Tropical Biomedicine 31(4): 654–662 (2014)

Detection and characterization of viruses causing hand, foot and mouth disease from children in Seri Kembangan, Malaysia

Beh Poay Ling[1], Farid Azizi Jalilian[2], Nabil Saad Harmal[1], Putri Yubbu[1] and Zamberi Sekawi[1*]
[1]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, UPM Serdang, Selangor, Malaysia
[2]Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, 69391, Banganjab, Ilam, Iran
[*]Corresponding author email: zamberi@upm.edu.my
Received 2 December 2013; received in revised form 10 February 2014; accepted 21 February 2014

Abstract. Hand, foot and mouth disease (HFMD) is a common viral infection among infants and children. The major causative agents of HFMD are enterovirus 71 (EV71) and coxsackievirus A16 (CVA16). Recently, coxsackievirus A6 (CVA6) infections were reported in neighboring countries. Infected infants and children may present with fever, mouth/throat ulcers, rashes and vesicles on hands and feet. Moreover, EV71 infections might cause fatal neurological complications. Since 1997, EV71 caused fatalities in Sarawak and Peninsula Malaysia. The purpose of this study was to identify and classify the viruses which detected from the patients who presenting clinical signs and symptoms of HFMD in Seri Kembangan, Malaysia. From December 2012 until July 2013, a total of 28 specimens were collected from patients with clinical case definitions of HFMD. The HFMD viruses were detected by using semi-nested reverse transcription polymerase chain reaction (snRT-PCR). The positive snRT-PCR products were sequenced and phylogenetic analyses of the viruses were performed. 12 of 28 specimens (42.9%) were positive in snRT-PCR, seven are CVA6 (58.3%), two CVA16 (16.7%) and three EV71 (25%). Based on phylogenetic analysis studies, EV71 strains were identified as sub-genotype B5; CVA16 strains classified into sub-genotype B2b and B2c; CVA6 strains closely related to strains in Taiwan and Japan. In this study, HFMD in Seri Kembangan were caused by different types of Enterovirus, which were EV71, CVA6 and CVA16.

INTRODUCTION

Hand, foot and mouth disease (HFMD) is caused by several types of Enterovirus from family Picornaviridae. The major causative agents of HFMD are enterovirus 71 (EV71) and coxsackievirus A16 (CVA16). Outbreaks of HFMD might be caused by other types of Enterovirus as well, such as coxsackievirus A4-A10, A24, B2-B5, and echovirus (Chen *et al.*, 2012b). Infants or children who are infected with the viruses might present with fever, mouth ulcers and vesicles on palms and soles (Chen *et al.*, 2012a). Although HFMD is a self-limiting disease, EV71 infections might cause fatal and severe neurological diseases, such as encephalitis, acute flaccid paralysis and meningitis (Chan *et al.*, 2012).

In 1969, EV71 was firstly isolated from the outbreak in California (Schmidt *et al.*, 1974). Ever since then, EV71 epidemics continue to be seen in Asian and European countries (Chan *et al.*, 2011). The incidence of EV71 infections was increased in the Asia Pacific region since 1997 (Solomon *et al.*, 2010). In 1997, fatalities caused by EV71 were reported in Sarawak (AbuBakar *et al.*, 1999) and Peninsula Malaysia (Lum *et al.*, 1998). Outbreaks with fatalities still continue to be seen in the following years. CVA16 was reported in Malaysia since 2000 and was seen

during the outbreak or inter-outbreak of EV71 (Podin *et al*., 2006). Outbreaks of CVA6 were reported since 2008 in several countries, such as Singapore (Wu *et al*., 2010), Taiwan (Wei *et al*., 2011), Japan (Fujimoto *et al*., 2012), Finland (Österback *et al*., 2009), Spain (Cabrerizo *et al*., 2013), and the United States (McIntyre *et al*., 2012).

EV71 is divided into genotypes; A (represented by prototype strain BrCr), B (B1-B5), C (C1-C3, C5) and D (known as C4) (Chan *et al*., 2010). For CVA16, it is classified into genotype A and B (B1, B2a, B2b, and B2c) (Zong *et al*., 2011). However, studies on CVA6 infections were limited. There is lack of data on genotype distribution of HFMD causing viruses in Malaysia. Therefore, the aims of this study are; to detect the causative agents of HFMD among the patients presenting with signs and symptoms of HFMD in Seri Kembangan, Malaysia, and to classify the identified viruses by using phylogenetic analysis.

MATERIALS AND METHODS

Specimen collection

A total of six clinics involved in this study. Patients below twelve years old with clinical case definition of HFMD; fever, mouth/tongue ulcers, and rashes/vesicles on palms and soles were included in the study. The specimens were collected from clinics in Seri Kembangan area in between December 2012 until July 2013. The specimens included throat swab, mouth ulcer swab, vesicles swab, rectum swab or combination of the swabs. The swabs were kept in viral transport medium (VTM), [Sigma Virocult (Large) Duo (Sigma, UK)], labelled and refrigerated before transported to virology laboratory in Universiti Putra Malaysia.

Specimen processing

The collected specimens were vortexed and filtered through 0.22 µm syringe filter. The filtrate of each specimen was kept in 2 ml screw-capped tube with label and stored in -80ºC until further analysis.

Viral RNA extraction

One hundred and forty ìl of filtered VTM was used to extract the viral RNA by using QIAamp Viral RNA Mini Kit (Qiagen, Germany). The extractions were performed according to the manufacturer's instructions. Extracted RNA was kept in -80ºC for further analysis.

Semi-nested reverse-transcription polymerase chain reaction (snRT-PCR) and sequencing

The cDNA synthesis was carried out by using Prime RT Premix (Genet Bio, Korea) with Random Hexamer Primer (Fermentas, Canada). The protocol was performed based on manufacturer's instructions. Synthesized cDNA was kept in -80ºC for further used.

For snRT-PCR of VP4, 5 ìl of cDNA was used in the PCR with ExPrime Taq™ Premix (Genet Bio, Korea). The PCR condition was described as initial denaturation 95ºC for 3 min, followed by 35 cycles of denaturation 95ºC for 1 min, annealing 45ºC for 1 min, and extension 72ºC for 1 min, and final extension 72ºC for 5 min with the primer set MD91 and OL68-1 (Table 1) (Ishiko *et al*., 2002). snRT-PCR was carried out by using 1 µl of PCR product with the same PCR condition above. The primer set used was MD91 and EVP4 (Table 1) (Ishiko *et al*., 2002).

snRT-PCR of VP1 was performed on the confirmed specimens by using different VP1 primer sets (Table 1) respectively (Chua *et al*., 2007; Chan *et al*., 2012). Five µl of cDNA was used with the PCR condition; initial denaturation 95ºC for 3 min, followed by 35 cycles of denaturation 95°C for 1 min, annealing 50ºC for 1 min, and extension 72ºC for 1 min, and final extension 72ºC for 5 min. The snRT-PCR condition was same as above by using 1 µl of PCR product in the reaction.

The amplicon was detected by using 1.5% (w/v) agarose gel in electrophoresis with 1X TAE buffer. Then, it was extracted and purified by using QIAquick gel purification kit (Qiagen, Germany). The purified product was sequenced with ABI PRISM 3730xl DNA sequencer (Applied Biosystems, USA).

Table 1. VP4 primers and VP1 primers of EV71, CVA16 and CVA6

PCR	Virus	Gene	Primer's name	Primer's sequences (5'-3')	Nucleotide position (nt)	Amplicon size (bp)	References
RT-PCR	All	VP4	MD91	CCTCCGGCCCCTGAATGCGGCTAAT	444-1197	754	(Ishiko *et al*., 2002)
			OL68-1	GGTAAYTTCCACCACCANCC			
	EV71	VP1	MJP-VP1F	ACCCTTGTGATACCATGGAT	2212-3446	1235	(Chua *et* al., 2007) with modification
			MJP-P1R	CGCGAGCTGTCTTCCCA			
	CVA16	VP1	CVVP1F	GAGGACATTGAGCAAAC	2408- 3393	986	(Chan *et al*., 2012) with modification
			CVVP1R	AGGTGCCGATTCACTAC			
	CVA6	VP1	CVA6F	GACACTGACGAGATCCAACA	2407-3422	1016	This study
			CVA6R	AGATGTCGGTTTACCACTC			
snRT-PCR	All	VP4	EVP4	CTACTTTGGGTGTCCGTGTT	541-1197	657	(Ishiko *et al*., 2002)
			OL68-1	GGTAAYTTCCACCACCANCC			
	EV71	VP1	EVVP1F	AGGGAGATAGGGTGGCAGAT	2441-3446	1006	(Chan *et al*., 2012) with modification
			MJP-P1R	CGCGAGCTGTCTTCCCA			
	CVA16	VP1	CV6F	GCGTCGTGTAATGCTAGTGAC	2597- 3393	797	(Chan *et al*., 2012) with modification
			CVVP1R	AGGTGCCGATTCACTAC			
	CVA6	VP1	CVA6F2	GCAAGCTCTAATGCTAGTGA	2590-3422	833	This study
			CVA6R	AGATGTCGGTTTACCACTC			

Phylogenetic analysis and statistical analysis

Complete VP4 sequences (207 bp) and partial VP1 sequences were trimmed respectively; EV71 with 826 bp (nt 2484-3309), CVA16 with 357 bp (nt 2711-3068), and CVA6 with 731 bp (nt 2627-3357). Multiple alignments were performed with the homologous sequences that was available from GenBank by using ClustalW. Phylogenetic analysis was constructed by neighbor-joining method and displayed by MEGA5.2. Bootstrap analysis was performed with 1000 replicates (Tamura *et al.*, 2011). The GenBank accession numbers of the VP4 and VP1 sequences are: KF772887-KF772898, KF772899-KF772910 respectively.

In the study, IBM SPSS statistic 21 was used to perform analysis on the data. Chi-square test was used and p value < 0.05 was considered significant.

RESULTS

A total of 28 specimens were collected from December 2012 until July 2013. A total of 13 (46.5%) male and 15 (53.6%) female patients participated in the study. Among 28 specimens, 12 (42.9%) of them were snRT-PCR positive. HFMD causing viruses were detected from seven (58.3%) male and five (41.7%) female patients but was not statistically significant (p>0.05). The age range of the patients was divided into four groups due to small sample size; 0 to 3 years old, 4 to 6 years old, 7 to 9 years old, and 10 to 12 years old. Seven (58.3%) of viruses were detected in the age range 0 to 3 years old, four (33.3%) in 4 to 6 years old, and one (8.3%) in 10 to 12 years old with no significant differences. The mean age with positive viruses detected was 3.6 year old with no significant difference. The swabs collected with positive snRT-PCR were; three (25%) throat/mouth ulcer, one (8.3%) throat/mouth ulcer/rectum, one (8.3%) vesicle, one (8.3%) throat, two (16.7%) mouth ulcer/vesicle, three (25%) mouth ulcer/rectum, and one (8.3%) throat/rectum. However, they were statistically not significant.

Among the 12 positive snRT-PCR specimens, seven (58.3%) of CVA6, two (16.7%) of CVA16 and three (25%) of EV71 were identified by sequencing the VP4 snRT-PCR product (657 bp). The confirmed viruses were subjected to snRT-PCR again with VP1 primer sets. Partial VP1 sequences of the viruses were sequenced and trimmed accordingly; EV71 with 826 bp (nt 2484-3309), CVA16 with 357 bp (nt 2711-3068), and CVA6 with 731 bp (nt 2627-3357).

Phylogenetic tree of three types of the viruses were conducted based on the partial VP1 sequences. According to the phylogenetic analysis, CVA6 strains were closely related with Taiwan and Japan strains (Figure 1). For the two CVA16 strains in this study, each of them was classified as genotypes B2b and B2c (Figure 2). The collected EV71 strains were identified as genotype B5 (Figure 3).

DISCUSSION

Chi-square test was used in the statistical analysis. Based on the test, there were no significant differences between snRT-PCR positive with gender, age range, and type of specimens. Those results might be due to small sample size. However, the data could be references for further study. VP1 was

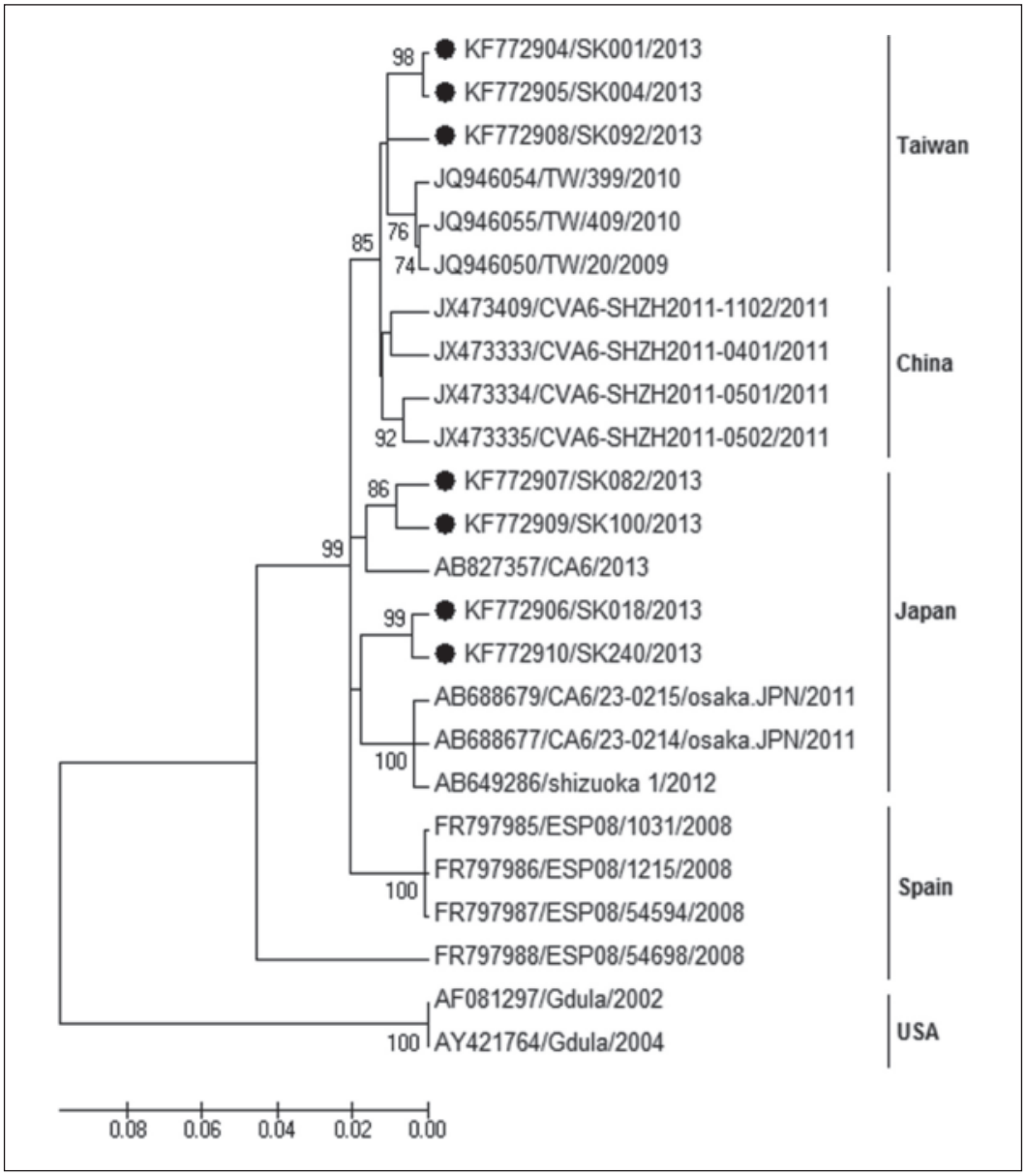

Figure 1. Phylogenetic tree of CVA6 VP1 was conducted by using partial VP1 sequences (nt 2627-3357) of the detected isolates. CVA6 strains from other countries were included to show the relationships between the detected CVA6. The dot indicates CVA6 strains in this study

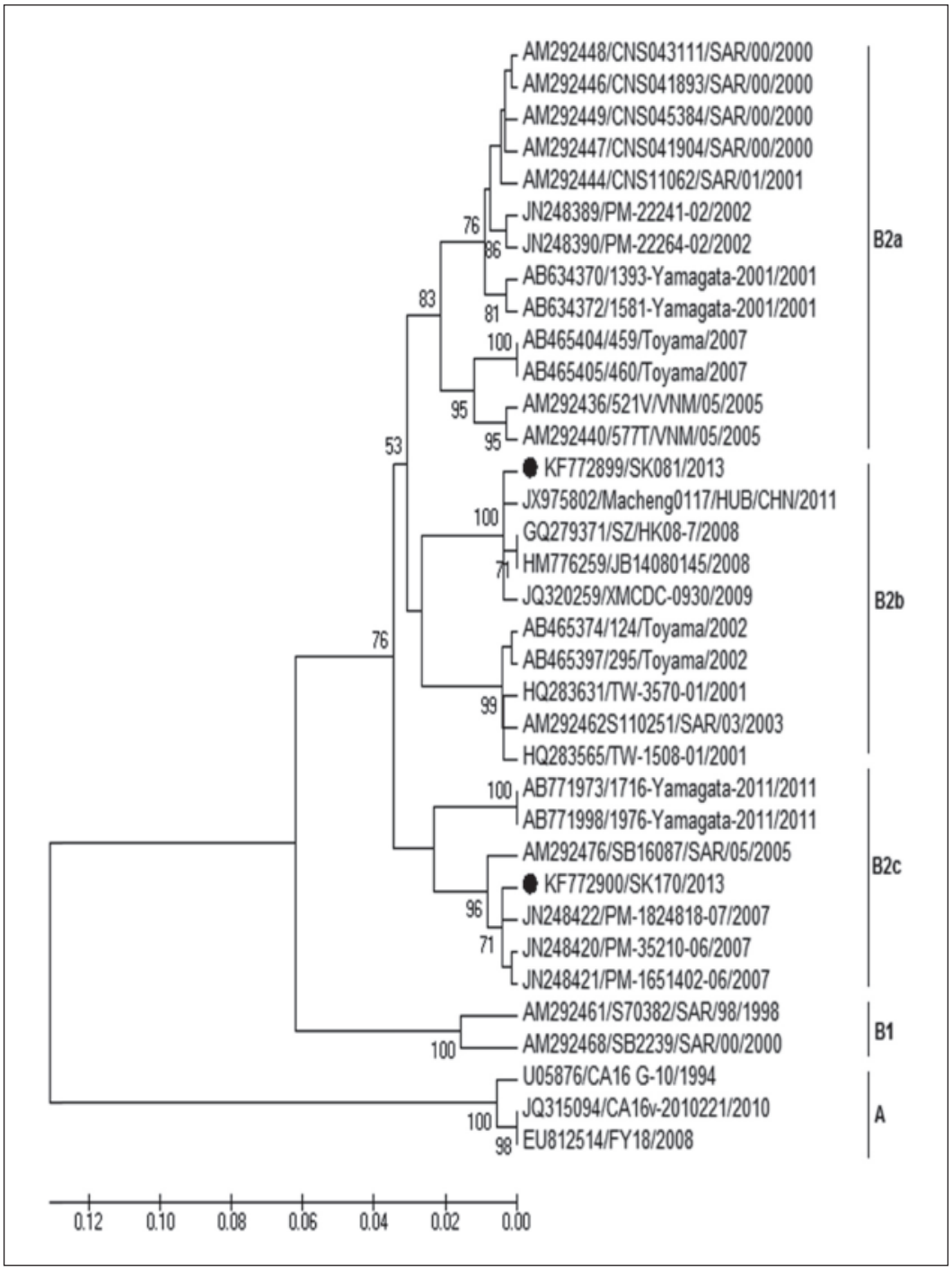

Figure 2. Phylogenetic tree of CVA16 VP1 was conducted by using partial VP1 sequences (nt 2711-3068) of the detected isolates. CVA16 strains from other countries were included to show the relationships between the detected CVA16. The dot indicates CVA16 strains in this study

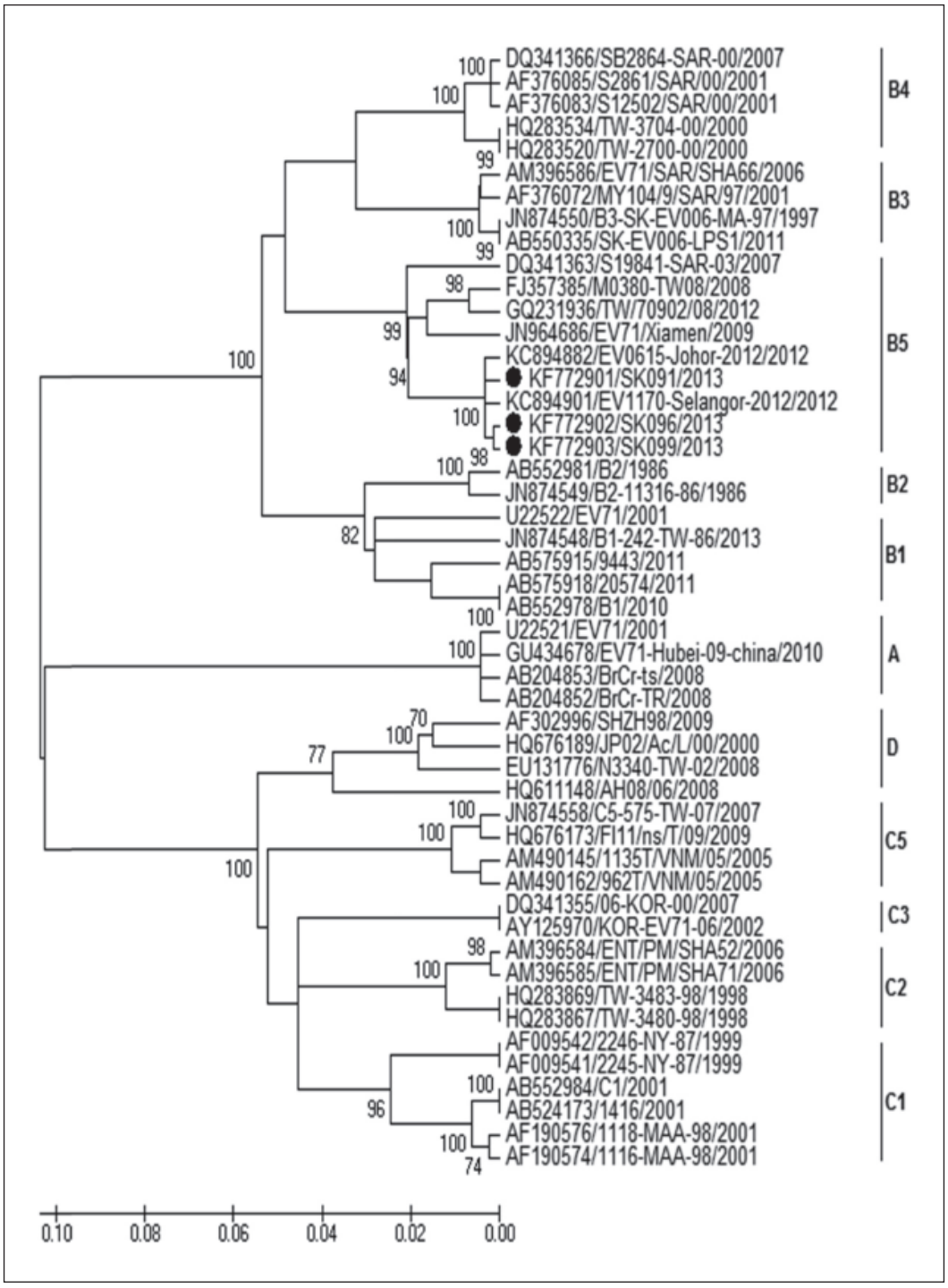

Figure 3. Phylogenetic tree of EV71 VP1 was conducted by using partial VP1 sequences (nt 2484-3309) of the detected isolates. EV71 strains from other countries were included to show the relationships between the detected EV71. The dot indicates EV71 strains in this study

chosen to construct phylogenetic analysis due to better reflect of genotyping (Chan *et al*., 2010).

The epidemics of HFMD caused by CVA6 were seen in 2008. The countries that reported CVA6 cases were Taiwan (Wei *et al*., 2011), Singapore (Wu *et al*., 2010), Finland (Österback *et al*., 2009), Japan (Fujimoto *et al*., 2012), and China (Lu *et al*., 2012). The study in Finland showed that CVA6 infections might cause onychomadesis. Positive samples of CVA6 were unable to be cultured (Österback *et al*., 2009). Therefore, the studies on CVA6 were limited. There was no published evidence of CVA6 outbreak in Malaysia. In this study, seven CVA6 strains were detected. Due to limitation on the studies, the detected CVA6 strains were compared with other strains from neighbouring countries. Three of them closely related with the strains from Taiwan in 2010 and another three of them were closely with Japan's strains in 2011.

Similarly with EV71, CVA16 was distributed in several countries as well, such as Spain (Cabrerizo *et al*., 2013), China (Chen *et al*., 2012a), Taiwan, Japan, Vietnam and Thailand (Zong *et al*., 2011). Subgenotype B1 of CVA16 was reported from 1998 and 2000 in Sarawak. In the period of 1997 to 2003, subgenotype B2a was dominant. After 2005, subgenotype B2c also detected (Chan *et al*., 2012). In this study, two CVA16 subgenotypes were classified as subgenotypes B2b and B2c. The subgenotype B2b was closely related to China strains isolated in 2011. However, subgenotype B2c was similar to the Malaysia strains isolated in 2007.

Since 1997, outbreaks of HFMD re-occurred in 2000, 2003, 2006 and 2008/2009 in Malaysia (Podin *et al*., 2006; Chan *et al*., 2011). Those outbreaks were caused by EV71 and CVA16. EV71 subgenotypes B3 and C2 were present in 1997, followed by subgenotypes B4 and C1 in 2001 and 2003. In 2006, subgenotype B5 caused outbreaks and continued circulating in Peninsula Malaysia and Sarawak (Chan *et al*., 2012). According to the study, subgenotype B5 was detected from the HFMD patients. These indicated that subgenotype B5 is still circulating in Malaysia. Based on phylogenetic analysis, detected B5 strains closely related to isolates from Selangor and Johor in 2012 (99% genetic identical in VP1 sequences). Those isolates had shown high homology to EV71 strains from China and Taiwan as well. EV71 is not only circulated in Malaysia, but also in other countries such as Europe (subgenotypes C1 and C2), Thailand (subgenotypes C1, C2, C4/D, C5), China (subgenotypes C4/D), and Japan (subgenotypes C2) (Chan *et al*., 2012).

There were some limitations in this study. Small amount of specimens were collected due to; no outbreaks in Seri Kembangan during specimens collection periods, and difficulties to convince participation from physicians and respondents. Small sample size might cause no significant difference in Chi-square test.

In this study, CVA6, CVA16 and EV71 were detected from patients presenting clinical signs and symptoms of HFMD in Seri Kembangan, Malaysia. Based on phylogenetic analysis studies; CVA6 strains were similar to strains in Taiwan and Japan, CVA16 strains were classified as subgenotype B2b and B2c, and EV71 strains were classified as subgenotype B5.

Acknowledgements. We would like to thank Klinik Y.F. Chew, Klinik Pakar Kanak-Kanak Dr. Chia, Klinik Hope, Poliklinik Penawar, Poliklinik Taman Impiyan, and CT Women and Child Specialist Clinic for their entire participation in this study. This study was funded by Research University Grant Scheme (RUGS), Universiti Putra Malaysia (04-02-12-1800RU).

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