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# Longitudinal study on enterovirus A71 and coxsackievirus A16 genotype/subgenotype replacements in hand, foot and mouth disease patients in Thailand, 2000–2017

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ABSTRACT

*Background:* Enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16) are the major causative agents of hand, foot and mouth disease (HFMD) worldwide, particularly in the Asia-Pacific region. Several strains have emerged, circulated, and faded out over time in recent decades. This study investigated the EV-A71 and CV-A16 circulating strains and replacement of genotypes/subgenotypes in Thailand during the years 2000–2017.

*Methods:* The complete VP1 regions of 92 enteroviruses obtained from 90 HFMD patients, one asymptomatic adult contact case, and one encephalitic case were sequenced and investigated for serotypes, genotypes, and subgenotypes using a phylogenetic analysis.

*Results:* The 92 enterovirus isolates were identified as 67 (72.8%) EV-A71 strains comprising subgenotypes B4, B5, C1, C2, C4a, C4b and C5, and 25 (27.2%) CV-A16 strains comprising subgenotypes B1a and B1b. Genotypic/subgenotypic replacements were evidenced during the study period. EV-A71 B5 and C4a have been the major circulating strains in Thailand for more than a decade, and CV-A16 B1a has been circulating for almost two decades.

*Conclusions:* This study provides chronological data on the molecular epidemiology of EV-A71 and CV-A16 subgenotypes in Thailand. Subgenotypic replacement frequently occurred with EV-A71, but not CV-A16. Monitoring for viral genetic and subgenotypic changes is important for molecular diagnosis, vaccine selection, and vaccine development.



## Introduction

Hand, foot and mouth disease (HFMD) is an acute febrile illness that mainly occurs in young children under 5 years of age, with characteristic vesicular eruptions on the hands, feet, mouth, and buccal cavity (Kashyap and Kashyap, 2015; Esposito and Principi, 2018). The disease can be caused by many enterovirus (EV) serotypes, but mainly by enterovirus A71 (EV-A71) and

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coxsackievirus (CV) A16 (CV-A16) (Ang et al., 2009; He et al., 2013; Huang et al., 2015a,b; Lei et al., 2015). Nevertheless, CV-A6 caused large epidemics in several Asia-Pacific countries from 2008 to 2017 and has subsequently become an endemic strain (Wu et al., 2010; Puenpa et al., 2013; Xu et al., 2015; Anh et al., 2018). Sporadic cases of HFMD associated with the other enteroviral serotypes have occasionally been reported, including CV-A4, CV-A5, CV-A6, CV-A7, CV-A9, CV-A10, CV-B1, CV-B2, CV-B3, CV-B5, and echovirus 7 (Blomqvist et al., 2010; Yang et al., 2011; Xu et al., 2013; Xu et al., 2015; Guo et al., 2015). Patients with HFMD mostly develop mild and self-limited symptoms, and serious complications are infrequently observed. Severe neurological symptoms are more common with EV-A71 and include aseptic meningitis, brain stem and/or cerebellar encephalitis, acute flaccid paralysis, neurogenic pulmonary edema and hemorrhage, and acute myocarditis (He et al., 2013; Lee, 2016). Approximately 10% of these severe complications have been observed with CV-A16 infection (Liu et al., 2015).

EV-A71 and CV-A16 viruses belong to the genus *Enterovirus* within the family *Picornaviridae*. The viral genome is a positive-sense, single-stranded RNA molecule of approximately 7.4 kilobases (kb) in length. The genome comprises one open reading frame (ORF) that encodes four structural or viral capsid proteins (VP1, VP2, VP3, and VP4) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Lei et al., 2015; Yuan et al., 2018). The VP1 region, the major neutralizing antigenic site, is more genetically variable than the other capsid proteins and is the most informative region for studying the evolutionary relationship among enteroviruses (Oberste et al., 1999; Tee et al., 2010; Yuan et al., 2018). Cardosa et al. (2003) suggested that VP1 and VP4 provide similar phylogenetic information, but the bootstrap values observed in the VP1 dendrograms provide greater confidence, particularly in the study of new genotypes.

Based on the VP1 nucleotide sequences, EV-A71 viruses are phylogenetically classified into three genotypes: A, B, and C. Genotype A contains only a single member, BrCr-CA-70, identified in a patient with encephalitis in California, USA, in 1970 (Brown et al., 1999). All of the current EV-A71 strains belong to genotypes B and C, which are further classified into multiple subgenotypes, i.e., B0, B1, B2, B3, B4 and B5, and C1, C2, C3, C4a, C4b and C5 (Huang et al., 2011; Yip et al., 2013; Yee and Laa Poh, 2017). Moreover, proposed genotypes D and G have been reported in India from patients with acute flaccid paralysis (Saxena et al., 2015), and the recent genotypes E and F were discovered in Africa and Madagascar, respectively (Bessaud et al., 2014). Similarly, based on the VP1 sequences, CV-A16 is classified into two genotypes, A and B. The prototype G-10 is the only member of genotype A, while genotype B is further divided into subgenotypes B1a, B1b, B1c and B2 (Zhang et al., 2010a; Chen et al., 2013; Mao et al., 2014). In the other classification system, which is based on VP4 sequences, CV-A16 strains isolated between 1951 and 2004 have been classified into three lineages: A, B, and C (Li et al., 2005).

HFMD was recognized as an emerging disease after it caused a large outbreak in Malaysia in 1997 (AbuBakar et al., 1999; Chan et al., 2000). HFMD has been a notifiable disease for the Ministry of Public Health in Thailand since 2001. The number of cases increases each year, which is partly due to the establishment of the reporting system. The disease reaches its peak in the rainy season between June and October annually. Small clusters of HFMD cases are observed each year and are mostly associated with EV-A71 or CV-A16 infections (Puenpa et al., 2011; Piriyapornpipat et al., 2014). An exception was found in 2011–2012 when CV-A6 infection was predominant over the other viral strains in many large outbreaks, before subsiding and persisting as a circulating strain (Puenpa et al., 2013, 2014, 2018; Linsuwanon et al., 2014), with a prevalence rate next to that of EV-A71 and CV-A16. The symptoms of CV-A6-associated HFMD are usually milder (Xu et al., 2015; Huang et al., 2015a,b).

EV-A71 has genetically evolved rapidly, and information about CV-A16 is seldom available. Hence this longitudinal study on genotypic/subgenotypic replacement of both EV-A71 and CV-A16 viruses between 2000 and 2017 was performed. Molecular epidemiological studies are useful for tracing the origin of outbreaks, supporting disease control activities, and selecting or developing vaccine strains.

## Materials and methods

### *Ethical approval*

The study was approved by the Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University.

### *The study viruses*

A total of 92 enteroviral strains were analyzed in this study. These viruses were mostly isolated from pediatric patients with a clinical diagnosis of HFMD ($n = 90$); the other two strains were obtained from an HFMD asymptomatic adult contact case and a pediatric patient with encephalitis.

### *Sequencing of the complete VP1 region*

Culture supernatants of enteroviral-infected Vero cells were processed for total RNA extraction using a QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The RNA extract was further utilized for the amplification of the complete VP1 region by reverse transcription PCR using the One-Step RT-PCR Kit (Qiagen). The primers for amplification of the EV-A71 VP1 included EV71VP1_f (5′-GCGGCAGCCCAGAAGAAC-3′) as the forward primer and EV71VP1_r (5′-CCACTCTAAAGTTGCCCAC-3′) as the reverse primer, whereas those for amplification of the CV-A16 VP1 were CA16VP1_f (5′-CTACAGCATATATCGTAGC-3′) as the forward primer and CA16VP1_r (5′-GCAGTGGTGGAGGAGAC-3′) as the reverse primer. These primer pairs yielded amplified products of 1017 base pairs (bp) in size for EV-A71 and 1128 bp for CV-A16, while the actual lengths of the complete VP1 sequences were 891 nucleotides long for both EV-A71 and CV-A16. The amplified products were purified using a QIAquick gel Purification Kit (Qiagen) and sent for DNA sequencing at 1st BASE Pte Ltd, Singapore, or Macrogen Inc., Seoul, Korea. All amplicons were subjected to bidirectional sequencing using their amplification primers together with the additional sequencing primers EV71f2 (5′-GGAGCATCATCAAATGC-3′) and CoxA16f2 (5′-GGTGGGGTAT GCTCAAC-3′) for EV-A71 and CV-A16, respectively. The sequencing data were assembled and analyzed using BioEdit Sequence Alignment Editor version 7.0.4.1 (Hall, 1999). Overlapping DNA sequences with at least 85% homology and a minimum of 20 overlaps were assembled using the CAP contig assembly program to generate consensus sequences.

### *Phylogenetic analysis*

The genotyping and subgenotyping of the EV-A71 and CV-A16 were performed based on the classification systems described previously by several groups of investigators (Cardosa et al., 2003; Mizuta et al., 2005; Huang et al., 2009; Tee et al., 2010; Yip et al., 2013; Mizuta et al., 2014; Zhang et al., 2010a; Chen et al., 2013). A total of 90 EV-A71 and 56 CV-A16 strains of known genotype and subgenotype obtained from various countries and in different years were used in the construction of the phylogenetic tree. The

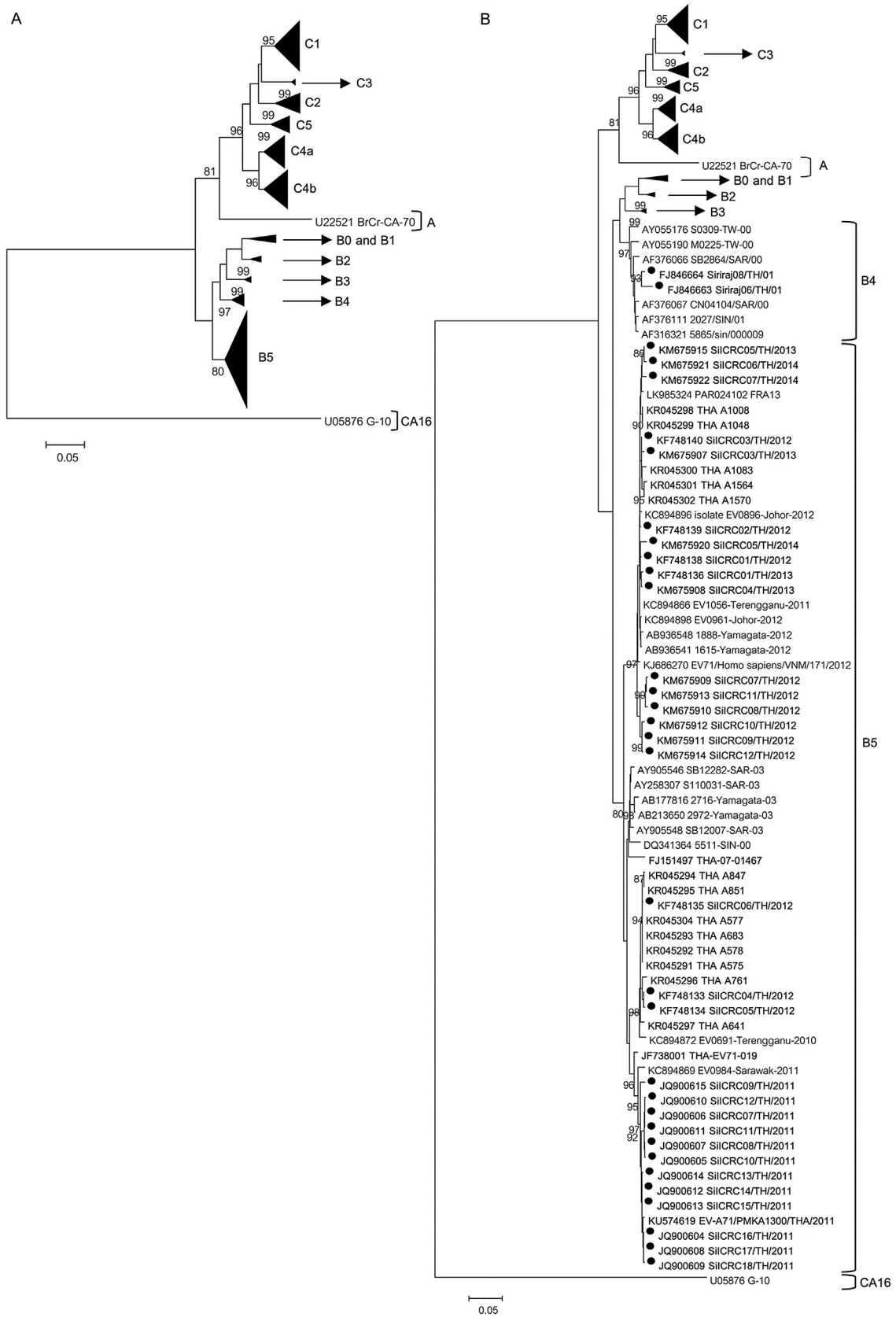


**Figure 1.** Phylogenetic analysis for subgenotyping of 101 enterovirus A71 (EV-A71) strains isolated in Thailand based on the VP1 sequences (A). The phylogenetic tree is extended to EV-A71 subgenotypes B4 and B5 (B); EV-A71 subgenotypes C1, C2, C3, and C5 (C); and EV-A71 subgenotypes C4a and C4b (D). The prototype coxsackievirus A16 (CV-A16) strain G-10 is used as the outgroup. Percentages of bootstrap values (1000 pseudoreplicates) ≥80% are shown at the nodes. Strains marked by a black circle indicate EV-A71 isolates obtained in this study, and the other Thai EV-A71 isolates are shown in bold.

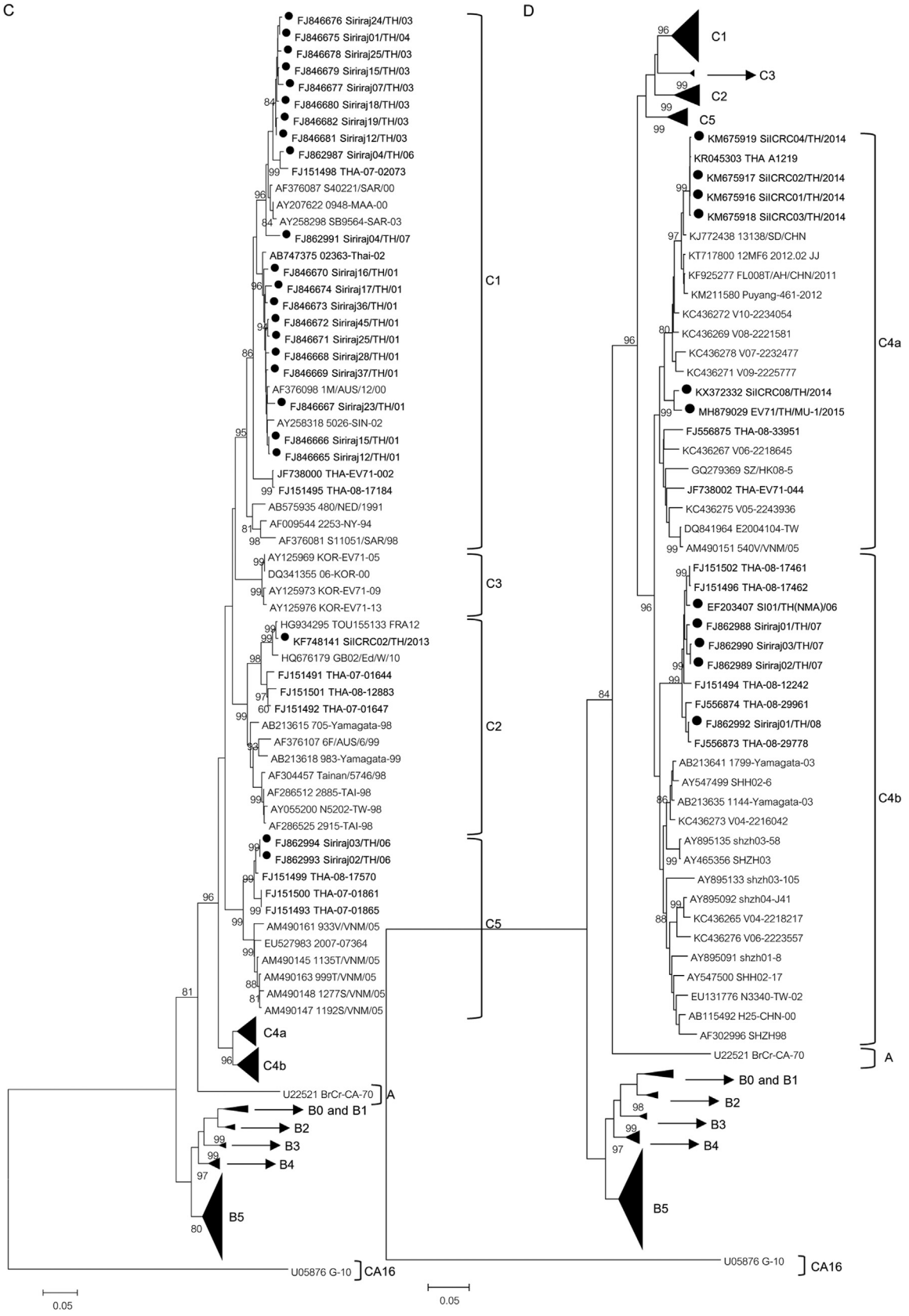


**Figure 1.** (Continued)

VP1 sequences in this study were aligned against the reference enteroviral strains retrieved from the GenBank database. An alignment was performed using ClustalW multiple sequence alignment in BioEdit program version 7.0.4.1, and a phylogenetic tree was constructed using MEGA 5.0 software (Tamura et al., 2011; available at http://megasoftware.net/) (Tamura et al., 2011). The evolutionary distances were estimated using the neighbor-joining method and maximum composite likelihood algorithm. The reliability of the neighbor-joining tree was estimated by bootstrapping analysis using 1000 replicate datasets. The supporting bootstrap value of greater than 80% was shown at the nodes of each cluster. The identities of VP1 nucleotide and deduced amino acid sequences were analyzed using ClustalW multiple sequence alignment and the sequence identity matrix applications in BioEdit program.

## Results

### *Enteroviral genotypic and subgenotypic identification*

Based on the complete VP1 sequences, the phylogenetic analysis identified 92 enteroviruses in this study: 67 (72.8%) EV-A71 and 25 (27.2%) CV-A16. These data show that EV-A71 was more commonly associated with HFMD than CV-A16. The complete VP1 sequences at a length of 891 nucleotides for both EV-A71 and CV-A16 were deduced into 297 amino acids. These sequences were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov); the GenBank accession numbers are given in the **Supplementary Material** (Tables S1 and S2).

### *Genetic analyses of the Thai EV-A71 and CV-A16 isolates*

Phylogenetic analysis was conducted with 92 EV sequences (67 EV-A71 and 25 CV-A16) obtained from 2000 to 2017 in this study, together with 37 sequences (34 EV-A71 and three CV-A16) of Thai isolates deposited in the GenBank database (**Supplementary Material**, Table S1 and S2). The EV-A71 phylogenetic analysis against the reference strains revealed multiple genotypic/subgenotypic replacements during the past two decades, as shown in Figure 1. EV-A71 B5 and EV-A71 C4a replaced the other subgenotypes (B4, C1, C2, C4b, and C5) and became the predominant viruses in the last 5 years (Table 1).

The EV-A71 VP1 nucleotide and deduced amino acid sequences were aligned and determined for similarity against the prototype viruses (EV-A71 genotype A strain BrCr-USA-1970 (accession number **U22521**), EV-A71 B4 strain 5865/sin/000009 (accession number **AF316321**), EV-A71 B5 strain 5511-SIN-00 (accession number **DQ341364**), EV-A71 C1 strain 480/NED/1991 (accession number **AB575935**), EV-A71 C2 strain Tainan/5746/98 (accession number **AF304457**), EV-A71 C4 strain SHZH98 (accession number **AF302996**), and EV-A71 C5 strain 2007-07364/TW/2007 (accession number **EU527983**)), as shown in the **Supplementary Material** (Table S3). Pairwise nucleotide identities within the subgenotype of the EV-A71 Thai isolates were in the range of 92.2–100%, and it was >96% for the deduced amino acid sequences. On the other hand, the sequence identities between members of each subgenotype and the prototype were in the range of 91.0–97.9% for nucleotide identities and 96.7–100% for amino acid identities. The variability of genetic differences between EV-A71 subgenotypes ranged from 7.9% to 19.1% for nucleotide sequences and from 0% to 5.0% for amino acid sequences (**Supplementary Material**, Table S4).

The analysis also found that more than 90% of the CV-A16 isolates (26 of 28 isolates) belonged to subgenotype B1a (Figure 2, Table 1). Pairwise alignment showed that all 28 CV-A16 isolates shared nucleotide and amino acid identities of 75.3–76.7% and 91.2–92.9%, respectively, to the CV-A16 prototype G-10, while the identities between the B1a and B1b subgenotypes were 88.8–93.3% for nucleotide sequences and 98.6–100% for amino acid sequences (**Supplementary Material**, Table S5).

These results indicate that the changes in amino acids were smaller than those in nucleotides because silent mutations were frequently observed.

## Discussion

The VP1 protein is not only the major neutralizing domain of enteroviruses but also the domain of high genetic variability for identifying an enterovirus serotype, genotype, and subgenotype (Cardosa et al., 2003; Perera et al., 2010; Zhou et al., 2011). Based on VP1 nucleotide sequences, the present study reported the emergence of several genotypes and subgenotypes of HFMD-associated EV-A71 and CV-A16 during the observation period of 18 years. It has previously been suggested that a minimum length of 350 nucleotides is required for enteroviral typing, while the complete VP1 sequences of 891 nucleotides in length is required for assigning a new EV genotype (Harvala et al., 2018). The complete VP1 nucleotide sequences contain more genetic information and are more reliable in the construction of phylogenetic trees than the shorter sequences. Hence, this study employed the complete length of the VP1 sequences, including those retrieved from the GenBank database, for the genetic analysis on the replacement of the EV-A71 and CV-A16 genotypes and subgenotypes in Thailand.

This phylogenetic analysis of EV-A71 VP1 nucleotide sequences revealed that all of the viruses investigated belonged to subgenotypes B4, B5, C1, C2, C4a, C4b, and C5. During the past two decades, several subgenotypes have emerged and disappeared over time, i.e., B4, C1, C2, C4b, and C5. The EV-A71 subgenotypes B5 and C4 have been co-circulating in Thailand since 2006. Previous

**Table 1**
Distribution and replacement of EV-A71 and CV-A16 subgenotypes between 2000 and 2017 in Thailand.

| Year[a] | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EV-A71 subgenotype | | B4 | | | | | | | | B4 | | | | | | | | |
| | | | | | | | B5 | B5 | B5 | | | B5 | B5 | B5 | B5 | B5 | B5 | B5 |
| | | C1 | C1 | C1 | C1 | | C1 | C1 | C1 | C1 | | | | | | | | |
| | | | | | | | | C2 | C2 | | | | | C2 | | | | |
| | | | | | | | | | C4a | C4a | | C4a | C4a | C4a | C4a | C4a | C4a | C4a |
| | | | | | | | C4b | C4b | C4b | | | | | | | | | |
| | | | | | | | C5 | C5 | C5 | | | | C5 | | | | | |
| CV-A16 subgenotype | B1a | B1a | B1a | | | B1a | | | | | B1a | B1a | B1a<br>B1b | B1a | B1a | B1a<br>B1b | B1a<br>B1b | B1a |

EV-A71, enterovirus A71; CV-A16, coxsackievirus A16.
[a] Note: EV-A71 and CV-A16 subgenotypes are those obtained from this study together with those from the National Institute of Health, Department of Medical Sciences, Ministry of Public Health.

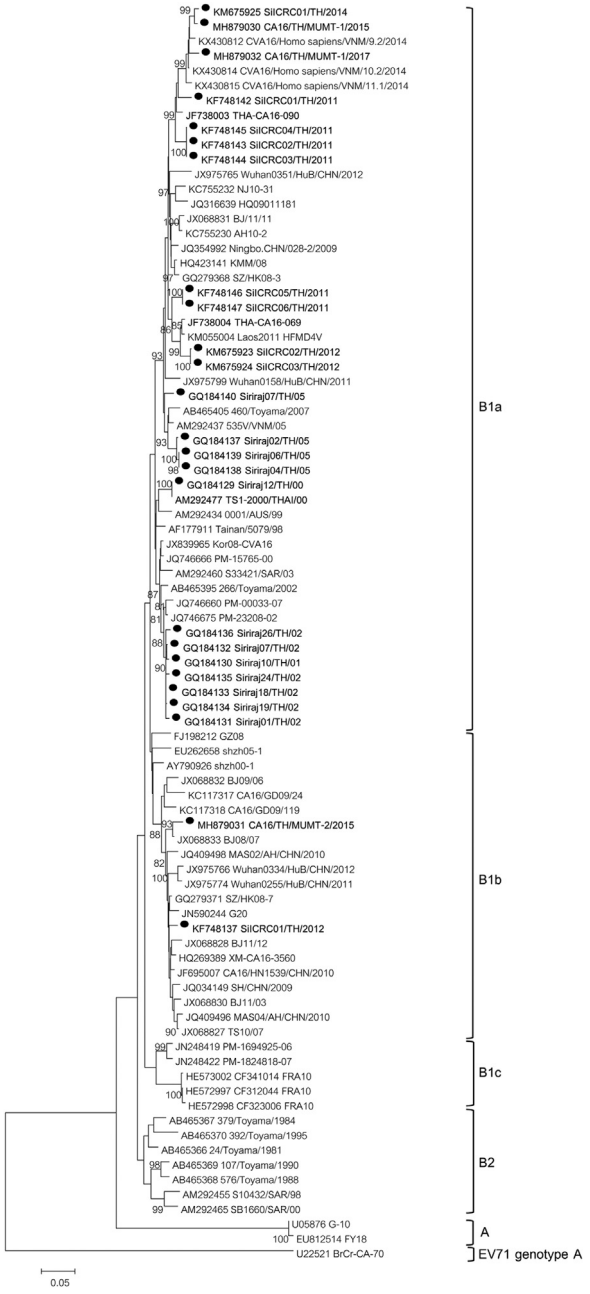


**Figure 2.** Phylogenetic analysis for subgenotyping of 28 coxsackievirus A16 (CV-A16) strains isolated in Thailand based on the VP1 sequences. The prototype enterovirus A71 (EV-A71) strain Br-CA-70 (genotype A) is used as the outgroup. Percentages of bootstrap values (1000 pseudoreplicates) ≥80% are shown at the nodes. Strains marked by a black circle indicate CV-A16 isolates obtained in this study, and the other Thai CV-A16 isolates are shown in bold.

long-term observations have demonstrated genotypic/subgenotypic replacements in some countries, e.g., Malaysia, Singapore, Taiwan, Japan, and China (Kung et al., 2007; Huang et al., 2011; Wu et al., 2013; Yip et al., 2013; Mizuta et al., 2014; NikNadia et al., 2016). The outbreak in Malaysia in 1997 was caused by a mixture of EV-A71 subgenotypes B3, B4, C1, and C2, of which B3 had the highest prevalence; however, subgenotypic replacements with B4 and C1 were observed in 2000 (Herrero et al., 2003; Huang et al., 2011; Yip et al., 2013; NikNadia et al., 2016). In addition, genotypic replacement with EV-A71 B5 was first documented in Malaysia in the 2003 outbreak (Ooi et al., 2007). In Taiwan, where the large outbreak in 1998 was caused by EV-A71 C2, a shift to the B4 subgenotype was recognized in the 2000 outbreak (Wang et al., 2002; Kung et al., 2007; Chia et al., 2014). There is no specific subgenotype predilection for disease fatality and transmissibility, even though some studies have suggested that severe disease has mostly been associated with subgenotype C4 (Duong et al., 2016).

Multiple introductions of several subgenotypes of EV-A71 from other countries into Thailand were likely. The first strain of EV-A71 C4b, SI01/TH(NMA)/06 with GenBank accession number **EF203407**, was isolated from a fatal encephalitic case in 2006, and had close genetic relatedness to the Shenzhen strain (Buathong et al., 2008). Subsequently, the prevalence of subgenotype C4b was replaced by C4a, which was first detected in 2008. Indeed, the EV-A71 C4a virus has been the most common and consistently found subgenotype in China since the late 1990s to the present day (Zhang et al., 2010b; Zhang et al., 2013). This subgenotype might have spread to Taiwan in 1998, Japan in 2002, Australia in 2004, Vietnam in 2005, and Cambodia in 2012 (Huang et al., 2011; Yip et al., 2013; Geoghegan et al., 2015; Duong et al., 2016; Yee and Laa Poh, 2017). Surprisingly, all EV-A71 B5 Thai isolates in 2011 and the minor isolates in 2012 were phylogenetically classified into different clusters from the major B5 isolates in 2012 and all the B5 isolates in 2013 and 2014. This finding implies that these B5 strains might have different origins. EV-A71 subgenotype B5 was predominantly found in Malaysia and Japan in 2003 (Mizuta et al., 2005; Ooi et al., 2007), Singapore in 2006 and 2008 (Ang et al., 2009; Wu et al., 2010; Huang et al., 2015a,b), and Taiwan in 2008 and 2011–2012 (Huang et al., 2009; Wu et al., 2013). The B5 subgenotype first appeared in China in 2008 (strain EV71/Xiamen/2009, GenBank accession number **JN964686**) (Zhang et al., 2013); nevertheless, C4 has been the most common subgenotype circulating in China since its emergence in 1998 up to the present day. The first B5-associated HFMD outbreak in Cambodia in 2012 might have spread from Thailand (Duong et al., 2016). Moreover, a subgenotype switch from C4 to B5 was documented in Vietnam in 2012 (Geoghegan et al., 2015). It is interesting that EV-A71 genotypic replacement has rarely occurred outside the Asia-Pacific countries. EV-A71 C1 and C2 have been the major subgenotypes reported in the European Union (EU) member states and England, Iceland, Norway, and Switzerland from 1998 to the present day (Bible et al., 2008; Hassel et al., 2015; Chang et al., 2016).

According to the virulence determinant described by previous groups of investigators based on the EV-A71 virus derived from site-directed mutagenesis, the amino acid substitution to glutamic acid at position 145 (145E) in the VP1 sequence has been shown to be related to neurovirulence and viremia in mouse and monkey models (Zaini and McMinn, 2012; Kataoka et al., 2015; Fujii et al., 2018), including resistance to neutralizing antibody in an in vitro assay (Fujii et al., 2018). An analysis for this determinant was also performed in the 67 EV-A71 isolates in the present study and it was found that all of them contained the amino acid residue 145E. Nevertheless, almost all viruses were isolated from mild HFMD cases (data not shown).

In addition to EV-A71, all of the Thai CV-A16 isolates were clustered in lineage B1 of the B genotype. Most of the isolates (26/28, 92.9%) belonged to subgenotype B1a, while only two isolates (2/28, 7.1%) belonged to subgenotype B1b. A previous study reported that CV-A16 subgenotypes B1a and B1b were the most prevalent strains in China (Chen et al., 2013; Yong et al., 2016). The present study revealed that CV-A16 viruses showed less genetic diversity than EV-A71 viruses. This serotype showed identities of 88.8–93.3% for nucleotide sequences and 98.6–100% for amino acid sequences across lineages B1a and B1b.

This 18-year molecular epidemiological study showed that EV-A71 and CV-A16 viruses have circulated widely in Thailand, similar to other countries in the Asia-Pacific region (Ang et al., 2009; Wu

et al., 2013; He et al., 2013; Mizuta et al., 2013; Yip et al., 2013; Zhang et al., 2013; Liu et al., 2014; Huang et al., 2015a,b; Nguyen et al., 2014). Occasionally, the other enterovirus serotypes, e.g., CV-A4, CV-B1, CV-B4, and CV-B5, were isolated from HFMD patients in our laboratory; however, EV-A71 and CV-A16 are still the most common viruses associated with the disease in Thailand at present, followed by CV-A6 (Puenpa et al., 2011; Linsuwanon et al., 2014; Puenpa et al., 2018). The dynamics of intra- and inter-genotypic replacements demonstrate the genetic evolution and fitness of the viruses to survive and replicate in their hosts. New variants may emerge from spontaneous mutation; in addition, the co-circulation of various enterovirus strains during the same period of time may result in co-infection and the emergence of new variants through the genetic recombination process (Huang et al., 2011; Yip et al., 2013; Liu et al., 2014; Guo et al., 2015). Host immunity is also a factor that drives viral genotypic shifts (Geoghegan et al., 2015; NikNadia et al., 2016). Monitoring for viral genetic change is important, not only regarding aspects of vaccine design and evaluation, but also in the molecular diagnosis of enterovirus-associated HFMD. During the monitoring period of 18 years, the molecular diagnosis protocol had to be changed a few times (unpublished data). The primers and probes should be verified annually against the circulating strains to assure their efficiency to detect the new variants that emerge over time.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

P.P. conceived and designed the research study and wrote the manuscript; P.N. performed the experiments, analyzed the data, and wrote the manuscript; A.K., J.P., and K.S. performed the experiments; K.C., A.M., A.T., and R.B. provided the clinical specimens; R.G. shared the epidemiological data. All authors read and approved the manuscript submission.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2018.12.020.

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