enterovirus and serving as an internal control, respectively.

Materials and Methods

Ethical considerations

The research protocol was approved by the institutional review board of the Ethical Committee of the Faculty of Medicine, Chulalongkorn University, Thailand (approval number IRB286/58). Patient identifiers including personal information (name, address) and hospitalization number were removed from these samples to protect patient confidentiality and neither did they appear in any part of document in this study. Permission for specimen utilization had been granted by the Director of King Chulalongkorn Memorial hospital. IRB waived the need for consent because the samples were de-identified.

Virus and samples

The specificity of the multiplex rRT-PCR assay was evaluated by cross reaction tests with known viral isolates and different panels of sequence confirmed known clinical samples with other genotypes of EV including: CV-A4 ($N = 4$), CV-A5 ($N = 2$), CV-A8 ($N = 7$), CV-A9 ($N = 3$), CV-A10 ($N = 10$), CV-A12 ($N = 2$), CV-A21 ($N = 1$), echovirus 25 ($N = 1$), CV-B1 ($N = 2$), CV-B2 ($N = 6$), CV-B4 ($N = 2$) and CV-B5 ($N = 1$). The assays were also evaluated against other viral pathogens as reference material including: influenza A (H3N2; $N = 5$ and H1N1; $N = 5$), influenza B ($N = 5$), respiratory syncytial virus (RSV) ($N = 5$), adenovirus ($N = 4$), rotavirus ($N = 5$), parechovirus ($N = 5$), and the non-specific pathogen specimens ($N = 100$), which were negative for pan-enterovirus, EV-A71, CV-A16 and CV-A6.

A total of 1,312 clinical specimens (stool/rectal swab, throat swab, vesicle fluid, serum, CSF) collected from HFMD suspected patients in Thailand during the period from

2012-2016 were utilized to test the multiplex real-time PCR. The samples were stored at -70 ⁰C until testing.

Design of primers and probes

In this study, a rapid and sensitive single-step multiplex real-time RT-PCR was developed and evaluated by using primers and TaqMan probes in two assays. The first assay comprises of primers and probes specific to VP1 genes of EV-A71, CV-A16 and CV-A6 in order to identify the three most predominant genotypes of enterovirus that cause of HFMD in Thailand. Primers and probes for differentiation between EV-A71, CV-A16 and CV-A6 were designed from conserved nucleotide sequences specific for the VP1 gene of EV-A71 for EV-A71 detection, specific for the VP1 gene of CV-A16 for CV-A16 detection and specific for the VP1 gene of CV-A6 for CV-A6 detection. For the other assays aimed at screening other HEV, the specific regions of 5´UTR hEV were chosen for designation of primers and probes to identify other hEV. Primers and probes for GAPDH detection were designed previously to correspond to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) nucleotide sequence.

In order to design primers and probes for the screening and typing of HEV, the nucleotide sequences of the VP1 genes of EV-A71, CV-A16 and CV-A6 and 5´UTR of other HEV available from the Genbank database were aligned using the CLUSTALX multiple alignment program. Both of primers and probes were analyzed using the primer design software (OLIGOS, Version 9.1 by Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland) and the software Primer Express 5.0 (Applied Biosystems) to ensure that they could be combined in a multiplex format and subjected to identical PCR conditions. The probes were labeled with minor groove binding black hole quencher (BHQ) at the 3´ end, and three different fluorescent reporter dyes (FAM, HEX and ROX) at the 5´ end, so that multiple pathogens of EV (EV-A71, CV-A16, CV-A6) could been

identified simultaneously in one-tube assay. All data related to the primers and probes are shown in table 9.

Nucleic acid extraction and reverse transcription

Viral genomic RNA was extracted from cell cultures infected with EV-A71 or clinical samples using a Viral Nucleic Acid Extraction Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's instructions and the extracted solution was stored at -70°C.

Reverse transcription was performed as follows: 5 µl of the extracted viral genomic RNA was mixed with 0.5 µg random primer, denatured at 70 °C for 5 min, and immediately cooled on ice for a final volume of 20 µl reaction mixture. For complementary DNA (cDNA) synthesis, 7.3 µl of nuclease-free water, 4 µl of 5x reaction buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTP Mix, 20 U of RNase inhibitor and 1 µl of ImProm-II^{TM} (Promega) reverse transcriptase were mixed.

Table 9. Multiplex PCR primers and TaqMan probes (135).

Target region	Primer/Probe	Sequence (5′-3′)	Position	Strand	Reference
GAPDH	GAPDH-F85	GTGAAGGTCGGAGTCAACGG	85 – 104	Sense	
	GAPDH-P121	HEX-CGCCTGGTCACCAGGGCTGC-BHQ1	121 – 140	Sense	(135)
	GAPDH-R191	TCAATGAAGGGGTCATTGATGG	191 – 169	Antisense	
5′UTR	pan-F453	TCCTCCGGCCCCTGAATG	453 - 470	Sense	-
	pan-P590	FAM-GCAGCGGAACCGACTACTTT-BHQ1	537 - 556	Sense	
	pan-R1	ATTGTCACCATAAGCAGCCA	587 - 606	Antisense	
VP1	EV-A71_F3079	ATGATGGGYACRTTCTCRGTG	3079 - 3099	Sense	-
EV-A71	EV-A71_P3153	FAM-GAGRATGAAGCAYGTCAGGGCRTGG -BHQ1	3153 - 3177	Sense	
	EV-A71_R3326	TTG CCC ACG TAA ATG GCC	3309 - 3326	Antisense	
VP1	CV-A16_F2830	ATGCGCTTTGATGCTGAATTCA	2830 - 2851	Sense	-
CV-A16	CV-A16_P2913	HEX-CCC RCC AGG GGC TCC RAA ACC-BHQ1	2913 - 2933	Sense	
	CV-A16_R3032	GACATGAARGGGACTGACACTTG	3010 - 3032	Antisense	
VP1	CVA6-F2586	GAGCRAGTTCYAATGCTAGTG	2586 - 2606	Sense	-
CV-A6	CVA6-P2642	ROX-CGAAACGGGGTYAATGARGCGAGTGTGG-BHQ2	2642 - 2669	Sense	
	CVA6-R2802	GAYAGCTCTAGTTTGCGCCG	2783 - 2802	Antisense	

Construction of plasmids as a positive control

Standard plasmids containing specific viral target fragments were used as template for optimization of each of the assays for screening pan-enterovirus and typing (EV-A71/CV-A6/CV-A16) of enteroviruses. Plasmid DNAs were constructed by insertion of the VP1 gene of EV-A71 (nt 832-1079; EV-A71B5_THA/CU1048/2012 and EV-A71C4_THA/CU562/2012), VP1 gene of CV-A6 (nt 146-359; CV-A6_THA/CU788/2012, KF661213), VP1 gene of CV-A16 (nt 2825-3027; CV-A16_THA/CU1012/2012, KF661132), GAPDH gene (nt 85-191), 5´UTR region (nt 453-R1; EV-A71B5_THA/CU1048/2012) into the pGEM-T Easy Vector (Promega, Madison, WI) by TA-cloning strategy. The resulting plasmid constructs were confirmed by PCR and DNA sequencing (First BASE Laboratories, Selangor Darul Ehsan, Malaysia).

RNA standard for sensitivity test

RNA standard for the sensitivity test was generated using the RiboMAX^{TM} Large Scale RNA Production System-T7 (Promega, Madison, WI) according to the manufacturer's instructions. After DNase digestion to remove residues of RT-PCR products, the transcribed RNA was purified twice using a Viral Nucleic Acid Extraction Kit according to the manufacturer's recommendation (RBC Bioscience, Taipei, Taiwan). The concentration of the transcribed RNAs was calculated by measuring absorbance at 260 nm. The RNAs standards of serial diluted concentrations (10^8 - 10 copies/µl) were used to evaluate the sensitivities and detection limits of the one-step multiplex rRT-PCR assay.

One-step multiplex rRT-PCR assay

To use differentially for each pathogen, five monoplex real-time RT-PCR assays were initially performed and followed by multiplexing. All real-time RT-PCR reactions were carried out in the LightCycler$^{®}$ Nano instrument capable of simultaneous detection of up

to three different fluorescence dyes. Thus, two multiplex assays were created and run separate in each of two tubes: screening (pan-EV, GAPDH) were tested in reaction 1; typing (EV-A71, CV-A16, CV-A6) were tested in reaction 2. The reaction mixture of a monoplex real-time RT-PCR in a total volume of 10 µl contained: RNA template, one-step RT-PCR reaction mix including RNase Inhibitor, Mg^{2+}, primers and probe. The reaction mixture of a multiplex real-time RT-PCR in a total volume of 20 µl contained: RNA template, one-step RT-PCR reaction mix including RNase Inhibitor, Mg^{2+}, two or three pairs of primers and two or three probes. Cycling was performed as follows: 30 min at 42ºC to activate RT, followed by initial denaturation for 3 min at 95ºC with a subsequent 40-45 cycles of amplification (denaturation at 95ºC for 15 s and annealing as well as extension at 55-62ºC for 20 s). Multiple fluorescent signals were obtained once per cycle upon completion of the extension step with detectors corresponding to FAM (518 nm), HEX (553 nm) and ROX (607 nm). Fluorescent signals were collected during annealing and extension steps and amplification data were analyzed using the Light Cycler® Nano SW1.1 software according to the manufacturer's instructions. To obtain the highest fluorescence signals and lowest threshold cycle (Ct), the final concentration of primers, probes and Mg^{2+} were optimized.

Statistical analysis

Reproducibility was computed using the Ct values, standard deviations, coefficients of variation of the standard curves and confidence intervals (CI) separately for each RNA dilution. Sensitivity, specificity, and positive and negative predictive values of the two assays were calculated from two-by-two contingency tables for each test with their 95 % confidence intervals (CIs). Data analysis was done using the Statistical Package for Social Sciences version 17.0 (SPSS Inc., Chicago, IL).

Results

Optimization of mono- and multiplex real-time RT-PCR conditions

To obtimize monoplex and multiplex real-time RT-PCR panels, several factors including primers and probes concentration, additional Mg^{2+} concentration and thermocycling condition were subjected by using the transcribed RNAs as positive control. A monoplex real-time RT-PCR assay and a multiplex assay for simultaneous detection of pan-enterovirus, EV-A71, CV-A16 and CV-A6 were carried out in use of SensiFAST Probe No-ROX One-Step kit (Bioline, Australia). The optimized reaction system of each monoplex real-time RT-PCR assay of 10 μl total volume consisted of 5 μl of 2x PCR buffer, 0.3 μM of forward and reverse primer, 0.1 μM of each probe, 0.1 μl of Reverse transcriptase and 0.2 μl of RNase inhibitor. The optimized reaction system of each tube reaction of multiplex real-time RT-PCR of 20 μl total volume consisted of 10 μl of 2x PCR buffer, 0.3 μM of forward and reverse primer, 0.075 μM of each probe, 0.1 μl of Reverse transcriptase and 0.2 μl of RNase inhibitor. The optimal reaction conditions of a one-step assay were used as follows: 42 °C for 30 min, 95 °C for 3 min, then 45 cycles of 15 s at 95 °C and 20 s at 60 °C. Fluorescence data was collected at 60 °C.

Interpretation of multiplex real-time RT-PCR detection

In first assay aimed at screening of panEV, the results were obtained by using multiple Taqman probes label with the FAM and HEX fluorescent signals corresponding to 5′UTR of panEV and GAPDH gene, respectively. The fluorescent signals obtained from the screening assay can be interpreted as shown in figure 16a. The results obtained from each detection channel revealed a clear signal without any unanticipated noises. PanEV yielded an amplification signal in the FAM channel and the internal control gene yielded detected in the HEX channel.

In the typing assay, the presence of EV-A71, CV-A16 and CV-A6 genotypes was detected by using TaqMan probes labeled with the FAM, HEX and ROX fluorescent dyes, respectively (figure 16b). Samples yielding a positive FAM signal have been interpreted as EV-A71 genotype, samples yielding a positive HEX signal as CV-A16 genotype and samples yielding a positive ROX signal as CV-A6 genotype.

Specificities and qualitative ability of assays

To estimate the specificity of the multiplex real-time RT-PCR assay, the cross-reactivity of the primers and probes was tested against other RNA viruses including influenza A virus (H3N2 and H1N1), influenza B virus, respiratory syncytial virus (RSV), adenovirus, rotavirus, human parechovirus, human rhinovirus and the unknown specimens, which were negative for enteroviruses. The results did not show any cross amplification signal when testing against other RNA viruses (data not shown).

Moreover, other genotypes of enterovirus including CV-A4, CV-A5, CV-A8, CV-A9, CV-A10, CV-A12, CV-A21, CV-B1, CV-B2, CV-B4, CV-B5 and echovirus 25 were also included in order to validate the assays in terms of specificity. The results show yielded only a FAM fluorescent signal of 5′UTR indicating panenterovirus but not typing EV-A71, CV-A16 and CV-A6 genotypes (Table 10), indicative of absence of cross-reactivity to other genotypes of enterovirus. These findings indicate high specificity of the primers and probes used in the multiplex real-time RT-PCR assay.
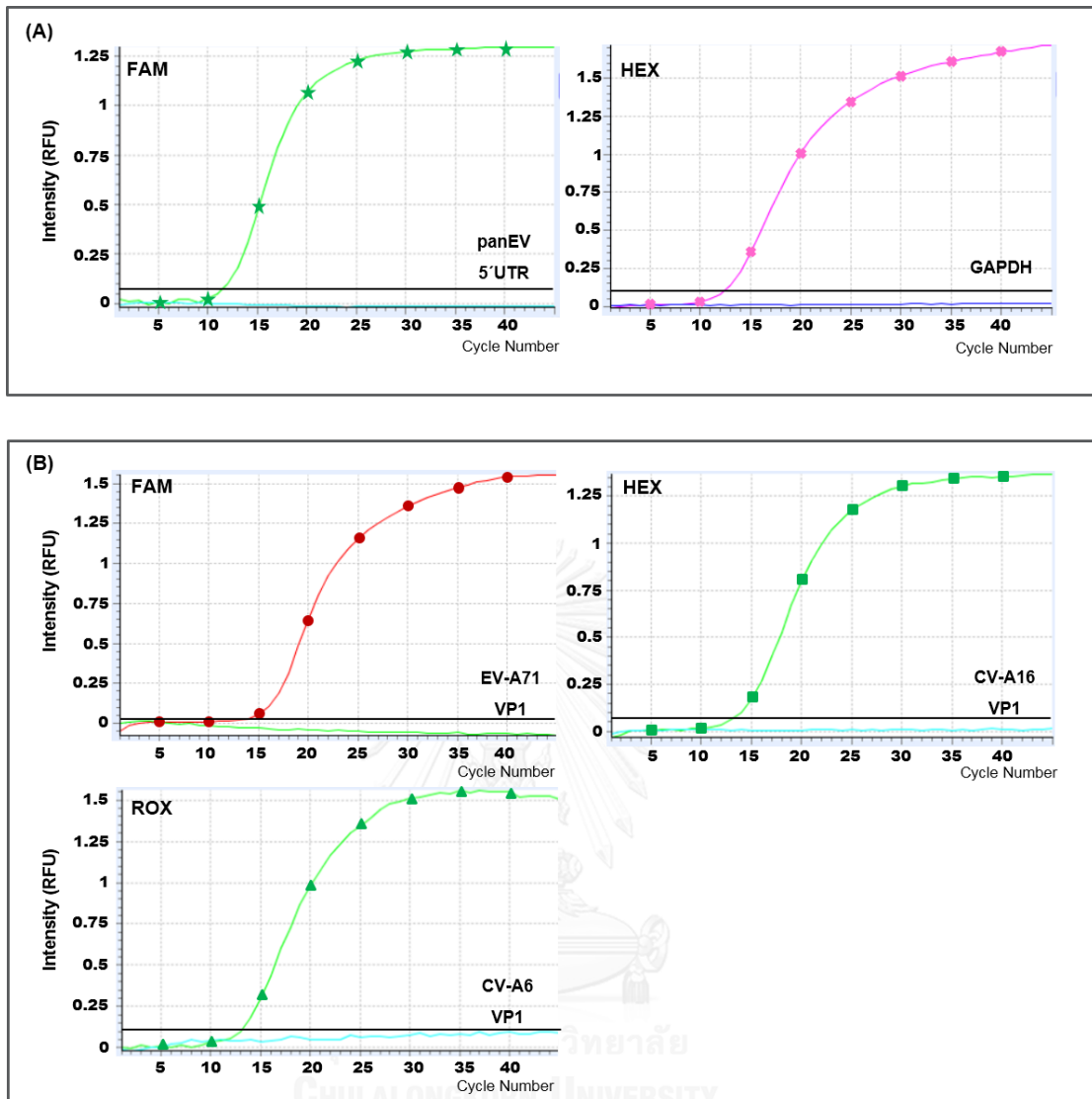
Figure 16. Interpretation of panEV (A) and hEV (B) detection by multiplex real-time RT-PCR assay. The panEV screening (A) shows fluorescent signals of FAM and HEX corresponded to 5UTR of panEV and GAPDH, respectively. The typing of hEV detection (B) shows fluorescent signals of FAM, HEX and ROX corresponded to EV-A71, CV-A16 and CV-A6, respectively.

Table 10. Specificity test of multiplex real-time RT-PCR.

Virus	Code	Fluorescent signal			
		FAM (panEV)	FAM (EV-A71)	HEX (CV-A16)	ROX (CV-A6)
Influenza A virus (H3N2)	B7858	-	-	-	-
Influenza A virus (H3N2)	B7885	-	-	-	-
Influenza A virus (H3N2)	B7914	-	-	-	-
Influenza A virus (H3N2)	B7919	-	-	-	-
Influenza A virus (H3N2)	B7922	-	-	-	-
Influenza A virus (H1N1)	B7256	-	-	-	-
Influenza A virus (H1N1)	B7491	-	-	-	-
Influenza A virus (H1N1)	B7652	-	-	-	-
Influenza A virus (H1N1)	B7678	-	-	-	-
Influenza A virus (H1N1)	B7731	-	-	-	-
Influenza B virus	B7283	-	-	-	-
Influenza B virus	B7284	-	-	-	-
Influenza B virus	B7294	-	-	-	-
Influenza B virus	B7508	-	-	-	-
Influenza B virus	B7636	-	-	-	-
Respiratory syncytial virus (RSV)	CU99	-	-	-	-
Respiratory syncytial virus (RSV)	CU161	-	-	-	-
Respiratory syncytial virus (RSV)	H3090	-	-	-	-
Respiratory syncytial virus (RSV)	CU205	-	-	-	-
Respiratory syncytial virus (RSV)	CU215	-	-	-	-
Adenovirus	B1435	-	-	-	-
Adenovirus	B1500	-	-	-	-
Adenovirus	B1522	-	-	-	-
Adenovirus	B1535	-	-	-	-
Rotavirus	B1755	-	-	-	-
Rotavirus	B1756	-	-	-	-
Rotavirus	B1757	-	-	-	-
Rotavirus	B1760	-	-	-	-
Rotavirus	B1762	-	-	-	-

Human parechovirus	B1304	-	-	-	-
Human parechovirus	B1415	-	-	-	-
Human parechovirus	B1610	-	-	-	-
Human parechovirus	B1630	-	-	-	-
Human parechovirus	B1652	-	-	-	-
Coxsackievirus A4 (KF661251)	CU283	+	-	-	-
Coxsackievirus A4 (KF661252)	CU369	+	-	-	-
Coxsackievirus A4 (KF661253)	CU571	+	-	-	-
Coxsackievirus A4 (KF661254)	CU672	+	-	-	-
Coxsackievirus A4 (KF661255)	CU983	+	-	-	-
Coxsackievirus A5 (KF661151)	CU200	+	-	-	-
Coxsackievirus A5 (KF661152)	CU291	+	-	-	-
Coxsackievirus A8 (KF661169)	CU178	+	-	-	-
Coxsackievirus A8 (KF661170)	CU261	+	-	-	-
Coxsackievirus A8 (KF661175)	CU314	+	-	-	-
Coxsackievirus A8 (KF661176)	CU349	+	-	-	-
Coxsackievirus A8 (KF661179)	CU381	+	-	-	-
Coxsackievirus A8 (KF661181)	CU389	+	-	-	-
Coxsackievirus A8 (KF661183)	CU457	+	-	-	-
Coxsackievirus A9 (KF661148)	CU646	+	-	-	-
Coxsackievirus A9 (KF661149)	CU948	+	-	-	-
Coxsackievirus A9 (KF661150)	CU981	+	-	-	-
Coxsackievirus A10 (KF661155)	CU254	+	-	-	-
Coxsackievirus A10 (KF661156)	CU255	+	-	-	-
Coxsackievirus A10 (KF661158)	CU320	+	-	-	-
Coxsackievirus A10 (KF661159)	CU833	+	-	-	-
Coxsackievirus A10 (KF661160)	CU843	+	-	-	-
Coxsackievirus A10 (KF661161)	CU902	+	-	-	-
Coxsackievirus A10 (KF661162)	CU957	+	-	-	-
Coxsackievirus A10 (KF661163)	CU958	+	-	-	-
Coxsackievirus A10 (KF661164)	CU787	+	-	-	-
Coxsackievirus A10 (KF661165)	CU963	+	-	-	-
Coxsackievirus A12 (KF661144)	CU281	+	-	-	-

Coxsackievirus A12 (KF661146)	CU767	+	-	-	-
Coxsackievirus A21 (KF661250)	CU976	+	-	-	-
Coxsackievirus B1 (KF661141)	CU617	+	-	-	-
Coxsackievirus B1 (KF661142)	CU753	+	-	-	-
Coxsackievirus B2 (KF661133)	CU760	+	-	-	-
Coxsackievirus B2 (KF661135)	CU197	+	-	-	-
Coxsackievirus B2 (KF661137)	CU727	+	-	-	-
Coxsackievirus B2 (KF661138)	CU954	+	-	-	-
Coxsackievirus B2 (KF661139)	CU992	+	-	-	-
Coxsackievirus B2 (KF661140)	CU993	+	-	-	-
Coxsackievirus B4 (KF661166)	CU202	+	-	-	-
Coxsackievirus B4 (KF661167)	CU540	+	-	-	-
Coxsackievirus B5 (KF661168)	CU256	+	-	-	-
Echovirus 25 (KF661108)	CU967	+	-	-	-

Sensitivities and detection limits of assays

To examine the actual detection limit of the multiplex real-time PCR assays, *in vitro* RNA transcripts of EV-A71, CV-A6, CV-A16 and panenterovirus were carried out with T7 RNA polymerase. A ten-fold serial dilution of each EVs RNA standard was then tested in the multiplex real-time PCR assays. The actual detection limit of the assay was determined to be 10 copies of synthetic RNA per reaction for EV-A71, CV-A6, CV-A16 and panenterovirus (Figure 17). The standard curves were generated using Ct values of each multiplex real-time RT-PCR assay and showed a linear relationship between the log of the viral titer and the Ct values for all assays (Figure 18). The multiplex rRT-PCR assays were linear with a dynamic range of detection between 10^8 and 10 copies/µl in 45 cycles for the standards of EV-A71 (correlation coefficient $R^2 = 0.9982$; slope = -3.57), CV-A6 ($R^2 = 0.9974$; slope = -3.53), CV-A16 ($R^2 = 0.9863$; slope = -3.26) and panenterovirus ($R^2 = 0.9740$; slope = -3.68). The amplification efficiency of developed multiplex rRT-PCR

assays for EV-A71, CV-A6, CV-A16 and panenterovirus was 90.6%, 92.0%, 100% and

87.0% respectively.
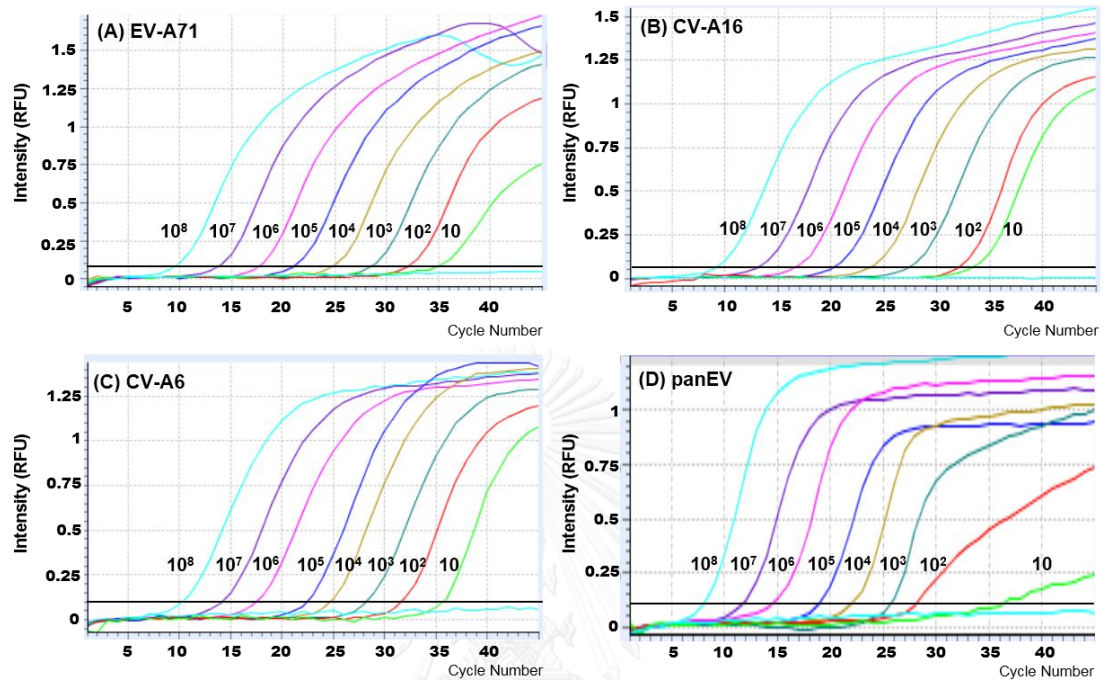
Figure 17. Sensitivity of the multiplex real-time RT-PCR assay for EV-A71 (A), CV-A16

(B), CV-A6 (C) and panEV (D), obtained from the amplification of 10-fold serially diluted

the transcribed RNA standards.
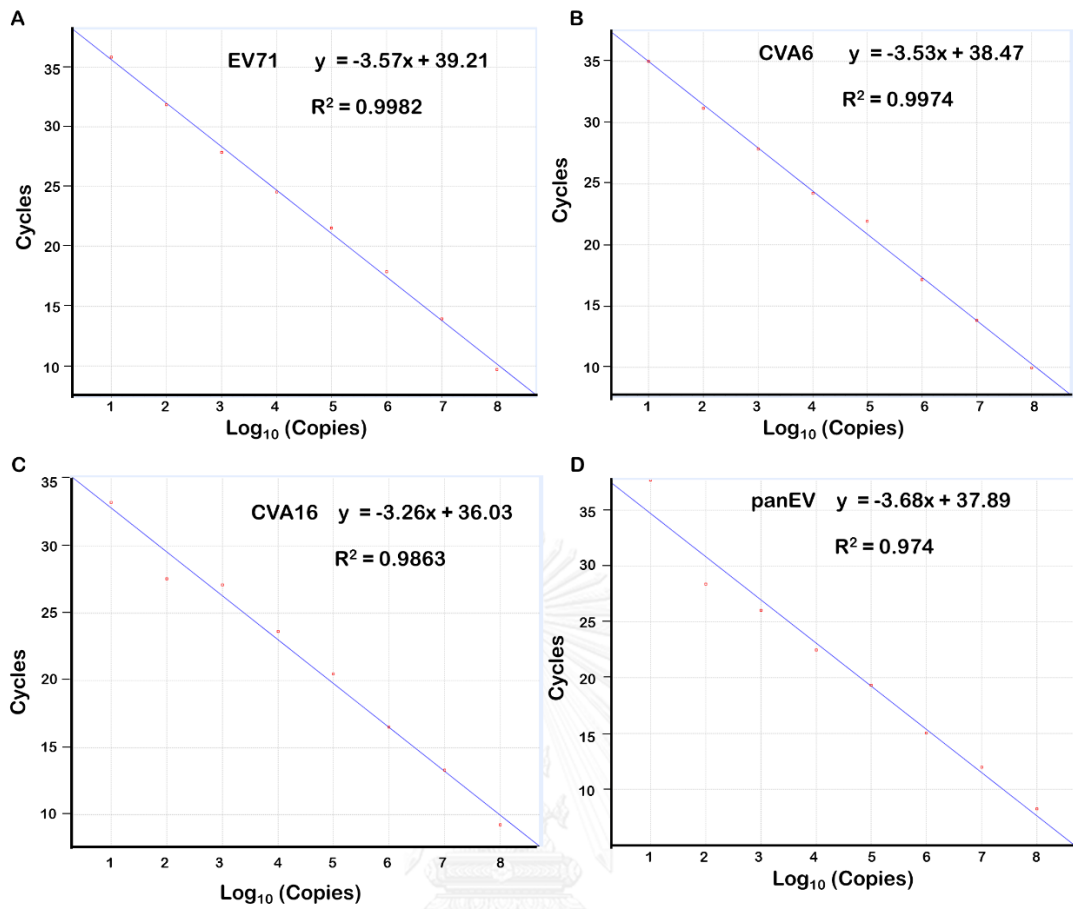
Figure 18. Standard curves of multiplex one-step real-time RT-PCR assays.

The multiplex one-step realtime TaqMan RT-PCR assays were tested using synthesized

in vitro target viral RNA transcripts ranging from 10^1 to 10^8 copies/μL.

Reproducibility of real-time RT-PCR assays

To determine intra-assay variation of the monoplex and multiplex rRT-PCR assay, serial dilution viral RNA standards (from 10^6 - 10^3 copies/reaction) were tested in triplicate within a single run (Table 11). The coefficients of variation (CV) and confidence intervals (CI) were calculated separately for each dilution. The mean CVs of intra-assay variability of the monoplex rRT-PCR were 0.98% (95% CI, -0.56–2.52%) for EV-A71, 1.30% (95% CI, 0.22–2.38%) for CVA6, 1.43% (95% CI, -1.09–3.94%) for CVA16 and 0.95% (95% CI, -0.11–2.01%) for panenterovirus. The mean CVs of intra-assay variability of the multiplex rRT-PCR were 1.63% (95% CI, 0.47–2.79%) for EV-A71, 1.92% (95% CI, 0.31–3.52%) for CVA6, 1.39% (95% CI, -1.14–3.91%) for CVA16 and 1.26% (95% CI, 0.08–2.43%) for panenterovirus.

Inter-assay variation of the monoplex and multiplex rRT-PCR assay were performed by testing for each standard dilution tested in three independent runs (Table 10). The mean CVs of inter-assay variability of the monoplex rRT-PCR were 2.91% (95% CI, 1.67–4.14%) for EV-A71, 2.48% (95% CI, 1.66–3.31%) for CVA6, 2.09% (95% CI, 0.32–3.87%) for CVA16 and 1.92% (95% CI, 0.36–3.48%) for panenterovirus. The mean CVs of inter-assay variability of the multiplex rRT-PCR were 2.24% (95% CI, -0.28–4.77%) for EV-A71, 3.11% (95% CI, 1.74–4.48%) for CVA6, 3.47% (95% CI, 1.77–5.16%) for CVA16 and 2.49% (95% CI, 1.54–3.43%) for panenterovirus. These results suggest that the singleplex and multiplex rRT-PCR assay were sufficiently reproducible.

Table 11. Intra- and inter-assay reproducibility of monoplex and multiplex real-time RT-PCR.

Virus Standard	Concentration (Copies/µl)	Intra-assay						Inter-assay					
		Monoplex RT-qPCR			Multiplex RT-qPCR			Monoplex RT-qPCR			Multiplex RT-qPCR		
		Mean Ct	SD	CV%	Mean Ct	SD	CV%	Mean Ct	SD	CV%	Mean Ct	SD	CV%
Enterovirus 71													
	1,000,000	18.53	0.15	0.81	20.47	0.49	2.39	20.13	0.67	3.33	17.4	0.6	3.45
	100,000	22.23	0.06	0.27	23.1	0.17	0.74	23.97	0.76	3.17	21.03	0.15	0.71
	10,000	27.17	0.12	0.44	27.23	0.55	2.02	28.2	0.95	3.37	25.1	0.46	1.83
	1,000	30.1	0.72	2.39	32.4	0.44	1.36	32	0.56	1.75	29.27	1.03	3.52
Coxsackievirus A6													
	1,000,000	18.9	0.1	0.53	21.47	0.72	3.35	19.2	0.61	3.18	17.53	0.59	3.37
	100,000	23.97	0.4	1.67	27.3	0.4	1.47	24.23	0.55	2.27	22.97	0.97	4.22
	10,000	27.5	0.56	2.04	31.37	0.32	1.02	28.13	0.55	1.96	24.63	0.59	2.40
	1,000	32.33	0.31	0.96	35.13	0.64	1.82	32.13	0.81	2.52	28.2	0.69	2.45
Coxsackievirus A16													
	1,000,000	19.7	0.1	0.51	20.53	0.06	0.29	20.2	0.52	2.57	17.67	0.83	4.70
	100,000	24.7	0.2	0.81	25.5	0.17	0.67	24.57	0.12	0.49	21.43	0.85	3.97
	10,000	28.6	0.17	0.59	29.33	0.25	0.85	29.47	0.67	2.27	25.8	0.61	2.36
	1,000	32.97	1.25	3.79	32.6	1.22	3.74	32.27	0.98	3.04	30.7	0.87	2.83
Panenterovirus													
	1,000,000	21.03	0.12	0.57	22.3	0.5	2.24	21.07	0.15	0.71	19.27	0.55	2.85
	100,000	24.33	0.06	0.25	26.03	0.15	0.58	25.07	0.71	2.83	22.37	0.42	1.88
	10,000	30.4	0.53	1.74	30.47	0.42	1.38	29.9	0.46	1.54	25.4	0.53	2.09
	1,000	33.87	0.42	1.24	35.23	0.29	0.82	34.67	0.9	2.60	28.87	0.9	3.12

Performance of multiplex real-time RT-PCR against conventional RT-PCR

To evaluate the performance of the multiplex rRT-PCR assay, the clinical specimens were tested and its sensitivity and specificity were compared with conventional RT-PCRs (Table 12). A total of 1,312 samples from suspected HFMD and herpangina patients in hospitals and 100 other respiratory samples were tested using he multiplex rRT-PCR assay and conventional RT-PCRs. Of the 1,312 samples tested, 723 (55.1%) samples were detected positive by multiplex rRT-PCR and 602 (45.9%) samples positive by conventional RT-PCRs. Overall, concordance between both methods was 83.6%. Similarly, both methods identified specimens that were positive for EV-A71 (87 samples), CV-A6 (206 samples), CV-A16 (101 samples) and panenterovirus (161 samples). The overall diagnostic sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the multiplex rRT-PCR assay were greater than those of conventional RT-PCRs as shown in table 12. For example, the sensitivity and

specificity for EV-A71 using multiplex rRT-PCR were 99.0% (95% CI, 94.4 - 100.0%) and 100% (95% CI, 96.4 - 100.0%) and conventional RT-PCRs were 90.7% (95% CI, 83.1 - 95.7%) and 98.0% (95% CI, 93.0 - 99.8%) respectively. For CV-A6, sensitivity and specificity were 93.1% (95% CI, 89.2 - 95.8%) and 97.0% (95% CI, 91.5 - 99.4%) for multiplex rRT-PCR while sensitivity and specificity were 86.5% (95% CI, 81.7 - 90.4%) and 84.0% (95% CI, 75.3 - 90.6%) for conventional RT-PCRs.

Table 12. Diagnostic performance of one step multiplex real-time PCR and conventional RT-PCR identification of hand foot mouth viruses.

Virus	Assay Performance (95% CI)			
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Enterovirus 71				
multiplex rRT-PCR	99.0 (94.4 - 100.0)	100.0 (96.4 - 100.0)	100.0 (96.2 - 100.00)	99.0 (94.6 - 100.0)
cRT-PCR	90.7 (83.1 - 95.7)	98.0 (93.0 - 99.8)	97.8 (92.2 - 99.7)	91.6 (84.6 - 96.1)
Coxsackievirus A6				
multiplex rRT-PCR	93.1 (89.2 - 95.8)	97.0 (91.5 - 99.4)	98.8 (96.5 - 99.8)	84.4 (76.4 - 90.5)
cRT-PCR	86.5 (81.7 - 90.4)	84.0 (75.3 - 90.6)	93.3 (89.4 - 96.1)	70.6 (61.5 - 78.6)
Coxsackievirus A16				
multiplex rRT-PCR	92.8 (87.4 - 96.3)	100.0 (96.4 - 100.0)	100.0 (97.4 - 100.00)	90.1 (83.0 - 95.0)
cRT-PCR	73.7 (65.9 - 80.5)	100.0 (96.4 - 100.0)	100.0 (96.8 - 100.00)	71.4 (63.2 - 78.7)
Panenterovirus				
multiplex rRT-PCR	93.5 (89.8 - 96.2)	94.0 (87.4 - 97.8)	97.6 (94.9 - 99.1)	84.7 (76.6 - 90.8)
cRT-PCR	67.9 (61.9 - 73.6)	80.0 (70.8 - 87.3)	89.9 (84.8 - 93.7)	48.8 (40.9 - 56.7)

Part 3: Molecular epidemiology and the evolution of human Coxsackievirus A6

Coxsackievirus A6 (CV-A6) is a group of genetically diverse RNA viruses belonging to the Picornaviridae family and the enterovirus (EV) species. The enterovirus species contains important pathogenic viruses such as poliovirus, hepatitis A virus, and EV-A71 viruses (1). CV-A6 has been classified in the genus Enterovirus species A which currently comprises a total of 20 types. EVs possess a positive-stranded RNA genome of approximately ~7.400 nucleotides (or 7.4 kb), encased by a highly structured icosahedral capsid. The viral genome is translated into a large polyprotein that is subsequently cleaved into structural (VP1 to VP4) and non-structural proteins (2A to 2C and 3A to 3D). The VP1 is the immunodominant structural protein and contains the most important serotype-specific neutralization epitopes. The degree of similarity of nucleotides and amino acid sequences of the VP1 region provides the primary tool for identification and assignment of new types within a species; novel EV variants showing less than 75% nucleotide sequence identity are classified as new types (136).

Infection with CV-A6 is frequently asymptomatic, but may also manifest as influenza like syndrome or as a self-limiting hand, foot and mouth disease (HFMD). Previously, HFMD typically occurred in young children and was predominantly caused by enterovirus 71 (EV-A71) and CV-A16. HFMD has only sporadically associated with other members of EV species A (6, 114, 137). More recently, CV-A6 was first identified as the cause of epidemic HFMD with unusual clinical manifestation in Finland in 2008 (113). Since then, HFMD cases associated with CV-A6 have been reported in several countries in Europe, Asia and USA (116-119, 138, 139), reflecting the rapid emergence of a more pathogenic form of this species A serotype. Lately, CV-A6 has been the most common EV type identified in several outbreaks of HFMD that have typically been characterized by

a more severe clinical spectrum (eczema coxsackium and erosive lesions) as well as subsequent benign onychomadesis (115, 125, 140-145).

Since 2008, the predominant causative pathogen of HFMD in Thailand was either EV-A71 or CV-A16, which, however changed from year to year. In 2012, a large-scale outbreak of HFMD took place in Thailand with the highest incidence rate in a decade. Meanwhile, CV-A6 had replaced EV-A71 and CV-A16 as the major EV types, and was identified in 33.5% of surveillance samples in Thailand in 2012 (119). Moreover, there was a high number of CV-A6-infected patients presented with influenza like symptoms (126).

Recently, (146) described a potential correlation between the emergence of new CV-A6 variants with altered clinical phenotypes and the appearance of novel recombinant forms (RFs) of the virus. The latter possessed phylogenetically distinct 3Dpol region sequences likely acquired from other species A serotypes through recombination. They found that several different RFs have circulated over the past decade worldwide. Notably, the emergence of the new recombinant form (RF-H) has been associated with clinically reported cases of HFMD presenting as eczema herpeticum in Edinburgh in 2014.

How widely RF-H and its predecessor, RF-A and other recently emerged RFs circulate in Europe is unknown, nor is it clear whether the greater community circulation and spiking incidence of HFMD in Thailand reflects the more widely spread of such recombinants. To address these questions, we utilized molecular epidemiology by analyzing VP1 sequence divergence and 3Dpol sequence grouping of a total of 151 CV-A6 isolates and samples collected in several European countries (Germany, Spain, Sweden and Denmark) and Thailand between 2013 and 2014.

Materials and Methods

Samples.

A total of 151 anonymized CV-A6-positive clinical samples from Denmark, Germany, Spain, Sweden and Thailand collected between years 2013 and 2014 were selected for genetic characterization. Sequences were named using the following convention: two-letter country code/sample code/3Dpol clade/year of collection (e.g. DK/M22416/G/2014 for sample code M22416 referred from Denmark, collected in 2014, and belonging to the 3Dpol clade G). 3Dpol clade assignments were identified from the results of phylogeny (see below). Sequences obtained in the current study were supplemented with 79 previously published nucleotide sequences of complete genomes from 6 countries, including the CV-A6 prototype strain Gdula. The respective samples were collected between 1949 and 2014. Details of the sequences used in the study including the countries of origin, sampling dates and accession numbers are provided in Appendex A Table 1.

Amplification of VP1 and 3Dpol regions.

Nested reverse transcription-PCRs (RT-PCRs) were carried out using newly designed primers listed in Appendix A table 2 to amplify a 1,331-bp region of the VP1 gene and a 1,200-bp region of the 3Dpol gene. The reverse transcription and first round PCR were performed using Superscript III One-Step RT-PCR system with Platinum Taq High Fidelity according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The following conditions were used: heating at 43 ˚C for 1 h, then 20 cycles of 53 ˚C for 1 min and 55 ˚C for 1 min, followed by 70 ˚C for 15 min and 94 ˚C for 2 min. PCR cycles included 40 cycles of 94 ˚C (30 s), 50 ˚C (30 s), and 68 ˚C (1 min 45 s) and a final extension at 68 ˚C for 5 min. One microliter of the first-round reaction was used as template in the second-round PCR containing second-round primers and GoTaq DNA polymerase (Promega,

Madison, WI) for 30 cycles of 94 ˚C for 30 s, 50 ˚C for 30 s and 72 ˚C for 90 s, followed by a final extension step for 5 min at 72 ˚C. Sequencing of the amplification products was carried out directly in both directions using ABI 7200 BigDye capillary sequencing (Applied Biosystems, Framingham, MA).

Complete genome sequencing.

The full-length genome of 39 CV-A6 variants representing four of the recombination groups were sequenced (RF-A, n = 21; RF-F, n = 10; RF-G, n = 2; and RF-H, n = 6) using primer sets (Appendix ATable 2) designed according to CV-A6-specific nucleotide sequences downloaded from GenBank. cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers and used as the template for nested PCR. The cycling conditions were identical to the second-round PCR mentioned above.

Sequence analysis.

Sequences were formatted, assembled and aligned using the SSE 1.2 sequence editor package (www.virus-evolution.org) (147). Phylogenetic trees were constructed using the Maximum-likelihood method and determined best-fit model of nucleotide substitution for each sequence data set using a correction value in the model selection procedure implemented in the MEGA program (v6) (148). In the analyses, Kimura two-parameter (K2P) with invariant sites (I) was selected as the substitution model for VP1 and 5UTR; K2P with I and gamma distribution (I-) for 3Dpol; K2P with I- for VP4/2 and 1000 bootstraps.

Rates of evolution and molecular clock phylogeny (149) were estimated from VP1 gene sequences using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST, version 1.8.0 (150). Bayesian MCMC analyses were performed

using a relaxed log-normal molecular clock model. The dynamic among study populations was estimated by performing a constant size and exponential growth models. Each Bayesian MCMC analysis was for 100,000,000 iterations and sampled every 10,000 states and discarding 10% of the chain as burn-in. Convergence of the chains and effective sample sizes of the estimates were checked using Tracer (http://beast.bio.ed.ac.uk/Tracer). The resulting tree of each run was summarized using Tree Annotator, and the maximum clade credibility tree was visualized with TreeAnnotator/FigTree.

Nucleotide sequence accession numbers.

All newly generated sequences obtained in this study were submitted to GenBank and were assigned the accession numbers KX212338 - KX212678.
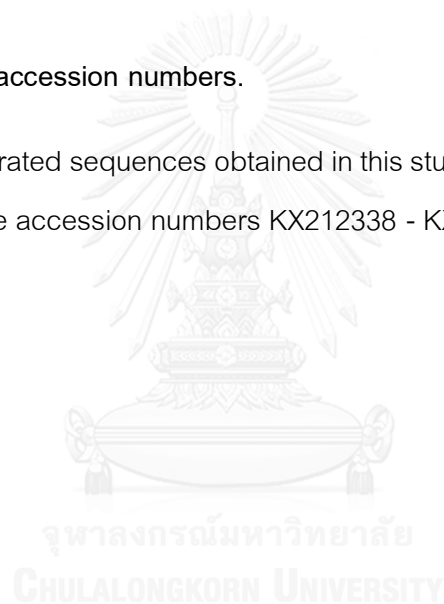
Results

Phylogeny of CVA6 VP1 and 3Dpol genome regions

CV-A6 variants associated with HFMD presenting with eczema herpeticum in Edinburgh in 2014 were clustered in a novel appearance clade (RF-H) distinct from other previously characterized recombination groups assigned in the 3D region (146). These variants were also phylogenetically distinct in the VP1 region (146). In the current study we have extended the analysis of VP1 and 3D regions to CV-A6 variants identified in wider geographical area including Denmark (n = 22), Germany (n = 4), Spain (n = 14), Sweden (n = 6) and Thailand (n = 105). CV-A6 sequences for the VP1 region between positions 2485 and 3816 (numbering based on the Gdula prototype strain; GenBank accession number AY421764) were obtained.

VP1 sequences from Denmark and Spain (2014) clustered within the same lineage (lineage I) with those analysed previously from the patients with eczema herpeticum presenting in 2014 in Edinburgh but distinct from previous outbreaks in Taiwan (lineage II), Thailand (lineage III), China (lineage VI) and Finland (lineage V) (Figure 19-20).

In order to investigate the occurrence of recombination, sequences from the 3Dpol region were obtained and analyzed for each of the 151 isolates included in the VP1 analysis. The data set was supplemented by 39 (near-) complete genome sequences of CV-A6 variants from Denmark (n = 8), Germany (n = 3), Spain (n = 3) and Thailand (n = 25). CV-A6 3Dpol clades comprising groups A, B, C, D, E, F, G, H, I, J and K were designated through identification of bootstrap-supported clades as described previously for other enteroviruses (151-153). Designations follow the nomenclature established in our previous analysis of Edinburgh CV-A6 isolates (146). The majority of variants in the study clustered within two of the previously assigned recombinant forms, RF-A (99/151) and RF-F (37/151) (Table 13). Four Danish variants clustered within clade RF-H, as did two of the

variants from Spain. Three variants from Denmark and Spain were assigned as RF-G (Figure 21). No sequences within this study clustered within the previously described RF-B, RF-C, RF-D and RF-E. None of the CV-A6 3Dpol groups contained sequences from other species A serotypes.

Phylogenetic analysis of the VP1, VP4/2 and the 5´UTR produced broadly similar groupings of CV-A6 variants in each region (Figure 20, 22 and 23). However, there were several examples of incongruent groupings that indicate the existence of further recombination within CV-A6. For example, RF-E variants grouped with RF-A and –H in VP1 region but changed position in the 5´UTR and VP4/2. In contrast to VP1, RF-A were interspersed in the 5´UTR and VP4/2 regions with the member of RF-J. The phylogenetic group formed of RF-K was monophyletic in the VP1, VP4/2 and the 5´UTR regions but the only one exception grouped with RF-D. These findings indicate the occurrence of multiple recombinant events between VP1, VP4/2 and the 5'UTR.
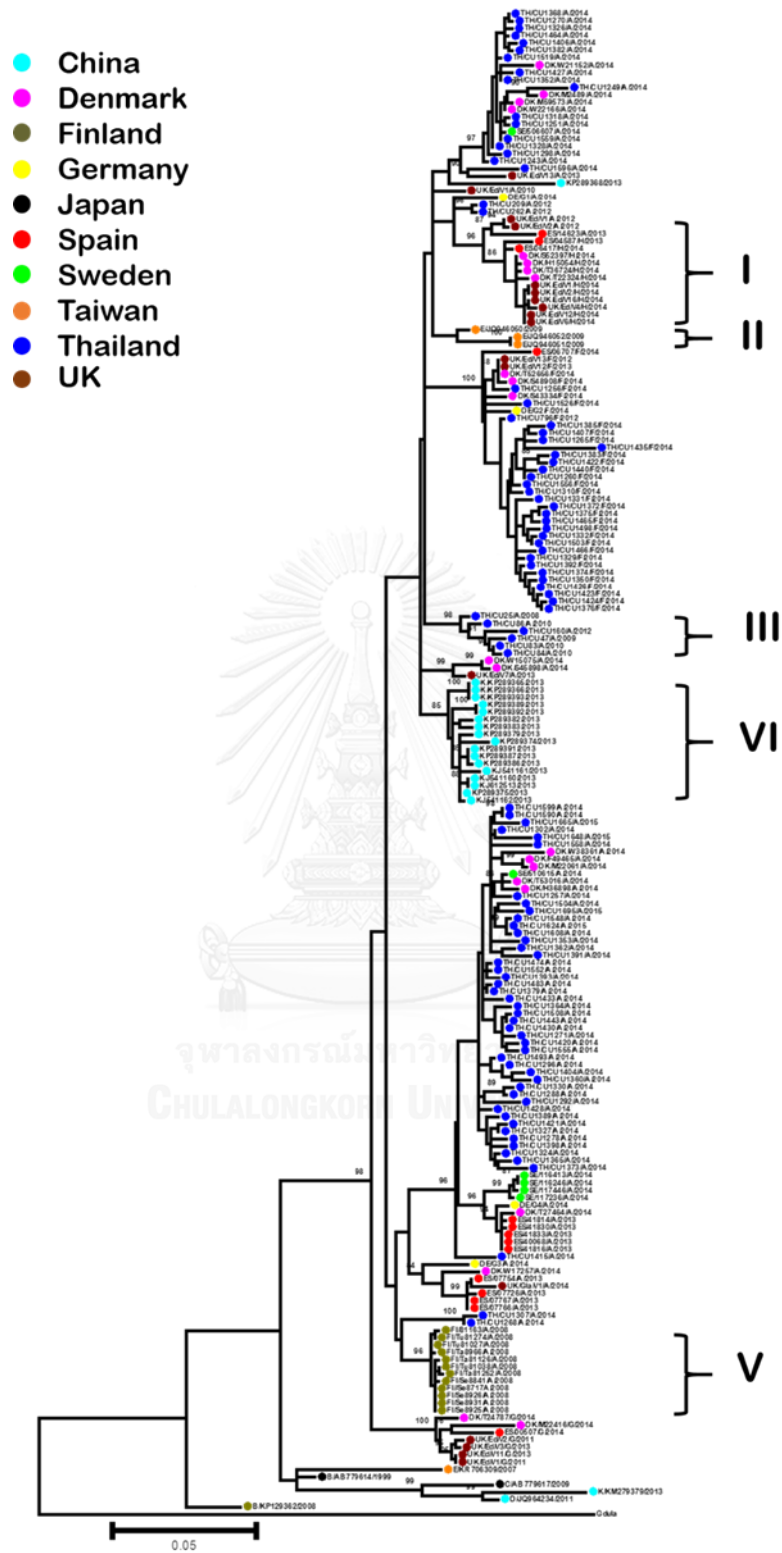
Figure 19. Phylogenetic analysis of VP1 sequences of CVA6 for study subjects and those previously determined. The tree display the color code for different countries.

Table 13. Recombination groups (RF-A to -K) based on phylogenetic analysis of the 3Dpol region.

Country	RF-A	RF-B	RF-C	RF-D	RF-E	RF-F	RF-G	RF-H	RF-J	RF-K	Total
All	105					37	3	6			151
Denmark	13					3	2	4			22
Germany	3					1					4
Spain	10					1	1	2			14
Sweden	6										6
Thailand	73					32					105
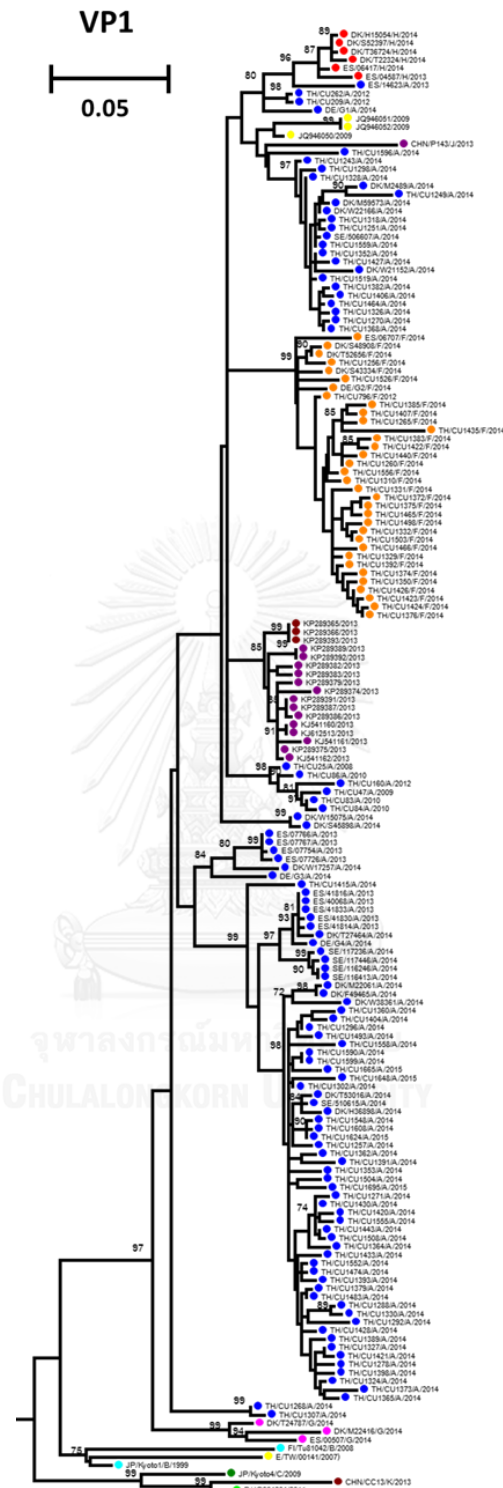
Figure 20. Phylogenetic analysis of VP1 sequences of CVA6 for study subjects and those previously determined (RF-B, -C, -D, and -E). Each sequence is labelled by country of origin, sample code, 3Dpol clade assignment and year of collection.
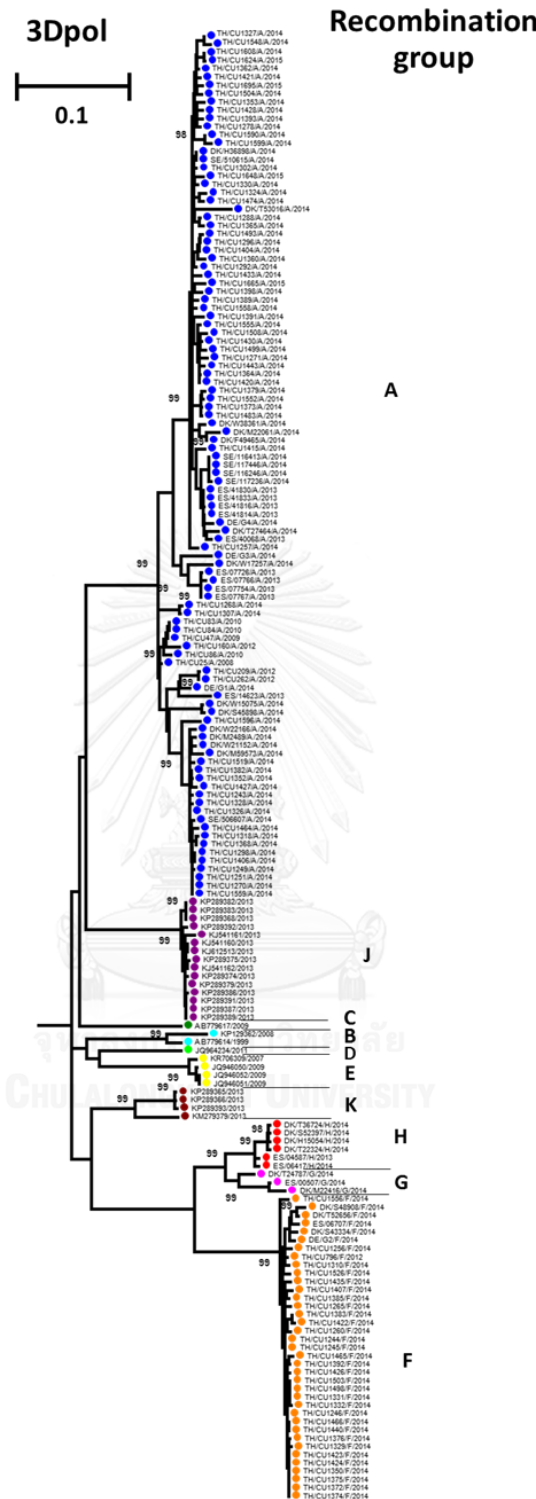
Figure 21. Phylogenetic analysis of 3D sequences of CVA6 for study subjects and those previously determined (RF-B, -C, -D, and -E). Each sequence is labelled by country of origin, sample code, 3Dpol clade assignment and year of collection.

Sequence diversity of CVA6

Sequence divergence in VP1 provides a proxy measure for the time of divergence of CV-A6 variants from which estimates of the life spans of individual recombinant forms can be estimated. By pairwise comparison of each CV-A6 variant with each other, there was a precise correlation between VP1 sequence distances and assignment to the same or a different RF group in 3Dpol; a divergence threshold of 0.1 divided the two sets of comparisons (Figure 24).

CV-A6 VP1 variability was restricted primarily to synonymous sites, which indicates that most changes in sequences occurred through neutral drift. Through molecular clock analysis, the nucleotide substitution rate and times to the most recent common ancestor (tMRCAs) of different regions and the assignment of 3Dpol clades of the greatest RFs (RF-A and RF-F) were estimated. The substitution rate of the whole data set of all VP1 sequences was estimated to be 8.1×10^{-3} substitutions/site/year (high-probability distribution [HPD] range, 6.0×10^{-3} to 10.5×10^{-3}); the date that the MRCA of all CV-A6 clusters existed was estimated to be 1947 (HPD, 1940 to 1949). The substitution rate for the whole data set of Asia VP1 sequence compared to Europe was more than 2.4 times faster at 9.5×10^{-3} substitutions/site/year (HPD, 6.8×10^{-3} to 12.4×10^{-3}) (Table 14). Despite the greater sequence variability among Asia, the most recent common ancestor (MRCA) of this group occurred at almost the same time with that of Europe. The estimated date of the MRCA of RF-A was 1999 (HPD, 1995 to 2003); since then, RF-A lineage has diverged into five recombination groups (RF-E, -F, -H, -J and -K). The VP1 lineage containing the RF-G recombinant form was first documented in 2011 and thereafter underwent the recombination event that exchanged the 3Dpol region sequences between 2004 and 2011. VP1 lineage 1 contains CV-A6 variants belonging to RF-F with the

recombination event dated between 2009 and 2012. A further RF group (H) that appeared in 2013 probably recombined between 2011 and 2013.

Using the substitution rates estimated from VP1 sequence analysis, an estimation of approximate half-lifes of CV-A6 lineages were calculated by combining the mean sequence divergence in VP1 at the 50% recombinant frequency threshold (0.05) with the substitution rate in VP1 (8.1 × 10^{-3} substitutions/site/year). The RF half-lifes of CV-A6 was approximately 3.1 years, thus within the range estimated from previous analyses of other enterovirus serotypes (Figure 25).

Table 14. Rates of sequence change and TMRCAs by MCMC analysis.

RF group and geographic set	RF	n[†]	Divergence[*]				dN/dS		MCMC (BEAST)			
			Nucleotide		aa				Substitution rate (10⁻³)[#]		TMRCA[‡]	
			VP1	3Dpol	VP1	3Dpol	VP1	3Dpol	VP1	3Dpol	VP1	3Dpol
Whole data set												
All	All	244	0.052	ND	0.014	ND	0.035	ND	8.1 (6.0-10.5)	ND	68.2 (66.2-75.4)	ND
Europe	All	79	0.051	ND	0.011	ND	0.027	ND	3.9 (2.5-5.5)	ND	16.9 (9.7-25.4)	ND
Asia	All	164	0.049	ND	0.015	ND	0.041	ND	9.5 (6.8-12.4)	ND	18.9 (16.2-23.3)	ND
Individual RF group												
All	RF-A	160	0.046	0.047	0.012	0.020	0.036	0.057	10.4 (7.6-13.8)	11.0 (8.2-14.4)	15.9 (12.3-20.4)	12.9 (12.2-14.4)
Asia	RF-A	109	0.044	0.045	0.014	0.017	0.041	0.051	9.2 (6.4-12.3)	8.2 (5.7-10.9)	16.1 (12.2-20.8)	12.7 (12.2-13.9)
Europe	RF-A	51	0.044	0.049	0.009	0.025	0.027	0.068	6.8 (4.4-9.4)	10.9 (5.9-16.5)	7.5 (6.2-9.0)	7.9 (6.3-10.6)
All	RF-F	36	0.016	0.013	0.010	0.006	0.097	0.055	1.4 (0.03-3.0)	6.5 (3.6-10.2)	17.1 (3.0-42.1)	3.5 (2.7-4.4)

*   Mean pairwise P distances. ND, not determined.

†   Number of sequences in each set analyzed.

\#   Mean value with the HPD interval in parentheses.

‡   Time before the present of the most recent common ancestor
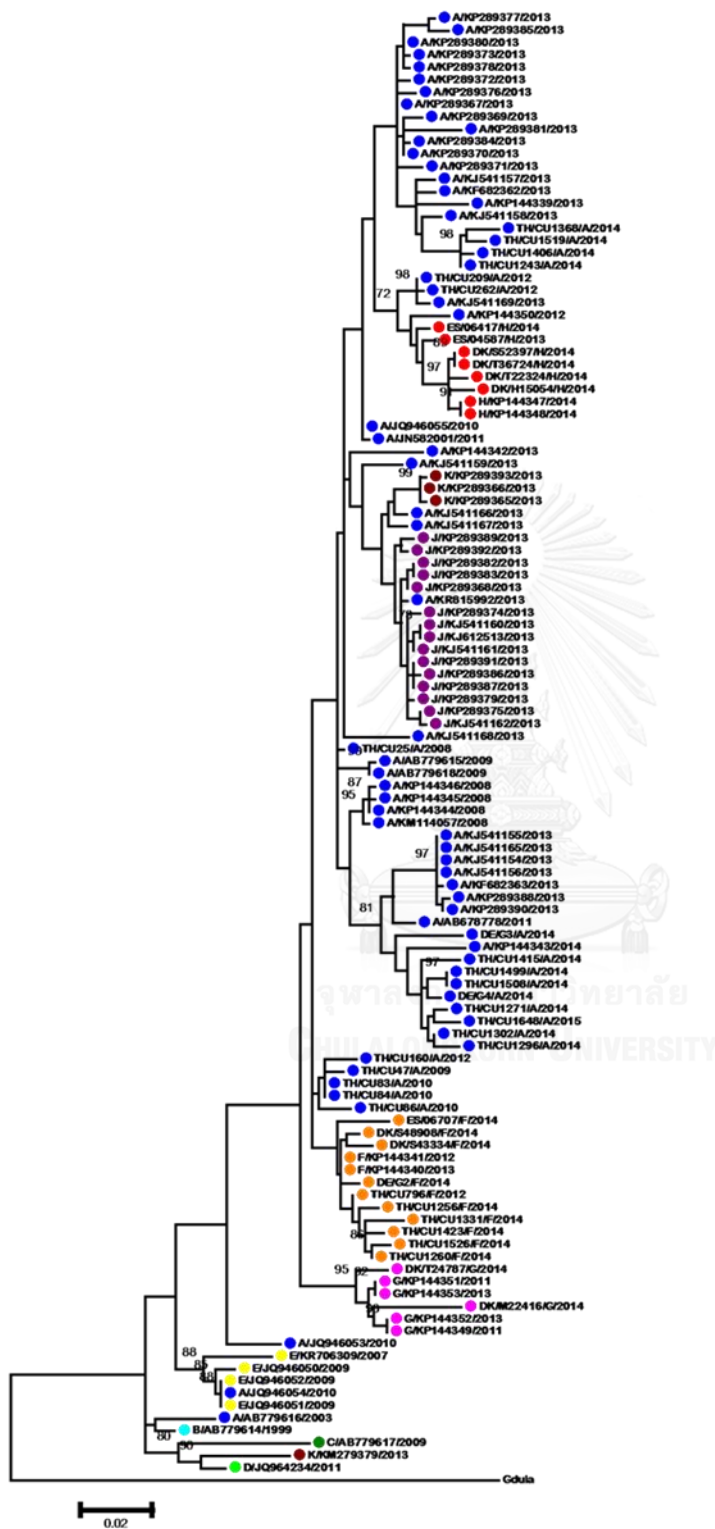
Figure 22. Phylogenetic analysis of 5´UTR of CVA6 for study subjects and those previously
determined (RF-B, -C, -D, and -E).
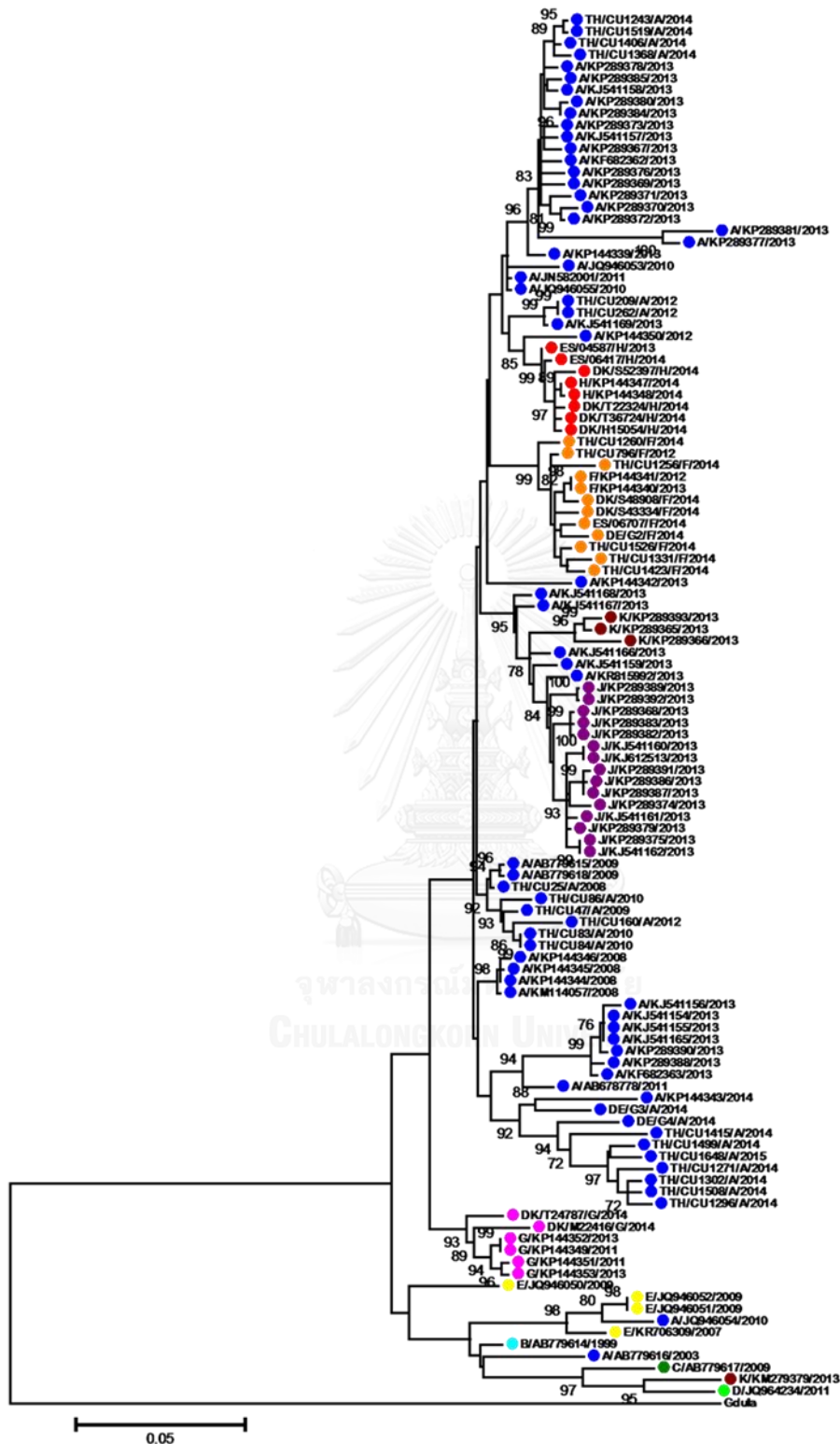
Figure 23. Phylogenetic analysis of VP4/2 sequences of CVA6 for study subjects and those previously determined (RF-B, -C, -D, and -E).
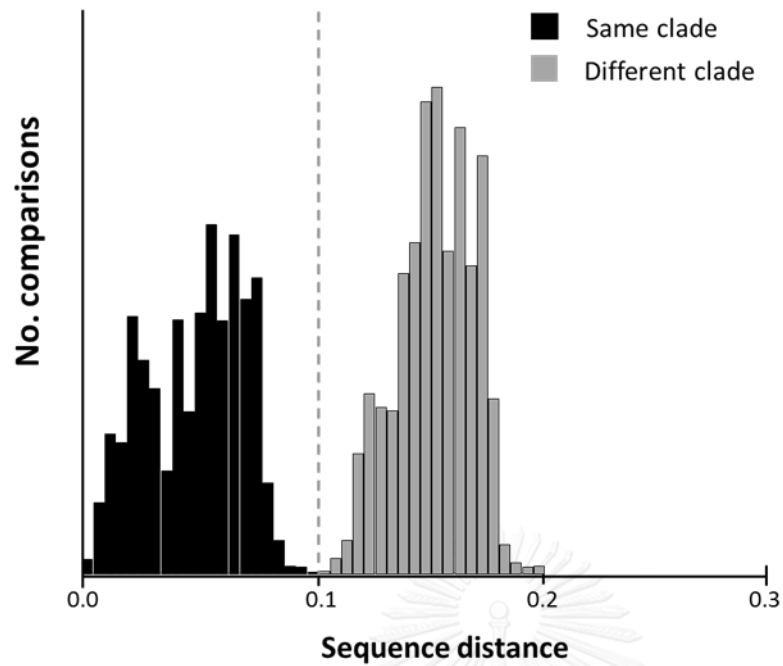
Figure 24. **Histogram of genetic distances between CVA6 sequences.**

The histogram shows the distribution of pairwise distances between sequences from this study and published CVA6 sequences in the 3Dpol region. The dashed line represents the threshold between a member of the same clades and between different clades.
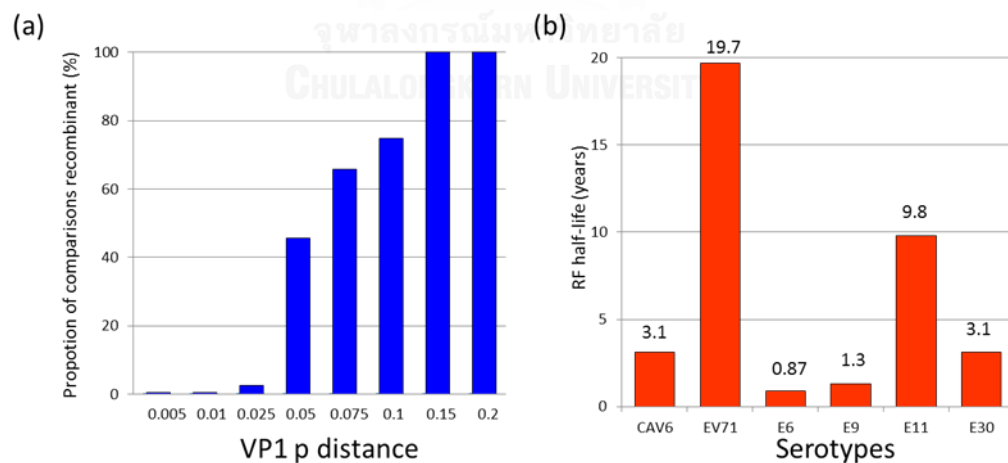
Figure 25. (A) Association between VP1 sequence divergence (shown on the x-axis) and the proportion of recombinant comparisons. (B) Comparison of mean half-lives of CVA6 with previously estimated by the same method.

To determine more precisely the timescale of recombination events underlying the appearance of each RF, datasets of VP1 gene sequences were analysed using the Bayesian Markov Chain Monte Carlo (MCMC) method to generate time-correlated phylogeny (Figure 26). While earlier recombination events could not be reconstructed in any detail due to inadequate sampling of CV-A6 before 2008, variants collected after this date were monophyletic falling into three further lineages with estimated dates of splitting betweeen 2004 and 2005. The oldest lineage comprised purely RF-G samples while the other two lineages contained samples belonging to RF-A. One comprised solely RF-A and persisted for at least 11 years (2005 to 2015). The other lineage contains RF-A sequences and samples isolated subsequently belonging to other RF groups (E, F, H, J and K). Variants within lineage 2 (RF-A) were those detected in the first HFMD outbreak in Finland in 2008 along with variants detected subsequently in Europe and Asia over the following 1 to 5 years. In lineage 1, the oldest variants were those originally described in Asia (Thailand and Japan) before 2012 and then spread into Europe and Asia between 2013 and 2014. Within this lineage, several separate recombination events generated RFs including an RF-F group described in Thailand in 2012 (119). The RFs (F, G, H and K) were monophyletic and likely originated from single recombination events, unlike RF-E and RF-J which were detected in more than one VP1 lineage. With the exception of RF-F which was isolated from both Europe and Asia, other RFs groups were detected only from Europe or Asia; G and H (Europe), E (Taiwan), J and K (China).
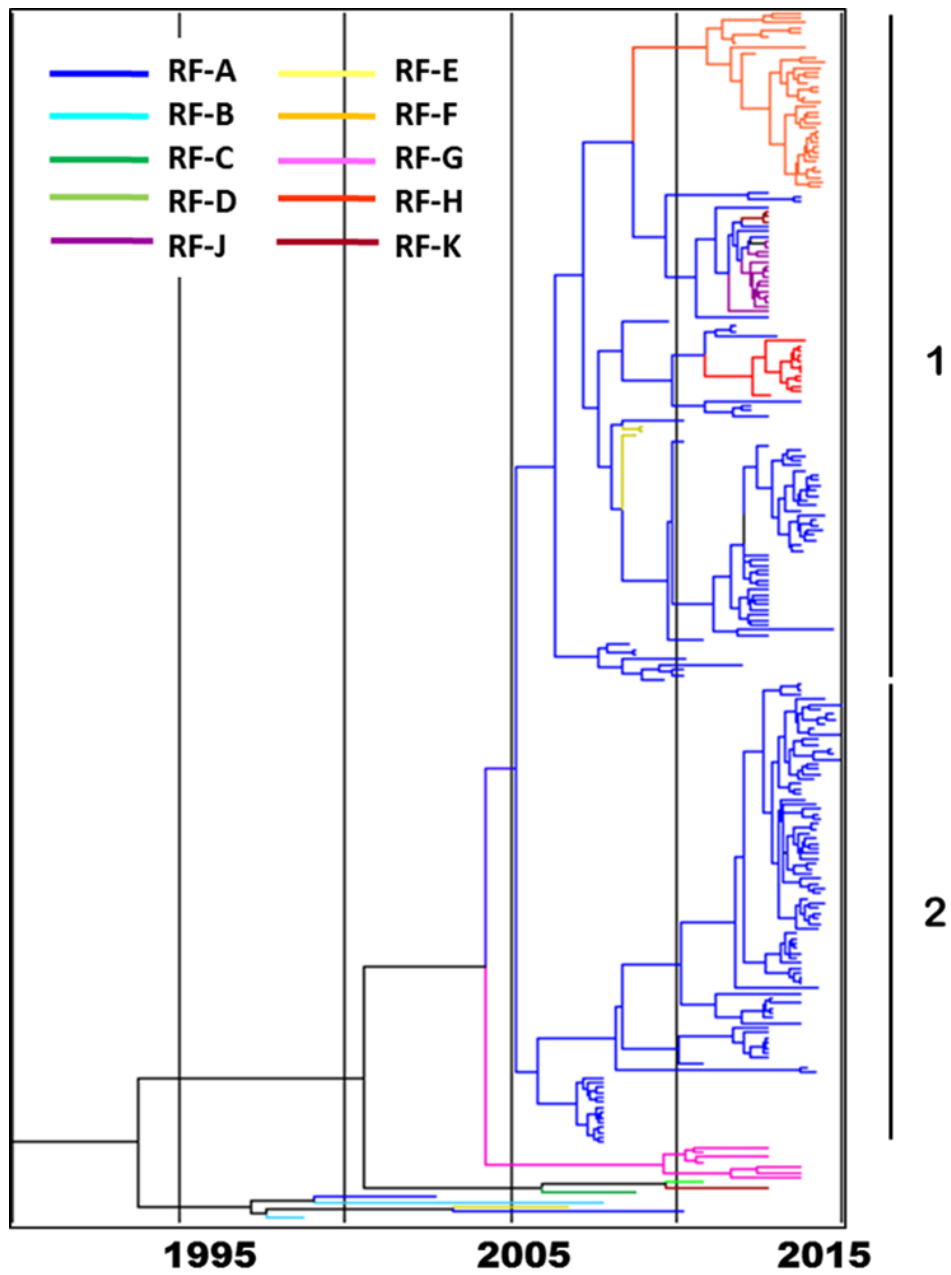
Figure 26. A dated phylogeny of VP1 sequences of CVA6 variants in this study and published sequences. Branch colors label recombination groups in each clade.

Having identified the likely time course and direction of the recombination events, divergence scan analyses were performed between the ancestral RF-A sequences with complete genome sequences generated in the current study (RF-F, -G, and -H) and the previous study (RF-J and RF-K) recombinant forms to identify recombination breakpoints (Figure 27). The sharp increase in sequence divergence at various points in the P2 region provided evidence for the occurrence of separate, individual recombination events for each RF. The first breakpoint was founded at the 2A protein-encoding region around nucleotide position 3500 (RF-G). The next breakpoints were located in 2B and at the border between 2B and 2C regions (RF-F, -J and -H). The last breakpoint of RF-K can be recognized at nucleotide position 5000 and included the 3' part of the 2C region.
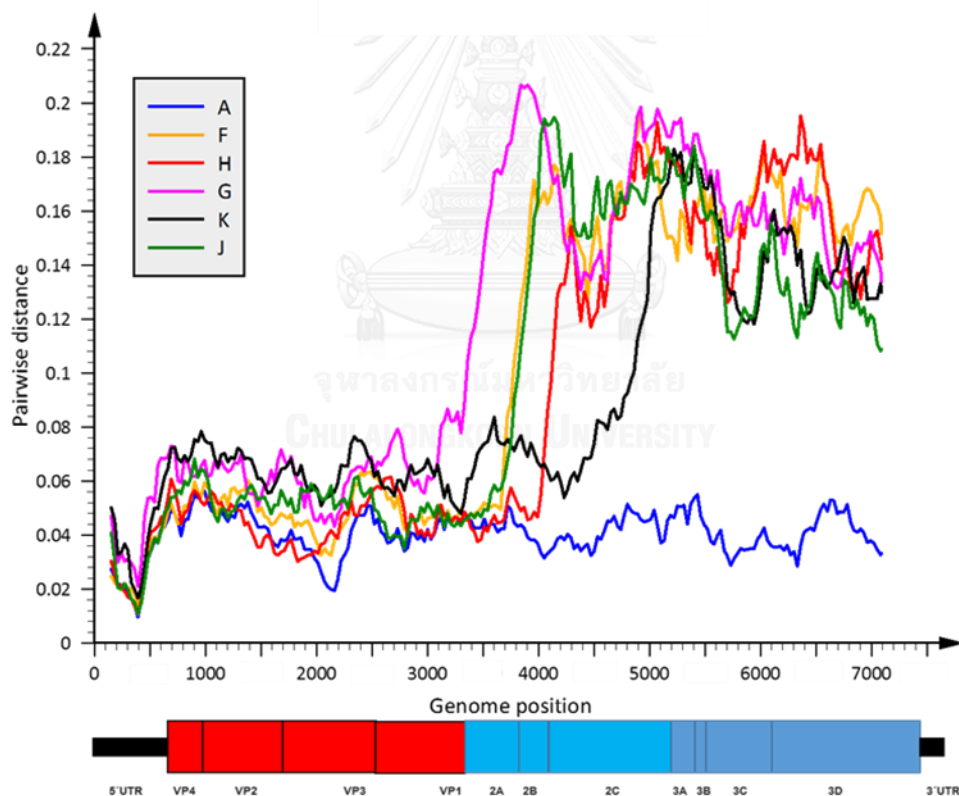
Figure 27. Divergence scan of nucleotide sequence between RF-A with other recombination groups (RF-F, H, G, K and J).

Discussion and conclusion

In Thailand, HFMD usually occurs during the rainy season (June–August); average incidence during 2007–2011 was 20.2 cases per 100,000 population (5, 6). In 2012, an extensive outbreak of HFMD occurred; the incidence rate was 3-fold higher than the average incidence rate of 58.15 cases per 100,000 population or >36.000 cases; the 2012 outbreak included 2 fatal cases of EV71 encephalitis. Our study is the first report investigating epidemiology of multiple enterovirus infections among Thai patients with HFMD, herpangina and ILI who visited various hospitals in Thailand, 2012. In recent years, HFMD outbreaks caused by other types, especially CV-A6 have increasingly been reported in several countries worldwide (112, 113, 115-118). EV-A71 and CV-A16 are found predominantly in HFMD patients in Thailand (5, 6). Awareness of information on the epidemiological profiles, their pathogenic role, geographic distributions of other enteroviruses in Thailand are also still lacking.

Our study included 1964 suspected cases representing 35.8% (704/1,964), 8.5% (166/1,964) and 55.7% (1,094/1,964) of HFMD, herpangina and influenza-like illness, respectively. During the study period, 33.5% (657/1,964) patients presented with EV infection. HEV-A species were the most common HEV types among the pathogens causing HFMD (54.4%), herpangina (94.1%) and ILI (2.4%), followed by HEV-B and HEV-C species, while no HEV-C species were detected in herpangina. The number of HFMD/herpangina suspected cases in 2012 was up to six-fold higher than the yearly number of cases reported during the study period between 2008 and 2011 (6). The number of enterovirus infection increased abruptly during the rainy season in 2012, particularly in July 48.9%, which was consistent with reports from Korea in 2009 (154). Most strains in this study were detected during this period. This result suggests that epidemiological surveillance and prevention should mainly focus on the period of June-

August of each year. The majority of reported cases were children of 1-3 years of age. The incidence rate of HFMD in infants less than six months was low, which might be due to the protective role of maternal antibodies (155). All patients associated with HFMD/Herpangina included in this study presented with mild disease and did not develop any more severe symptoms.

According to previous studies, EV-A71 and CV-A16 were the major enteroviruses causing epidemics of HFMD in Thailand. EV-A71 subgenotype C4 was the predominant strain circulating during the 2008-2009 epidemic followed by CV-A16 and CV-A10 (5). Although CV-A16 was the most prevalent genotype in 2010, EV-A71 has been found to be an emerging causative agent in 2011 (6). In the present study, we found CV-A6 to be the dominant cause associated with HFMD. CV-A16 and EV-A71 are also very frequently identified types of human enterovirus. CV-A4, CV-A5, CV-A8-10, CV-A12, CV-B1, CV-B2, CV-B4 and CV-B5 are potential causes of HFMD. In Thailand, high prevalence of CV-A6 infections has not been reported yet. As reported in previous studies (112, 156-158), dual infections with CV-A6 and CV-A10 were predominant causes of HFMD/herpangina outbreaks in many countries during 2008-2010. Our study has established CV-A6 as the predominant cause of HFMD. Although CV-A6 has been a predominant emerging pathogen since 2012, no patients infected with CV-A6 died.

As reported in a previous study, herpangina is associated with different strains of enteroviruses, such as CV-A2 in Taipei 2008 (159), CV-A5 in Korea 2009 (160), CV-A6 and CV-A10 in France 2010 (158). In addition, in Japan during 2000-2005, there have been reports of enterovirus infections caused by CV-A2, CV-A4, CV-A6, CV-A8 and CV-A10 (103). Our study has established CV-A8 as the most prevalent cause of herpangina followed by CV-A6.

Our study investigated distinct clusters of CV-A6, EV-A71, CV-A6 and CV-B2 strains in relation to their geographic origins. Phylogenetic analysis showed that the Thai CV-A6 strains were closely related to strains isolated from Shanghai provinces of China 2012. Furthermore, on the basis of the VP1 sequences of EV-A71, the C4 subgenotype was previously identified as the most prominent EV-A71 subgenotype circulating in Thailand, and subgenotypes C1, C2 and B5 were also found (5, 6). Based on the results of phylogenetic analysis, we reported that most EV-A71 strains circulating in Thailand 2012 belonged to the B5 subgenotype, with 2 strains of subgenotype C4. Subgenotype B5 and subgenotype C4 were first described in Japan in 2003 and Taiwan in 1998 (86, 161). The Thai CV-A8 strains belonged to two evolutionary clusters with high nucleotide homology, closely related to the strains found in India and Japan, whereas all Thai CV-B2 strains were most closely related to the strains in China and Korea. Strains diversity may result from the diverse residues in the VP1 BC loop region, which is implicated with viral antigenicity. Substitutions resulting in conformational changes in this region may play an important role in the adaptation of enteroviruses (162, 163). We found amino acid sequence substitutions at 5 positions in CAV6 (89, 95, 97-99) and 5 positions in CAV8 (92, 93, 96, 97 and 100).

Conclusively, first, during the 2012 enterovirus outbreak, most HFMD was caused by CV-A6, whereas herpangina was mainly caused by CV-A8. Second, surveillance of epidemiology and monitoring of the HFMD/Herpangina outbreaks should be continued very carefully to see whether the circulating enterovirus genotypes have a serious impact on Thailand's public health. Third, this is the first report describing the circulation of multiple enteroviruses in Thailand.

Our previous study investigated the substantial increasing of CV-A6 infection among Thai children with HFMD in 2012 (119, 126). In recent years, sporadically detected

CV-A6 virus has been identified as causative agents in Europe, Asia and the USA since it has been reported with a change in its clinical phenotype in 2008 and declining EV-A71 and CV-A16. CV-A6 is becoming an increasingly crucial agent as it was the only pathogen documented differences in symptomatology from HFMD disease including frequently associated with high fever as shown in previous study (126). The benefits of using real time PCR technology in routine diagnostics include rapid, capacity for high throughput, high specificity and quantitative ability (164). Several conventional RT-PCR and real time PCR assays for EV-A71, CV-A6 and CV-A16 detection have also been reported, but none of them were not developed for simultaneous detection and differentiation of these viruses. In the current study, we developed and validated multiplex real-time PCR systems for rapid detection and typing of EV-A71, CV-A6 and CV-A16 associated with HFMD diseases.

The diagnostic tool for clinical tests need for rapid results with high enough sensitivity to identified the infectious agent in the early stage of disease to utilize in management and treatment of viral infection. With the multiplex rRT-PCR developed in this study, the amount of RNA transcript standard can be definitely detected at least 10 copies of EV-A71, CV-A6, CV-A16 and panenterovirus per reaction. The sensitivities of singleplex or multiplex real-time PCR assays can vary widely depending on the target region and EVs genotypes. Previous comparison of conventional gel electrophoresis based multiplex PCR and microarray to detect EVs RNA which had a detection limit of 100 copies and 1 copy respectively (129). Moreover, a GeXP analyzer-based multiplex RT-PCR assay also had the detection limit for EV-A71, CV-A16 and panenterovirus of 10 copies/ul (165). Despite the sensitivity of multiplex real-time PCR assay evaluated in this study has similar or higher values compared to other assays while can detect more pathogens.

The targets for the primers and probes for each of the selected agents were designed within viral protein 1 (VP1) for EV-A71, CV-A6, CV-A16 and 5´UTR for panenterovirus. Nevertheless, it is feasible that mutations in the primer and probe regions may evolve, due to the high evolutionary rates proper of RNA viruses (166). There was no amplification signals obtain from specimens of other RNA viruses such as influenza A (H3N2 and H1N1), influenza B, respiratory syncytial virus (RSV), adenovirus, rotavirus, parechovirus, indicating this method was dependable in specificity.

In conclusion, the multiplex rRT-PCR assays described above provide the highly sensitive detection and rapid simultaneous typing of EV-A71, CV-A6 and CV-A16 thus rendering it feasible and attractive for large scale surveillance of EVs associated with HFMD outbreaks.

CV-A6 infection represents a significant global public health concern and has become one of the most frequently detected enteroviral cause of HFMD. This study sought to understand how widespread the new recombinant forms of CV-A6 have become in Europe and other geographical regions through analysis of the molecular epidemiology and dynamics of recombination of CV-A6 variants collected within the last 8 years. Differences in the phylogenetic trees of VP1 and 3Dpol regions provided a strong evidence of recombination in several strains. Specifically, three CV-A6 variants did not cluster within the same recombinant group on the VP1 phylogenetic tree. The TW/00141/2007 strain was assigned as RF-E, but grouped with RF-B. The CHN/P143/2013 strain was assigned as RF-J, but grouped with RF-A. Finally, the CHN/CC13/2013 strain was assigned into RF-K, but grouped with RF-D. The patterns of phylogenetic discordance observed from this study suggests the occurrence of recombination in CV-A6 consistent with previous findings for other enteroviruses (151-153, 167-169).

As previously described (151), the RF lifespans of CV-A6 were calculated by measuring the relationship between VP1 divergence and the likelihood of recombination. The RF half-life of CV-A6 was closer to that estimated previously for E-30 (3.1 years), much shorter than that of EV-A71 and E-11 whereas the value was higher those of E-6 and E-9 (151-153, 167). Based on analysis of Bayesian MCMC methods, the rate of evolutionary change for CV-A6 in the VP1 region of 8.1×10^{-3} substitutions per site per year (Table 14) fell within the middle of the range described for other enteroviruses, as examples, CV-A24 (1.2×10^{-3}), CV-A21 (3.1×10^{-3}), EV-C96 (3.3×10^{-3}), EV-C99 (3.7×10^{-3}), CV-B5 (4.2×10^{-3}), E-11 (4.8×10^{-3}), EV-D68 (4.93×10^{-3}), EV-D70 (5×10^{-3}), CV-A4 (5.5×10^{-3}), E-9 (5.8×10^{-3}), EV-A71 ($3.1-7.2 \times 10^{-3}$), CV-A2 (8.3×10^{-3}), E-30 (8.8×10^{-3}), CV-A16 (9.1×10^{-3}), poliovirus (10.3×10^{-3}), E-6 (11.2×10^{-3}), CV-A10 (14.1×10^{-3}) (151, 152, 167, 169-179).

Phylogenetic reconstruction was used to analyze trait evolution such as temporal and geographical correlates of individual recombination events. The most frequently detected recombinant form, RF-A, showed decades-long circulation and was the ancestor of five separate recombinant groups (RF-E, F, H, K and J) that have emerged in the past 5-10 years. The first outbreak of CV-A6-HFMD in Finland in 2008 was associated with RF-A with the subsequent appearance of this RF across of Europe and Asia between 2013 and 2014. The more recent emergence of RFs in Europe and Asia between 2013 and 2014 originated from descendants of multiple VP1 lineages that have diverged from RF-A variants circulating in Asia (Thailand and Japan) between 2008 and 2010. Recombination events have previously been recorded widely in other enteroviruses and were found to play a significant role in the evolution of these genomic viruses and the recombination breakpoints detected in this study (2A - 2C regions) are well-known recombination hotspots in enteroviruses (180). Regarding CV-A6, breakpoints within VP3

and between 5´UTR and VP1 have been detected in the genomes of RF-E variants in Taiwan (146).

This study reports a detailed, multi-centre investigation of the emergence of an enterovirus serotype associated with epidemics of HFMD. It catalogues the complexity of the evolutionary processes associated with its geographical expansion and the occurrence of a number of recombination events each involving replacement of close to complete non-structural gene blocks at varying times since the founder recombinant form, RF-A was first described in 2008 (113). Non-structural (NS) region sequences of most RFs have not been described in association with other species A serotypes including any of the RFs described for EV-A71 (152). However, there was some evidence for a limited degree of re-circulation within the recombination pool of NS region sequences; RFs -E and -K appear at two different positions in the VP1 phylogenetic tree (Fig. 1a). Furthermore, a limited degree of sharing of NS regions sequences between different species B serotypes has been documented (181).

Nevertheless, the typical pattern for an RF was its rapid emergence, variable penetrance into the sampled virus population and relatively rapid extinction, within years rather than decades, based on average recombination half-lives documented for CV-A6 and other EV types (Fig. 4b). This patterns is well attested in the turnover of RFs of CV-A6. While we remain relatively ignorant of the reasons for RF turnover, whether driven by immunological, host adaptive factors or transmissibility, or alternatively whether it occurs as a consequence of population bottlenecks and replacements without a fitness component, molecular epidemiological studies such as these will be of value in gaining a longer term, better understanding of the nature of enterovirus evolution and their clinical outcomes.

APPENDIX A

Table S1: Sequences information

Isolate	Country	City	Year	RF group	Accession number		
					Complete genome	VP1	3D
Gdula	US	NS	1949	I	AY421764	-	-
Fin/Se8926	Finland	NS	2008	A	KP144346	-	-
TS6/Fin/Se8925/2008	Finland	NS	2008	A	-	KP129346	KP129366
Fin/Se8717	Finland	NS	2008	A	KP144344	-	-
V2/Fin/Ta81126/2008	Finland	NS	2008	A	-	KP129337	KP129357
Fin/Se8841	Finland	NS	2008	A	KP144345	-	-
TS5/Fin/Se8931/2008	Finland	NS	2008	A	-	KP129344	KP129364
TS2/Fin/Tu81038/2008	Finland	NS	2008	A	-	KP129341	KP129361
TS1/Fin/Ta81252/2008	Finland	NS	2008	A	-	KP129336	KP129356
TA1/Fin/Ta8966/2008	Finland	NS	2008	A	-	KP129339	KP129359
V4/Fin/Tu81274/2008	Finland	NS	2008	A	-	KP129340	KP129360
V1/Fin/81163/2008	Finland	NS	2008	A	-	KP129335	KP129355
V5/Fin/Se8928/2008	Finland	NS	2008	A	-	KP129345	KP129365
TS4/Fin/Tu81027/2008	Finland	NS	2008	A	-	KP129343	KP129363
Finland/2008	Finland	NS	2008	A	KM114057	-	-
Shizuoka 18	Japan	Shizuoka	2011	A	AB678778	-	-
V1/Gla/UK/2014	UK	Glasgow	2014	A	KP144343	-	-
Kyoto2	Japan	Kyoto	2003	A	AB779616	-	-
Kyoto3	Japan	Kyoto	2009	A	AB779615	-	-
Kyoto5	Japan	Kyoto	2009	A	AB779618	-	-
V1/Ed/UK/2012	UK	Edinburgh	2012	A	KP144350	-	-
V2/Ed/UK/2012	UK	Edinburgh	2012	A	-	KP129347	KP129367
V1/Ed/UK/2010	UK	Edinburgh	2010	A	-	KP129348	KP129368
TW/409/10	Taiwan	NS	2010	A	JQ946055	-	-
TW/1537/2011	Taiwan	NS	2011	A	JN582001	-	-
TW/391/10	Taiwan	NS	2010	A	JQ946053	-	-
TW/399/10	Taiwan	NS	2010	A	JQ946054	-	-
V7/Ed/UK/2013	UK	Edinburgh	2013	A	KP144342	-	-
V13/Ed/UK/2013	UK	Edinburgh	2013	A	KP144339	-	-

CU25/THA/2008	Thailand	Bangkok	2008	A	KX212500	KX212346	KX212536
CU47/THA/2009	Thailand	Bangkok	2009	A	KX212498	KX212344	KX212534
CU83/THA/2010	Thailand	Bangkok	2010	A	KX212495	KX212341	KX212531
CU84/THA/2010	Thailand	Bangkok	2010	A	KX212496	KX212342	KX212532
CU86/THA/2010	Thailand	Bangkok	2010	A	KX212499	KX212345	KX212535
TH/CU1499/A/2014	Thailand	Bangkok	2014	A	KX212503	KX212338	KX212528
TH/CU1508/A/2014	Thailand	Bangkok	2014	A	KX212504	KX212403	KX212593
TH/CU1420/A/2014	Thailand	Bangkok	2014	A	-	KX212380	KX212570
TH/CU1555/A/2014	Thailand	Khon kaen	2014	A	-	KX212404	KX212594
TH/CU1430/A/2014	Thailand	Bangkok	2014	A	-	KX212384	KX212574
TH/CU1271/A/2014	Thailand	Bangkok	2014	A	KX212505	KX212405	KX212595
TH/CU1443/A/2014	Thailand	Bangkok	2014	A	-	KX212385	KX212575
TH/CU1364/A/2014	Thailand	Bangkok	2014	A	-	KX212379	KX212569
TH/CU1379/A/2014	Thailand	Khon kaen	2014	A	-	KX212409	KX212599
TH/CU1483/A/2014	Thailand	Khon kaen	2014	A	-	KX212410	KX212600
TH/CU1552/A/2014	Thailand	Khon kaen	2014	A	-	KX212408	KX212598
TH/CU1433/A/2014	Thailand	Bangkok	2014	A	-	KX212417	KX212607
TH/CU1474/A/2014	Thailand	Bangkok	2014	A	-	KX212387	KX212577
TH/CU1393/A/2014	Thailand	Bangkok	2014	A	-	KX212378	KX212568
TH/CU1327/A/2014	Thailand	Bangkok	2014	A	-	KX212391	KX212581
TH/CU1421/A/2014	Thailand	Bangkok	2014	A	-	KX212396	KX212586
TH/CU1695/A/2015	Thailand	Bangkok	2015	A	-	KX212422	KX212612
TH/CU1278/A/2014	Thailand	Bangkok	2014	A	-	KX212419	KX212609
TH/CU1428/A/2014	Thailand	Bangkok	2014	A	-	KX212399	KX212589
TH/CU1389/A/2014	Thailand	Bangkok	2014	A	-	KX212381	KX212571
TH/CU1288/A/2014	Thailand	Bangkok	2014	A	-	KX212373	KX212563
TH/CU1330/A/2014	Thailand	Bangkok	2014	A	-	KX212375	KX212565
TH/CU1365/A/2014	Thailand	Bangkok	2014	A	-	KX212374	KX212564
TH/CU1398/A/2014	Thailand	Bangkok	2014	A	-	KX212418	KX212608
TH/CU1324/A/2014	Thailand	Bangkok	2014	A	-	KX212386	KX212576
TH/CU1373/A/2014	Thailand	Khon kaen	2014	A	-	KX212407	KX212597
TH/CU1292/A/2014	Thailand	Bangkok	2014	A	-	KX212402	KX212592
TH/CU1257/A/2014	Thailand	Bangkok	2014	A	-	KX212383	KX212573
DK/M22061/A/2014	Denmark	NS	2014	A	-	KX212411	KX212601
DK/F49465/A/2014	Denmark	NS	2014	A	-	KX212412	KX212602
DK/W38361/A/2014	Denmark	NS	2014	A	-	KX212413	KX212603
TH/CU1353/A/2014	Thailand	Khon kaen	2014	A	-	KX212420	KX212610
TH/CU1362/A/2014	Thailand	Bangkok	2014	A	-	KX212395	KX212585

TH/CU1391/A/2014	Thailand	Bangkok	2014	A	-	KX212406	KX212596
TH/CU1504/A/2014	Thailand	Bangkok	2014	A	-	KX212421	KX212611
DK/H36898/A/2014	Denmark	NS	2014	A	-	KX212416	KX212606
DK/T53016/A/2014	Denmark	NS	2014	A	-	KX212414	KX212604
TH/CU1608/A/2014	Thailand	Bangkok	2014	A	-	KX212393	KX212583
TH/CU1624/A/2015	Thailand	Bangkok	2015	A	-	KX212394	KX212584
TH/CU1548/A/2014	Thailand	Bangkok	2014	A	-	KX212392	KX212582
TH/CU1590/A/2014	Thailand	Bangkok	2014	A	-	KX212397	KX212587
TH/CU1599/A/2014	Thailand	Bangkok	2014	A	-	KX212398	KX212588
TH/CU1665/A/2015	Thailand	Bangkok	2015	A	-	KX212388	KX212578
TH/CU1302/A/2014	Thailand	Bangkok	2014	A	KX212501	KX212389	KX212579
TH/CU1648/A/2015	Thailand	Bangkok	2015	A	KX212502	KX212415	KX212605
TH/CU1558/A/2014	Thailand	Khon kaen	2014	A	-	KX212390	KX212580
TH/CU1296/A/2014	Thailand	Bangkok	2014	A	KX212506	KX212400	KX212590
TH/CU1493/A/2014	Thailand	Bangkok	2014	A	-	KX212401	KX212591
TH/CU1360/A/2014	Thailand	Bangkok	2014	A	-	KX212376	KX212566
TH/CU1404/A/2014	Thailand	Bangkok	2014	A	-	KX212377	KX212567
DK/T27464/A/2014	Denmark	NS	2014	A	-	KX212426	KX212616
ES/41833/A/2013	Spain	Teruel	2013	A	-	KX212425	KX212615
ES/40068/A/2013	Spain	Mallorca	2013	A	-	KX212427	KX212617
ES/41830/A/2013	Spain	Teruel	2013	A	-	KX212424	KX212614
ES/41816/A/2013	Spain	Teruel	2013	A	-	KX212423	KX212613
ES/41814/A/2013	Spain	Teruel	2013	A	-	KX212429	KX212619
DE/G4/A/2014	Germany	NS	2014	A	KX212508	KX212428	KX212618
TH/CU1415/A/2014	Thailand	Bangkok	2014	A	KX212507	KX212382	KX212572
ES/07754/A/2013	Spain	Salamanca	2013	A	-	KX212430	KX212620
ES/07766/A/2013	Spain	Salamanca	2013	A	-	KX212433	KX212623
ES/07767/A/2013	Spain	Salamanca	2013	A	-	KX212431	KX212621
ES/07726/A/2013	Spain	Salamanca	2013	A	-	KX212432	KX212622
DK/W17257/A/2014	Denmark	NS	2014	A	-	KX212434	KX212624
DE/G3/A/2014	Germany	NS	2014	A	KX212509	KX212435	KX212625
TH/CU1268/A/2014	Thailand	Bangkok	2014	A	-	KX212436	KX212626
TH/CU1307/A/2014	Thailand	Bangkok	2014	A	-	KX212437	KX212627
ES/14623/A/2013	Spain	Mallorca	2013	A	-	KX212351	KX212541
DE/G1/A/2014	Germany	NS	2014	A	-	KX212350	KX212540
DK/M2489/A/2014	Denmark	NS	2014	A	-	KX212354	KX212544
TH/CU1249/A/2014	Thailand	Bangkok	2014	A	-	KX212365	KX212555
DK/M59573/A/2014	Denmark	NS	2014	A	-	KX212352	KX212542

DK/W22166/A/2014	Denmark	NS	2014	A	-	KX212371	KX212561
TH/CU1251/A/2014	Thailand	Bangkok	2014	A	-	KX212357	KX212547
TH/CU1559/A/2014	Thailand	Bangkok	2014	A	-	KX212366	KX212556
DK/W21152/A/2014	Denmark	NS	2014	A	-	KX212353	KX212543
TH/CU1368/A/2014	Thailand	Bangkok	2014	A	KX212494	KX212368	KX212558
TH/CU1464/A/2014	Thailand	Bangkok	2014	A	-	KX212358	KX212548
TH/CU1270/A/2014	Thailand	Bangkok	2014	A	-	KX212369	KX212559
TH/CU1326/A/2014	Thailand	Bangkok	2014	A	-	KX212361	KX212551
TH/CU1382/A/2014	Thailand	Bangkok	2014	A	-	KX212359	KX212549
TH/CU1406/A/2014	Thailand	Bangkok	2014	A	KX212493	KX212364	KX212554
TH/CU1352/A/2014	Thailand	Bangkok	2014	A	-	KX212360	KX212550
TH/CU1427/A/2014	Thailand	Khon kaen	2014	A	-	KX212356	KX212546
TH/CU1519/A/2014	Thailand	Bangkok	2014	A	KX212492	KX212370	KX212560
TH/CU1318/A/2014	Thailand	Bangkok	2014	A	-	KX212367	KX212557
TH/CU1328/A/2014	Thailand	Bangkok	2014	A	-	KX212362	KX212552
TH/CU1298/A/2014	Thailand	Bangkok	2014	A	-	KX212363	KX212553
TH/CU1243/A/2014	Thailand	Bangkok	2014	A	KX212491	KX212355	KX212545
TH/CU1596/A/2014	Thailand	Bangkok	2014	A	-	KX212372	KX212562
DK/W15075/A/2014	Denmark	NS	2014	A	-	KX212348	KX212538
DK/S45898/A/2014	Denmark	NS	2014	A	-	KX212349	KX212539
TH/CU160/A/2012	Thailand	Bangkok	2012	A	KX212497	KX212343	KX212533
TH/CU209/A/2012	Thailand	Bangkok	2012	A	KX212489	KX212339	KX212529
TH/CU262/A/2012	Thailand	Bangkok	2012	A	KX212490	KX212340	KX212530
SE/116246/A/2014	Sweden	Stockholm	2014	A	-	KX212488	KX212673
SE/116413/A/2014	Sweden	Stockholm	2014	A	-	KX212483	KX212674
SE/117236/A/2014	Sweden	Stockholm	2014	A	-	KX212484	KX212675
SE/117446/A/2014	Sweden	Stockholm	2014	A	-	KX212485	KX212676
SE/506607/A/2014	Sweden	Stockholm	2014	A	-	KX212486	KX212677
SE/510615/A/2014	Sweden	Stockholm	2014	A	-	KX212487	KX212678
P115/2013/China	China	Wenzhou	2013	A	KP289367	-	-
P169/2013/China	China	Wenzhou	2013	A	KP289369	-	-
P2/2013/China	China	Wenzhou	2013	A	KP289370	-	-
P223/2013/China	China	Wenzhou	2013	A	KP289371	-	-
P225/2013/China	China	Wenzhou	2013	A	KP289372	-	-
P246/2013/China	China	Wenzhou	2013	A	KP289373	-	-
P345/2013/China	China	Wenzhou	2013	A	KP289376	-	-
P358/2013/China	China	Wenzhou	2013	A	KP289377	-	-
P360/2013/China	China	Wenzhou	2013	A	KP289378	-	-

P362/2013/China	China	Wenzhou	2013	A	KP289380	-	-
P366/2013/China	China	Wenzhou	2013	A	KP289381	-	-
P6/2013/China	China	Wenzhou	2013	A	KP289384	-	-
P66/2013/China	China	Wenzhou	2013	A	KP289385	-	-
P702/2013/China	China	Wenzhou	2013	A	KP289388	-	-
P731/2013/China	China	Wenzhou	2013	A	KP289390	-	-
SZc173/13	China	Shenzhen	2013	A	KF682362	-	-
SZc294/13	China	Shenzhen	2013	A	KF682363	-	-
5084/SH/CHN/2013	China	Shanghai	2013	A	KJ541154	-	-
PF19/SH/CHN/2013	China	Shanghai	2013	A	KJ541155	-	-
5047/SH/CHN/2013	China	Shanghai	2013	A	KJ541156	-	-
4645/SH/CHN/2013	China	Shanghai	2013	A	KJ541157	-	-
5069/SH/CHN/2013	China	Shanghai	2013	A	KJ541158	-	-
4592/SH/CHN/2013	China	Shanghai	2013	A	KJ541159	-	-
PF001/SH/CHN/2013	China	Shanghai	2013	A	KJ541165	-	-
1827/SH/CHN/2013	China	Shanghai	2013	A	KJ541166	-	-
1232/SH/CHN/2013	China	Shanghai	2013	A	KJ541167	-	-
3913/SH/CHN/2013	China	Shanghai	2013	A	KJ541168	-	-
4368/SH/CHN/2013	China	Shanghai	2013	A	KJ541169	-	-
12743/GZ/CHN/2013	China	Guangzhou	2013	A	KR815992	-	-
TS3/Fin/Tu81042/2008	Finland	NS	2008	B	-	KP129342	KP129362
Kyoto1	Japan	Kyoto	1999	B	AB779614	-	-
Kyoto4	Japan	Kyoto	2009	C	AB779617	-	-
HN421	China	Henan	2011	D	JQ964234	-	-
TW/295/09	Taiwan	NS	2009	E	JQ946052	-	-
TW/273/09	Taiwan	NS	2009	E	JQ946051	-	-
TW/20/09	Taiwan	NS	2009	E	JQ946050	-	-
TW-2007-00141	Taiwan	NS	2007	E	KR706309	-	-
V13/Ed/UK/2012	UK	Edinburgh	2012	F	KP144341	-	-
V12/Ed/UK/2013	UK	Edinburgh	2013	F	KP144340	-	-
DK/S48908/F/2014	Denmark	NS	2014	F	KX212510	KX212438	KX212628
DK/T52656/F/2014	Denmark	NS	2014	F	-	KX212439	KX212629
TH/CU1256/F/2014	Thailand	Bangkok	2014	F	KX212519	KX212460	KX212650
DK/S43334/F/2014	Denmark	NS	2014	F	KX212511	KX212441	KX212631
DE/G2/F/2014	Germany	NS	2014	F	KX212512	KX212442	KX212632
ES/06707/F/2014	Spain	Mallorca	2014	F	KX212513	KX212440	KX212630
TH/CU1383/F/2014	Thailand	Bangkok	2014	F	-	KX212463	KX212653
TH/CU1422/F/2014	Thailand	Bangkok	2014	F	-	KX212464	KX212654

TH/CU1440/F/2014	Thailand	Bangkok	2014	F	-	KX212467	KX212657
TH/CU1260/F/2014	Thailand	Bangkok	2014	F	KX212518	KX212462	KX212652
TH/CU1385/F/2014	Thailand	Bangkok	2014	F	-	KX212461	KX212651
TH/CU1407/F/2014	Thailand	Bangkok	2014	F	-	KX212470	KX212660
TH/CU1435/F/2014	Thailand	Bangkok	2014	F	-	KX212471	KX212661
TH/CU1265/F/2014	Thailand	Bangkok	2014	F	-	KX212469	KX212659
TH/CU1310/F/2014	Thailand	Bangkok	2014	F	-	KX212468	KX212658
TH/CU1526/F/2014	Thailand	Bangkok	2014	F	KX212517	KX212473	KX212663
TH/CU1392/F/2014	Thailand	Bangkok	2014	F	-	KX212447	KX212637
TH/CU1466/F/2014	Thailand	Bangkok	2014	F	-	KX212446	KX212636
TH/CU1329/F/2014	Thailand	Bangkok	2014	F	-	KX212458	KX212648
TH/CU1331/F/2014	Thailand	Bangkok	2014	F	KX212515	KX212448	KX212638
TH/CU1498/F/2014	Thailand	Bangkok	2014	F	-	KX212452	KX212642
TH/CU1332/F/2014	Thailand	Bangkok	2014	F	-	KX212451	KX212641
TH/CU1503/F/2014	Thailand	Bangkok	2014	F	-	KX212453	KX212643
TH/CU1465/F/2014	Thailand	Bangkok	2014	F	-	KX212445	KX212635
TH/CU1423/F/2014	Thailand	Khon kaen	2014	F	KX212516	KX212459	KX212649
TH/CU1426/F/2014	Thailand	Khon kaen	2014	F	-	KX212450	KX212640
TH/CU1424/F/2014	Thailand	Khon kaen	2014	F	-	KX212449	KX212639
TH/CU1350/F/2014	Thailand	Bangkok	2014	F	-	KX212455	KX212645
TH/CU1376/F/2014	Thailand	Bangkok	2014	F	-	KX212457	KX212647
TH/CU1372/F/2014	Thailand	Bangkok	2014	F	-	KX212465	KX212655
TH/CU1375/F/2014	Thailand	Bangkok	2014	F	-	KX212456	KX212646
TH/CU1374/F/2014	Thailand	Bangkok	2014	F	-	KX212466	KX212656
TH/CU1556/F/2014	Thailand	Khon kaen	2014	F	-	KX212472	KX212662
TH/CU796/F/2012	Thailand	Bangkok	2012	F	KX212514	KX212347	KX212537
V2/Ed/UK/2011	UK	Edinburgh	2011	G	KP144351	-	-
V3/Ed/UK/2013	UK	Edinburgh	2013	G	KP144353	-	-
V11/Ed/UK/2013	UK	Edinburgh	2013	G	KP144352	-	-
V1/Ed/UK/2011	UK	Edinburgh	2011	G	KP144349	-	-
DK/M22416/G/2014	Denmark	NS	2014	G	KX212526	KX212475	KX212665
ES/00507/G/2014	Spain	Mallorca	2014	G	-	KX212474	KX212664
DK/T24787/G/2014	Denmark	NS	2014	G	KX212527	KX212476	KX212666
V12/Ed/UK/2014	UK	Edinburgh	2014	H	-	KP129352	KP129374
V1/Ed/UK/2014	UK	Edinburgh	2014	H	-	KP129354	KP129373
V2/Ed/UK/2014	UK	Edinburgh	2014	H	-	KP129353	KP129372
V16/Ed/UK/2014	UK	Edinburgh	2014	H	KP144347	-	-
V4/Ed/UK/2014	UK	Edinburgh	2014	H	KP144348	-	-

V6/Ed/UK/2014	UK	Edinburgh	2014	H	KP129370	-	-
DK/S52397/H/2014	Denmark	NS	2014	H	KX212524	KX212477	KX212667
DK/T22324/H/2014	Denmark	NS	2014	H	KX212525	KX212480	KX212670
DK/H15054/H/2014	Denmark	NS	2014	H	KX212522	KX212479	KX212669
DK/T36724/H/2014	Denmark	NS	2014	H	KX212523	KX212478	KX212668
ES/06417/H/2014	Spain	Vigo	2014	H	KX212521	KX212482	KX212672
ES/04587/H/2013	Spain	Madrid	2013	H	KX212520	KX212481	KX212671
P143/2013/China	China	Wenzhou	2013	J	KP289368	-	-
P278/2013/China	China	Wenzhou	2013	J	KP289374	-	-
P309/2013/China	China	Wenzhou	2013	J	KP289375	-	-
P361/2013/China	China	Wenzhou	2013	J	KP289379	-	-
P406/2013/China	China	Wenzhou	2013	J	KP289382	-	-
P426/2013/China	China	Wenzhou	2013	J	KP289383	-	-
P674/2013/China	China	Wenzhou	2013	J	KP289386	-	-
P695/2013/China	China	Wenzhou	2013	J	KP289387	-	-
P728/2013/China	China	Wenzhou	2013	J	KP289389	-	-
P786/2013/China	China	Wenzhou	2013	J	KP289391	-	-
P794/2013/China	China	Wenzhou	2013	J	KP289392	-	-
5039/SH/CHN/2013	China	Shanghai	2013	J	KJ541160	-	-
5056/SH/CHN/2013	China	Shanghai	2013	J	KJ541161	-	-
PF3/SH/CHN/2013	China	Shanghai	2013	J	KJ541162	-	-
PF1/SH/CHN/2013	China	Shanghai	2013	J	KJ612513	-	-
P289/2013/China	China	Wenzhou	2013	K	KP289365	-	-
P423/2013/China	China	Wenzhou	2013	K	KP289366	-	-
P874/2013/China	China	Wenzhou	2013	K	KP289393	-	-
CC13/57	China	Changchun	2013	K	KM279379	-	-

NS: non specified

Table S2: Primer set used for whole genome amplification of human coxsackievirus A6
by nested RT-PCR

No.	Primer	Sequence (5' to 3')	Position
1	CAV6-F39/OS	ACT GGG CGC YAG CAC ACT GAT TC	39 - 61
	CAV6-R1673/OAS	AGT TAR TGT RAT TGG YAC YTC TGT	1650 - 1673
	CAV6-F55/IS	CTG ATT CTA YGG AAY CTT TGT GCG	55 - 78
	CAV6-R1567/IAS	GGA AGY GCR TTR ACA TAT GGC AT	1545 - 1567
2	CAV6-F1266/OS	TCG GGY TTC TGY ATG CAY GTT CA	1266 - 1288
	CAV6-R2806/OAS	GAY AGT TCT AGY TTG CGC CGC TG	2784 - 2806
	CAV6-F1290/IS	TGY AAY GCR AGC AAR TTC CAT CA	1290 - 1312
	CAV6-R2727/IAS	CCG AGT CCT TYA CCT CCA CAA C	2706 - 2727
3	CAV6-F2458/OS	CRA ATG CDG TGG AAA GYG CTG T	2458 - 2479
	CAV6-R3832/OAS	CCT TTG ATR TAA TCW GAY ACD CC	3810 - 3832
	CAV6-F2485/IS	GCR CTY GCT GAY ACC ACA ATA TC	2485 - 2506
	CAV6-R3816/IAS	GAC ACC CTG YTC CAT RGC TTC	3795 - 3816
4	CAV6-F3498/OS	GCT CAR GGA TGT GAY ACY ATT GC	3498 - 3520
	CAV6-R4564/OAS	CTA GAG TGR TAY TTR TCR GCT AT	4542 - 4564
	CAV6-F3603/IS	GTC TTY GTG GAA GCT AGT GAG TA	3603 - 3625
	CAV6-R4463/IAS	ACG GTG TTT GCT CTT GAA CTG CAT	4440 - 4463
5	CAV6-F4107/OS	AGY GCA TCN TGG CTH AAG AAG TT	4107 - 4129
	CAV6-R5482/OAS	TGA TCY GTY TGV ACY TGC CTR AT	5460 - 5482
	CAV6-F4214/IS	TRT ACC AGC AGC TAA AGA GAA GGT	4214 - 4237
	CAV6-R5330/IAS	GTA GAT RAC ATA CAC CAR TGA RAC	5307 - 5330
6	CAV6-F4994/OS	ATC CAA RGT BAG RTA YAG TGT GGA	4994 - 5017
	CAV6-R6422/OAS	GAG RTC AAR DCC ATA CTT RTC CAT	6399 - 6422
	CAV6-F5061/IS	GCY ATT GGN AAC ACA ATC GAA GC	5061 - 5083
	CAV6-R6364/IAS	GGG TCY AAR ATG TCY CTC TTC TT	6342 - 6364

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VITA

Name: Miss Jiratchaya Puenpa

Birthday: August 8, 1987   Birth place: Bangkok, Thailand

Education:

2013-2016   Ph.D., Biomedical Science, Faculty of Medicine, Chulalongkorn University

2010-2012   M.Sc., Biomedical Science, Faculty of Medicine, Chulalongkorn University

2005-2008   B.Sc., Biochemistry, Faculty of Science, Chulalongkorn University

Research Experiences:

2015   Visiting scholar at the Roslin Institute, University of Edinburgh, UK

Publications:

1.   Puenpa J, Vongpunsawad S, Osterback R, Waris M, Eriksson E, Albert J, Midgley S, Fischer TK, Eis-Hübinger AM, Cabrerizo M, Gaunt E, Simmonds P, Poovorawan Y. Molecular epidemiology and the evolution of human coxsackievirus A6. J Gen Virol. (pending)

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