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SHORT COMMUNICATION

High prevalence of coxsackievirus A2 in children with herpangina in Thailand in 2015

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Abstract Coxsackievirus (CV) is a member of the genus *Enterovirus* and the family *Picornaviridae*. CV infection can cause herpangina, a disease characterized by multiple ulcers on the tonsils and soft palate affecting mostly young children. CV strains are categorized by serotypes. Unfortunately, serotypes responsible for infections in patients are often undetermined. This knowledge gap partly contributes to the ineffective prevention and control of CV-associated herpangina in Southeast Asia. To characterize the viral etiology of children presented with herpangina, 295 throat swabs were tested for human enterovirus infection. Using RT-PCR specific for the viral 5′UTR/VP2 and the VP1 regions, two most frequent CV types found in these samples were CV-A2 (33.33%, 40/120) and CV-A4 (15.8%, 19/120). Phylogenetic analysis of the VP1 gene demonstrated that the CV-A2 strains in this study not only were closely related to those previously identified in Asia and Europe, but the majority clustered into a distinct group. Thus, infection predominantly by CV-A2 and CV-A4 caused herpangina in 2015 in Thailand.

Human enteroviruses are commonly associated with a wide spectrum of acute and chronic diseases affecting the gastrointestinal tract [7]. Infection can be asymptomatic or manifests in fever, multiple oral ulcers, diarrhea, vomiting, and vesicular rash on the hands, feet and mouth. In some cases, infection can lead to acute flaccid paralysis, severe complications of the nervous system, myocarditis, and pulmonary edema. Herpangina, a primarily pharyngeal infection in children caused by human enterovirus of the family *Picornaviridae*, is characterized by multiple oral ulcers predominantly on the soft palate and the posterior of the oral cavity. Although symptoms often spontaneously resolve within 1–2 weeks, infection contributes significantly to childhood morbidity in the Asia–Pacific region and elsewhere around the world [12].

Enterovirus 71 and several coxsackievirus (CV) serotypes (CV-A2, -A4, and -A10) are frequently implicated in herpangina and hand-foot-mouth disease. Their predominance and prevalence varied with geographical locations, seasonality, and population susceptibility [1, 4, 14]. Determinants of clinical manifestation and disease severity have been linked to specific serotypes and co-infection status, which can sometimes lead to death [10]. Surveillance of outbreaks is therefore important in determining the viral etiology and transmission among young children who are most at risk.

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

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Chulalongkorn University (IRB number 286/58). All specimens were de-identified and anonymous, therefore the IRB waived the need for consent. Inclusion criteria for herpangina symptoms include the appearance of oral ulcers in the mouth but not elsewhere on the body. Approximately 86% of the children were ≤5 years-old (mean age 3.32).

Viral RNA was extracted using the Exgene Viral DNA/RNA kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. We initial screened for enterovirus genome region spanning the conserved 5′ untranslated region (5′UTR) and VP4/VP2 gene using conventional RT-PCR as previously described [6]. Subsequently, partial VP1 region was amplified with degenerate primers to identify the enterovirus species [8]. Nucleotide sequences were edited and analyzed with Chromas Lite (version 2.01), BioEdit (version 7.0.4.1), and BLAST (http://blast.ncbi.nlm.nih.gov/). Nucleotide sequences of the CV-A2 identified in this study were deposited in GenBank (accession numbers KX021203-KX021265). Moreover, CV-A2 strains were verified by an additional semi-nested PCR using degenerate primers CA2_F2763 (5′-TGG GAT ATA GAY ATA ATG GGG TA-3′), CA2_R3256 (5′-GCR GTG TAR TTT GGG AAA TTC TT-3′), and CA2_R3029 (5′-AAA AGT GGG RTA WCC ATC ATA GAA-3′) followed by sequencing. Reconstruction of the phylogeny trees was done using the neighbor-joining method and Maximum Composite Likelihood model with MEGA v.5.0 [13]. Pairwise deletions were used for missing data and the robustness of the tree was determined by bootstrapping with 1000 pseudo-replicates.

In all, 120 samples (40.7%) tested positive for human enterovirus, of which 35.8% (43/120) were CV-A2, 15.8% (19/120) were CV-A4, 10.8% (13/120) were CV-A16, and 10% (12/120) were CV-A6 (Fig. 1). The remaining samples tested positive for other human enterovirus of species A, B and C. The majority of positive samples were obtained in the rainiest months (July–September).
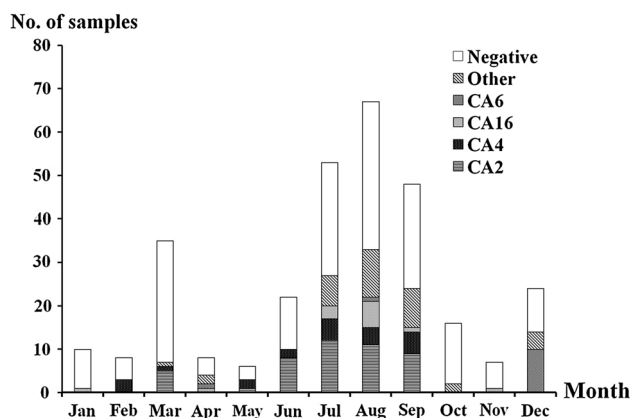

Fig. 1 Distribution of herpangina samples tested positive for coxsackievirus in this study

Consequently, most CV-A2 were found in June to September. Interestingly, CV-A6 comprised the majority of the virus identified in December.

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

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

Outbreaks of human enterovirus infection represent significant socio-economic burden to countries in resource-limited setting. In 2012, the unprecedented predominance of CV-A6 circulation in Thailand illustrated the ability for CV to rapidly and efficiently spread [11]. Other countries in the region have continued to report outbreaks of herpangina associated with CV-A2 and CV-A4 including Taiwan in 2008, mainland China in 2009–2014, and Korea in 2009. Unlike the more severe infections caused by CV-A16 and enterovirus 71, very few or no fatalities have been associated with CV-A2 and CV-A4 infection. The viral strains in this study were very similar to those identified in previous years in East Asia probably due to continued virus co-circulations in the region.

VP1 is the determinant of viral pathogenesis and virulence, especially the antigenic BC-loop region [5]. In addition to the error-prone replication mechanism of the human enterovirus, frequent recombination resulting from the exchange of structural and non-structural genes has allowed the virus to escape acquired host-immunity. The transmission of this rapidly evolving virus is thought to be mediated by the continuous interaction between spatiotemporal dispersion and natural selection process. This
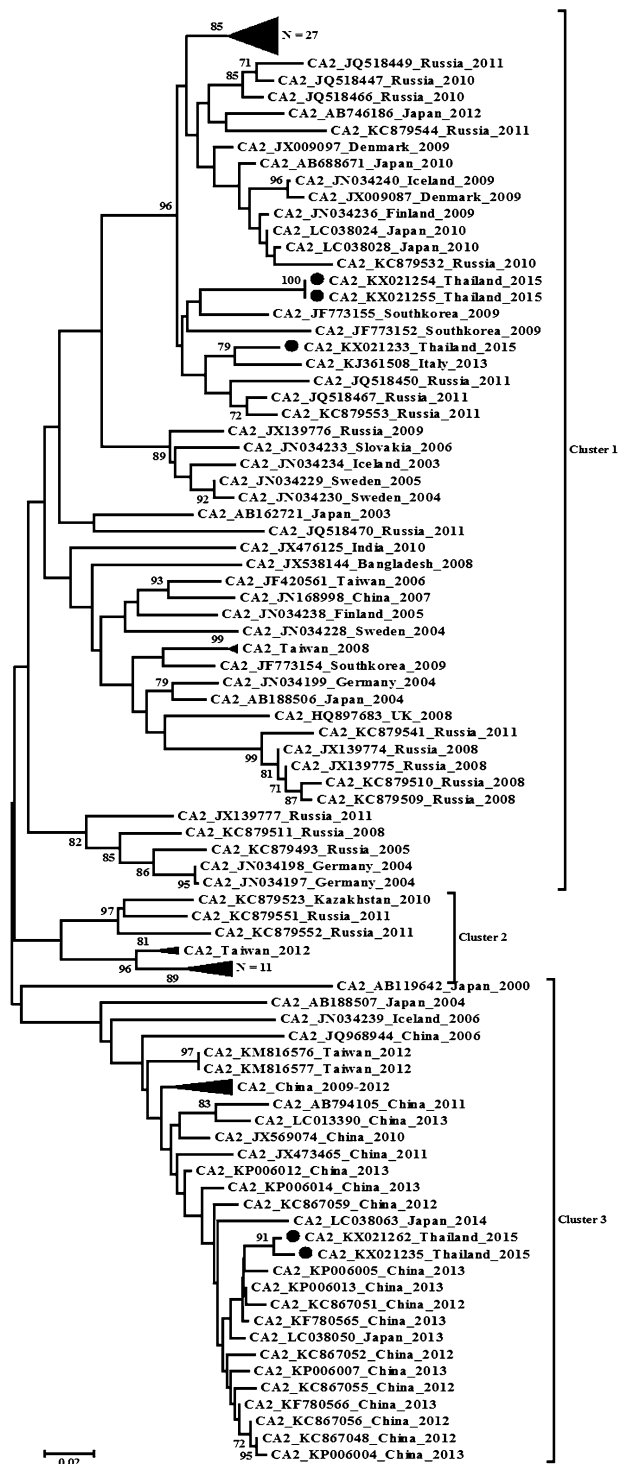
Fig. 2 Phylogenetic analysis of the partial VP1 nucleotide sequences of CV-A2. Phylogenetic trees were constructed by the neighbor-joining method implemented in MEGA v.5. Strains identified in this study are denoted as *black dots* or *triangles* (when many strains clustered together). Bootstrap resampling values >70 are indicated at the nodes. The *scale bar* indicates the number of substitutions per site

hypothesis is supported by studies demonstrating the rapid turnover of the CV-A4 [2] and the genetic divergence of CV-A6 [3], which has resulted in viral variants associated with novel, often more severe, clinical findings. Variations among subtypes may therefore increase viral diversity and result in a continued prevalence of CV. Examining the evolutionary rates of emerging CV variants will be useful when combined with vigilant epidemiological surveillance. Although we were unable to identify disease etiology in over half of the samples ($\sim 60\%$), past studies have also reported the inability to amplify the viral nucleic acid in a significant number of samples [9]. However, this is not expected to influence the diversity of the CV species identified. Frequent CV infection in many countries therefore requires good public health measures to reduce the incidence of herpangina and the socioeconomic impact of this disease.

Compliance with ethical standards

Conflict of interest None.

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