PREVALENCE OF HUMAN ENTEROVIRUS AMONG PATIENTS WITH HAND, FOOT, AND MOUTH DISEASE AND HERPANGINA IN THAILAND, 2013

John Mauleekoonphairoj, Jiratchaya Puenpa, Sumeth Korkong, Sompong Vongpunsawad and Yong Poovorawan

Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Abstract. Human enterovirus (EV) infection causes hand, foot, and mouth disease (HFMD) and herpangina (HA). We studied the prevalence of enterovirus (EV) among patients with HFMD and HA in Thailand during 2013. We conducted a study in archived specimens of patients sent for screening for enterovirus. A total of 203 clinical specimens from 184 individuals with painful blister in the oropharynx and on the palms, soles, knees, elbows or buttock were examined by semi-nested polymerase chain reaction (PCR) for the 5'UTR and VP1 genes of EV. Eighty-six samples were positive: EV71 was detected in 14 (30%), CV-A8 in 12 (26%) and CV-A16 in 10 (21%). Classification of EV species detected revealed that 46 specimens were EV-A, 14 specimens were EV-B, 1 specimen was EV-D, and 16 specimens were positive for unclassified enterovirus. The majority of individuals with EV infection were aged 2-6 years. Multiple EV-A serotypes were detected among HFMD and HA patients in our study.

Keywords: enterovirus, prevalence, Thailand

INTRODUCTION

Human enteroviruses (EV) cause hand, foot, and mouth disease (HFMD) and herpangina (HA) especially among young children (Lee *et al*, 2009; Chatproedprai *et al*, 2010; Solomon *et al*, 2010). Enterovirus 71 (EV71) and coxsackievirus A16 (CV-A16) are two serotypes of enterovirus commonly associated with HFMD outbreaks (Puenpa *et al*, 2013; Linsuwanon *et al*, 2014). In recent years other EV serotypes have been linked to HFMD, such as CV-A6 and CV-A10 (Blomqvist *et al*, 2010). During 2012 in Thailand, there was a 3-fold increase in the incidence of HFMD compared to the average incidence during 2007-2011 in which the majority of cases were caused by CV-A6 (Puenpa *et al*, 2013). There is no effective antiviral treatment or vaccine to prevent EV infection, although EV surveillance can limit the spread of the virus through early intervention (Podin *et al*, 2006; Ang *et al*, 2009).

Enteroviruses are single-stranded, positive-sense RNA viruses with approximately a 7.4 kb genome and are members

Correspondence: Prof Yong Poovorawan, Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.
Tel: 66 (0) 2256 4909; Fax: 66 (0) 2256 4929
E-mail: yong.p@chula.ac.th

of the genus *Enterovirus* and the family Picornaviridae (van der Sanden *et al*, 2009). The viral genomic RNA is encapsulated in a small, non-enveloped, icosahedral particle containing 60 identical subunits of the capsid protein (Plevka *et al*, 2012). Each subunit is assembled from four structural proteins (VP1 through VP4) (Brown and Pallansch, 1995). VP1, VP2, and VP3 proteins are exposed to the external environment, while VP4 is completely internalized. All structural proteins are encoded by the P1 region of the genome; non-structural proteins located in the P2 and P3 regions encode for the 2A-C and 3A-D genes, respectively (Solomon *et al*, 2010).

There are four human EV species (EV-A through EV-D) based on molecular and biological properties of the virus (Nasri *et al*, 2007). A member of the EV species A, EV71 can be subdivided into types A, B, and C by the VP1 gene, with >15% divergence separating each type (Brown *et al*, 1999). EV-71 type A consists of only the BrCr strain (Brown and Pallansch, 1995). Types B and C can be further separated into B1 to B5 and C1 to C5, respectively (Huang *et al*, 2008; Tan *et al*, 2011). CV-A16 is comprised of A and B genotypes, with genotype B being further subdivided into types B1 and B2 (Perera *et al*, 2007; Zhang *et al*, 2010).

Although HFMD is generally self-limited, severe complications can involve the central nervous system, such as aseptic meningitis, acute flaccid paralysis and encephalitis (McMinn, 2002). As part of the continuing effort in the EV surveillance following the severe HFMD outbreaks in 2012, we assessed the prevalence of EV in Thailand during 2013 among patients with suspected and confirmed HFMD and HA.

MATERIALS AND METHODS

Sample collection

We conducted a retrospective study of 203 archived specimens from 184 individuals previously sent for screening for enteroviruses to the Center of Excellence in Clinical Virology, King Chulalongkorn Memorial Hospital, as part of the enterovirus surveillance. The specimens were stool, rectal swab, throat swab, nasal swab, nasopharyngeal (NP) suction, vesicle fluid, and cerebrospinal fluid of patients suspected to have HFMD and HA. Multiple samples were obtained from the same individual in some cases. HFMD was defined as a patient with painful blisters in the oropharynx and on the palms, soles, knees, elbows or buttocks. HA was defined as having painful blisters only in the mouth, predominantly on the soft plate. Samples were sent from various parts of Thailand (Bangkok, Chon Buri, Khon Kaen and Uttaradit) between January and December 2013.

This study was approved by the institutional review board of the Faculty of Medicine, Chulalongkorn University (IRB 374/57). Permission was obtained from the director of King Chulalongkorn Memorial Hospital to perform the study. Samples were de-identified and anonymous, so informed consent was not obtained from subjects.

Detection and typing of enterovirus

Viral nucleic acid was extracted from the samples using a Viral Nucleic Acid Extraction Kit following the manufacturer's instructions (RBC Bioscience, Taipei, Taiwan). Complementary DNA amplification was performed using random hexamers. The VP1 gene was amplified using specific primers for EV71/CA16, CA6, and CA8 (Linsuwanon *et al*, 2014; Puenpa *et al*,

2014). COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) for EV were also used (Nix *et al*, 2006). For the latter, cDNA synthesis was performed using primers AN32, AN33, AN34, and AN35. In the subsequent nested PCR, primers 222 and 224 were used in the first round, and AN88 and AN89 were used in the second round. Amplicons 350-400 base-pairs in size were then purified (GeneAll Biotechnology, Seoul, Korea) and sequenced (1stBASE Laboratories, Seri Kembangan, Malaysia). Molecular typing relied on the partial VP1 sequence with the highest score of nucleotide similarity using the Basic Local Alignment Search Tool (BLAST) found on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/).

Sequence analysis

Sequences were analyzed using the Seqman program, part of DNASTAR Software (v5.0). Nucleotide identities were calculated with the BioEdit Sequence Alignment Editor (v7.0.9.0). Phylogenetic trees were constructed from Clustal W alignments of partial nucleotide sequences obtained from the CODEHOP results using the neighbor-joining method and Kimura's two-parameter distance model implemented in MEGA (version 5.0). Pair-wise deletions were used for missing data. A bootstrap of 1,000 replicates was used in construction of phylogenetic tree and strong tree topology supported by a bootstrap value ≥ 70%.

RESULTS

In this study, individuals with confirmed or suspected HFMD or HA were aged 16 days to 50 years (mean age 5.5 ± 7.1 years; median age 4 years). The male-to-female ratio was 1:1.1. Fifty-three percents of subjects (112/184) had HFMD and 19% (36/184) had HA, the clinical diagnosis for the remaining cases was either incomplete or inconclusive.

Eighty-six clinical specimens tested positive for EV; EV71 was most frequently detected (30%, *n*=14), followed by CV-A8 (26%, *n*=12) and CV-A16 (21%, *n*=10). Among positive samples, 63 specimens were EV-A, 14 specimens were EV-B, and 1 specimen was EV-D. Sixteen enterovirus specimens were unclassified. EV-C was not identified in any of the samples tested.

The number of specimens during the studied year (2013) peaked during January-March and July-September (Fig 1). These two periods account for 33% and 33% of all positive specimens recieved, respectively. During January-March, CV-A8 (*n*=12) and echovirus 16 (*n*=7) were the most prevalent, while different serotypes of enteroviruses were detected during July-September. EV-A infection occurred throughout the year, while EV-D infection was only detected during August.

EV was detected in 60 HFMD and 14 HA patients. The majority of HFMD (88%) and HA(58%) cases were due to EV-A (Fig 2, i). The only EV-C identified in this study was from an HA patient (data not shown). The majority (44%) of HFMD and HA infection occurred among subject aged 2-6 years (Fig 2, ii). HFMD occurred in all age groups, but there were no HA cases reported among individuals aged < 6 months or > 18 years.

Phylogenetic analysis showed distinct separations among the three species of EV detected: EV-A, EV-B and EV-D (Fig 3). One Thai EV71 strain (EV_1085) was likely subgenotype B5, while another (EV_1075) was closely related to subgenotype C4. All Thai CA16 strains were clustered into subgenotype B1. All CV-A8

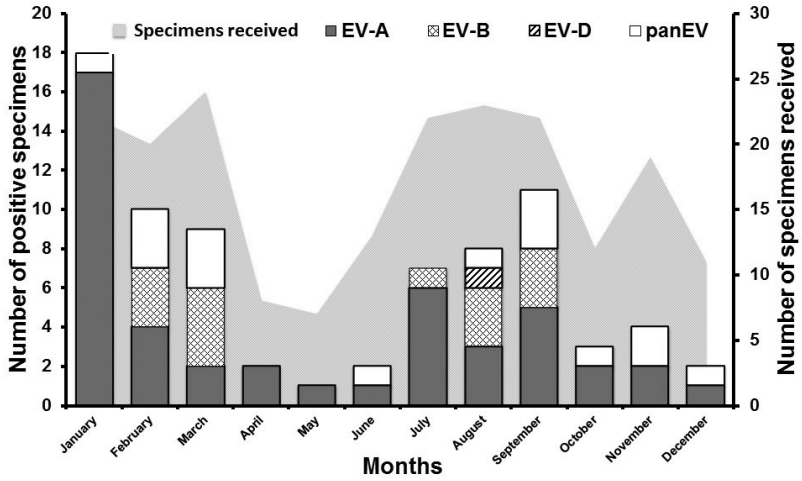

Fig 1–Distribution of HFMD and HA cases during 2013 and enterovirus (EV) species detected by month. Gray background indicates the number of samples received. Bar graph denotes EV species (A, B, D) identified. Unclassified pan-enterovirus assay-positive samples are uncolored.

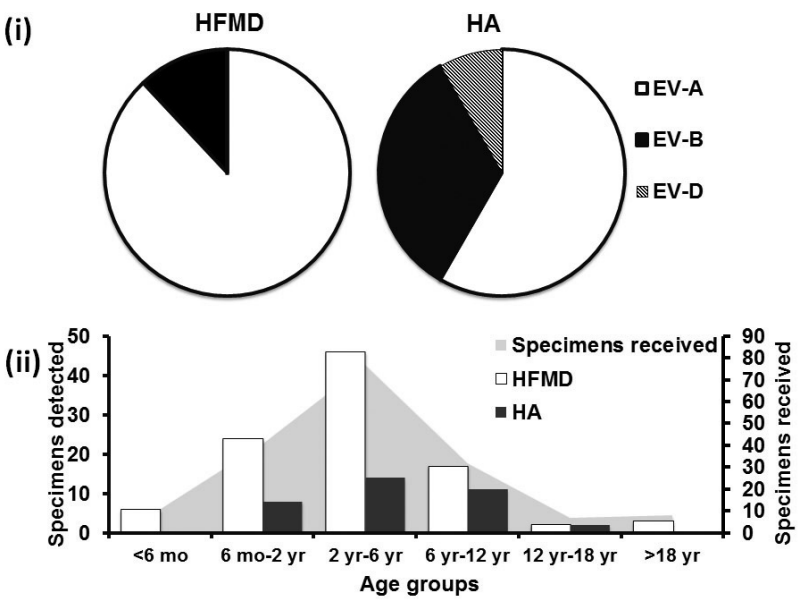

Fig 2–Enterovirus species among HFMD and HA cases. Proportions of EV species were identified (i) and the distribution of disease by age group (ii). Gray background indicates cases by age.

strains were detected in January (nucleotide identities 96.3%-100%). Approximately 50% (n=7) of all EV-B isolates detected were echovirus 16 and were found during February-March (nucleotide identities 96.3%-100%). The only EV-D detected was EV68.

DISCUSSION

HFMD and HA are caused by EV infection; outbreaks have been reported from multiple countries and are a major public health problem in Thailand (Chan *et al*, 2003; Puenpa *et al*, 2013; Linsuwanon *et al*, 2014; Oliveira *et al*, 2014; Zander *et al*, 2014). This study reported the prevalence of EV circulating during 2013 identified in HFMD and HA patients. Numerous enterovirus serotypes were detected during this period with EV71 (n=14), CV-A8 (n=12) and CV-A16 (n=10) being the three most common serotypes identified.

In our finding from another study from Thailand (Puenpa *et al*, 2014) conducted during 2012 when CV-

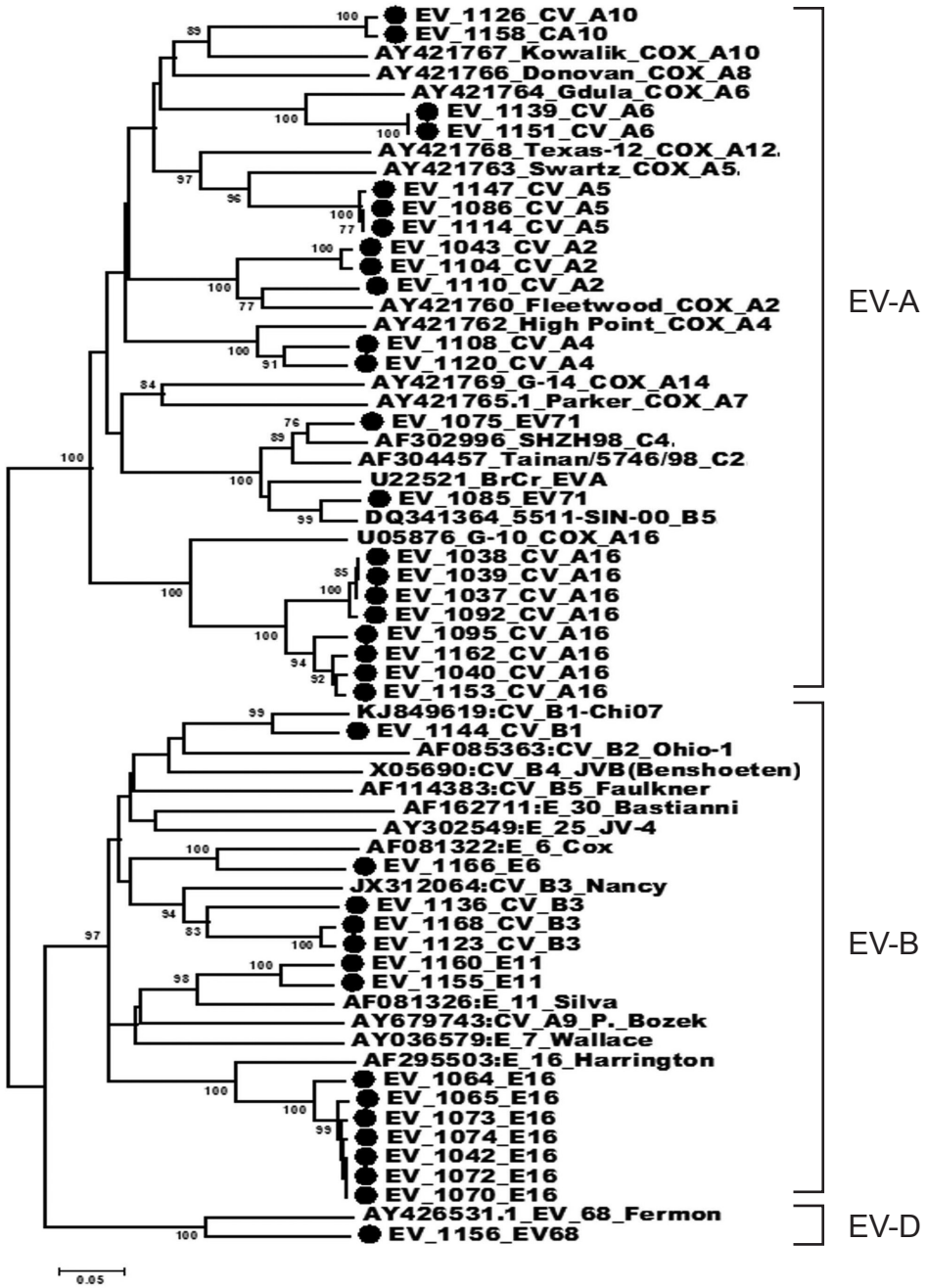

Fig 3–Phylogenetic tree of enterovirus strains detected by CODEHOP analysis. Prototypic strains of each serotype obtained from GenBank were used as references. Trees were constructed with MEGA (v5.0) using the neighbor-joining method and the maximum likelihood with 1,000 bootstrap replicates corrected using the Kimura-two-parameter substitution model. Bootstrap values ≥ 70% are indicated at the nodes. The scale bar indicates the number of substitutions per site. Strains from this study are denoted by a closed circle.

Table 1
Enterovirus species and numbers of specimens detected (n=61).

Enterovirus species	Serotypes	Specimens detected (%)
A	EV71	14 (23)
	CV-A8	12 (20)
	CV-A16	10 (16)
	CV-A6	3 (5)
	CV-A5	3 (5)
	CV-A2	2 (3)
	CV-A10	1 (2)
	CV-A4	1 (2)
B	ECHOVIRUS 16	7 (11)
	CV-B3	3 (5)
	ECHOVIRUS 11	2 (3)
	CVB1	1 (2)
	ECHOVIRUS 6	1 (2)
D	EV D68	1 (2)

A6 was the predominant strain detected. The difference between their 2012 study and our 2013 study may be due to immunity acquired against CV-A6 during 2012 resulting in fewer cases due to this strain during our 2013 study. The above 2012 study found CV-A8 to be more common in HA but we found it to be more common among HFMD patients (Puenpa *et al,* 2014).

Although members of EV species A (CV-A6, CV-A10, CV-A16, and EV-71) have been most frequently implicated in HFMD and HA, an increasing number of studies in recent years have reported an association between EV species B and HFMD (Hu *et al,* 2012; Wei *et al,* 2012). In our study 16% of all EV positive specimens were EV-B. EV-B was the most common EV found in stool specimens from healthy children in another study (Wu *et al,* 2013). One patient in our study with HA and influrenza-like illness had EV-D68. Since EV-D68 had been primarily associated with respiratory disease but not HA, we concluded EV-D68 did not cause HA.

Our study had several limitations. Since it only surveyed enteroviruses during 2013 from 4 provinces in Thailand; therefore it does not represent Thailand as whole. The study group was small so less common EV serotypes may have gone undetected. Phylogenetic analysis relied on the partial nucleotide sequences obtained from CODEHOP and did not include those obtained from using serotype-specific primers because the sequence regions differed so there is potential for misidentification. The patient charts were not reviewed so we were not able to correlate symptoms with serotypes and were forced to rely on reported data. Our study does provide more data regarding EV and HFMD and HA which can be used in determining trends in EV infection.

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