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Research Note

Causative agents of hand, foot and mouth disease in University Malaya Medical Centre, Kuala Lumpur, Malaysia in 2012-2013

Aw-Yong, K.L.[1], Sam, I.C.[1], Koh, M.T.[2] and Chan, Y.F.[1*]
[1]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]Department of Paediatrics, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[*]Corresponding author e-mail: chanyf@ummc.edu.my
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Abstract. Hand, foot and mouth disease (HFMD) is a childhood illness, commonly caused by enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16). In recent years, unusual HFMD outbreaks caused by coxsackievirus A6 (CV-A6) have been reported. From May 2012 to September 2013, enteroviruses were detected in 25 HFMD patients in University Malaya Medical Centre, Kuala Lumpur, Malaysia. The predominant serotypes were EV-A71 (48%) and CV-A6 (48%), followed by CV-A16 (4%). CV-A6 patients (mean age, 2.1) were significantly younger than EV-A71 patients (mean age, 3.3). There were no significant differences observed in clinical features between EV-A71 and CV-A6 patients. Since enteroviruses are difficult to differentiate clinically, the conserved 5' untranslated region (5' UTR) was used to identify enterovirus serotypes. Phylogenetic analysis of 5' UTR showed distinct clustering of viruses as EV-A71, CV-A16 and CV-A6. Further genotyping with capsid genes showed that all the EV-A71 sequences belonged to subgenotype B5, while the CV-A16 sequence belonged to subgenotype B2b. CV-A6 sequences were clustered into genotypes D1 and D2, with recent isolates from Seri Kembangan, Malaysia and China. In summary, 59.5% of HFMD cases in our centre in 2012-2013 were caused by EV-A71, CV-A16 and the newly emerging CV-A6. This study also demonstrated that 5' UTR is suitable for preliminary identification of enteroviruses during HFMD outbreaks, but specific capsid genes such as VP1 and VP4/VP2 are required for further genotyping. Apart from measures to control the spread of the virus during an outbreak of HFMD, identification of EV-A71 as the etiological agent is important as EV-A71 is a major cause of severe neurological complications and potentially fatal.

INTRODUCTION

Hand, foot and mouth disease (HFMD) is a common illness frequently occurring in children less than 5 years old and characterized by fever, vesicular lesions on the palms and feet, and oral ulcers. The predominant etiological agents of HFMD are enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16), from the family *Picornaviridae* and genus *Enterovirus*. HFMD is often mild and self-limiting; however, EV-A71 infection may result in severe neurological diseases such as encephalitis, meningitis and acute flaccid paralysis, which may be fatal (reviewed in Chan *et al.*, 2011).

Over the last decade, EV-A71 has caused large epidemics in the Asia-Pacific region (reviewed in Solomon *et al.*, 2010). CV-A16 may co-circulate during EV-A71 outbreaks or inter-outbreak periods (Podin *et al.*, 2006). Since 2008, unusual HFMD outbreaks caused by CV-A6 have been reported in Finland (Blomqvist *et al.*, 2010; Osterback *et al.*,

2009), Singapore (Wu *et al.*, 2010), Taiwan (Lo *et al.*, 2011; Wei *et al.*, 2011), China (He *et al.*, 2013; Lu *et al.*, 2012), Japan (Fujimoto *et al.*, 2012), France (Mirand *et al.*, 2012), Spain (Montes *et al.*, 2013) and Thailand (Puenpa *et al.*, 2013). In many of these outbreaks, more severe skin manifestations were observed (Fonseca *et al.*, 2014; Puenpa *et al.*, 2013).

EV-A71 is classified into three genotypes, A, B (subgenotypes B1-B5) and C (C1-C5), based on phylogenetic analysis of the structural VP1 gene (Brown *et al.*, 1999; Chan *et al.*, 2010). CV-A16 is classified into genotypes A and B (B1, B2a-B2c) (Zong *et al.*, 2011; Chan *et al.*, 2012). However, limited information is available for CV-A6 genotype distribution. In the present study, we examined throat swabs from HFMD patients from May 2012-September 2013 to analyse the circulating genotypes of both EV-A71 and CV-A16, and determine the occurrence of CV-A6 in Malaysia.

Throat swabs were obtained from children clinically diagnosed with HFMD in Paediatric Emergency, University Malaya Medical Centre (UMMC), Kuala Lumpur from May 2012 to September 2013. Demographic and clinical data were reviewed retrospectively. This study was approved by the centre's Medical Ethics Committee (reference number: 932.17). Data were analysed using IBM SPSS statistics 22.0 (IBM, New York, USA). The Fisher's exact test or chi-square test was used to compare categorical variables. A $p < 0.05$ was considered statistically significant.

Viral RNA was extracted from 140 µl viral transport medium with throat swab specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). For enterovirus detection, the 5' untranslated region (5' UTR, positions 127 to 553) was amplified with the primers CoxbanS (5'-GTAMCYTTGTRCGCCWGTTT-3') and CoxbanR (5'-GAAACACGGACACCCAAA GTA-3') (Arola *et al.*, 1995) with the following parameters: reverse transcription at 42°C for 60 min, reverse transcriptase inactivation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 68°C for 1 min, and final extension of 68°C for 7 min. Genotyping of EV-A71, CV-A16 and CV-A6 were performed as previously described (Chan *et al.*, 2012; Puenpa *et al.*, 2013; Yoke-Fun and AbuBakar, 2006). The amplicons were purified with Expin Combo GP (GeneAll, Korea), and sequenced by First BASE Laboratories (Selangor, Malaysia). Sequencing results were subjected to BLAST analysis to identify the enterovirus serotypes.

Sequences were trimmed and assembled using Geneious R7 (Biomatters Ltd., New Zealand), and aligned with relevant sequences from GenBank. The best substitution model was determined using jModelTest v2 (Posada, 2008). Phylogenetic trees were constructed using the Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST, version 1.7.4 (Drummond and Rambaut, 2007) run for 30 million generations with a 10% burn-in. All runs reached convergence with estimated sample sizes of >200. The tree prior was coalescent GMRF Bayesian Skyride and the clock model was uncorrelated lognormal relaxed. The maximum clade credibility tree was viewed using FigTree v1.4.0 (Rambaut, 2012). The sequences reported in this study have been deposited into GenBank with accession numbers KJ815033-KJ815044 and KT908004-KT908038 (Table 1).

Enteroviruses were detected in 25 out of 42 (59.5%) throat swab specimens collected from children with suspected HFMD from May 2012 to September 2013. Sequencing revealed that the predominant serotypes were EV-A71 (n=12; 48%) and CV-A6 (n=12; 48%), followed by CV-A16 (n=1; 4%). Demographic and clinical data of 11 EV-A71 and 11 CV-A6 patients were available for comparison (Table 2). The EV-A71-infected children (mean, 3.3 years) were significantly older than the CV-A6-infected patients (mean, 2.1 years; p=0.001). There was a non-significant trend towards hospitalization for CV-A6 patients, with 4 patients admitted for 0.5 ± 0.82 days. No EV-A71-infected patients required hospital admission as these are older children. Both sets of patients had the typical clinical features of HFMD, with no significant

Table 1. Nucleotide accession numbers of sequences reported in the present study

Isolate name	Accession number			Genotype
	5' UTR	VP4	VP1	
EV-A71				
MY-3898-12	KT908004	KT908026	NA	B5
MY-2949-12	NA	KT908027	NA	B5
MY-8352-12	KT908005	KT908028	NA	B5
MY-5105-12	KT908006	KT908029	NA	B5
MY-5313-12	KT908007	KT908030	NA	B5
MY-5390-12	KT908008	KT908031	NA	B5
MY-4703-12	KT908009	KT908032	NA	B5
MY-4285-12	KT908010	KT908033	NA	B5
MY-9836-12	KT908011	KT908034	NA	B5
MY-6937-12	KT908012	KT908035	NA	B5
MY-0757-12	KT908013	KT908036	NA	B5
MY-6407-13	KT908014	KT908037	NA	B5
CV-A16				
MY-2235-12	KT908015	NA	KT908038	B2b
CV-A6				
MY-6046-12	KT908016	NA	KJ815033	D1
MY-8586-12	KT908017	NA	KJ815034	D1
MY-2429-12	KT908018	NA	KJ815035	D1
MY-0446-12	NA	NA	KJ815036	D1
MY-3626-12	KT908019	NA	KJ815037	D2
MY-8299-13	KT908020	NA	KJ815038	D2
MY-6716-13	NA	NA	KJ815039	D2
MY-4657-13	KT908021	NA	KJ815040	D2
MY-0017-13	KT908022	NA	KJ815041	D2
MY-9784-13	KT908023	NA	KJ815042	D2
MY-8698-13	KT908024	NA	KJ815043	D2
MY-0319-13	KT908025	NA	KJ815044	D1

NA, not available

differences in clinical presentation. The single CV-A16-infected patient was not included in the analysis.

Phylogenetic analysis of 5' UTR showed that our sequences were grouped into EV-A71 (n=11), CV-A16 (n=1) and CV-A6 (n=10) (Figure 1). Three could not be sequenced, and were not analysed. EV-A71 was divided into 2 groups, in genotypes B and C (Figure 1). The segregation is probably due to recombination among enteroviruses. Next, we genotyped the viruses by sequencing the VP4 gene for EV-A71, and VP1 gene for both CV-A16 and CV-A6. The sequences of EV-A71 (n=12) and CV-A16 (n=1) were aligned with other previously published sequences from Malaysia. Phylogenetic analysis showed that all EV-A71 were grouped in subgenotype B5 (Figure 2A) and the CV-A16 grouped in subgenotype B2b (Figure 2B). Limited CV-A6 sequences from China, France, Japan, Spain, Taiwan and Malaysia were available for genotyping, which grouped CV-A6 into three major clusters, denoted as B, C (C1-C2) and D (D1-D2) (He *et al.*, 2013). Our CV-A6 were clustered into genotypes D1 (n=5) and D2 (n=7), and were closely related to isolates from Seri Kembangan, Malaysia and China (Figure 2C). Co-circulation of multiple clusters was observed in countries like Malaysia, China, and France.

Table 2. The demographic and clinical characteristics of patients with EV-A71 and CV-A6 infection

	EV-A71 (n=11)		CV-A6 (n=11)		p value
	n	%	n	%	
Demographic					
Male: female ratio	1:1.8		2.7:1		0.198
Ethnicity:					0.495
(a) Malay	9	81.8	8	72.7	
(b) Chinese	1	9.1	–	–	
(c) Indian	1	9.1	2	18.2	
(d) Others	–	–	1	9.1	
Mean age ± SD at enrolment (years)	3.29 ± 0.85		2.08 ± 2.29		0.001*
Clinical manifestations					
Length of history:					0.650
(a) 1 – 2 days	7	63.6	5	45.5	
(b) 3 – 4 days	3	27.3	5	45.5	
Hospitalized	0	0	4	36.4	0.090
Fever	8	72.7	10	90.9	0.586
Mouth/throat ulcers	11	100	9	81.8	0.476
Hand lesions:					0.442
(a) Palms	8	72.7	7	63.6	
(b) Dorsum and palms	2	18.2	–	–	
(c) No lesions	1	9.1	1	9.1	
Foot lesions	6	54.5	9	81.8	0.361

Prior to 2012, EV-A71 and CV-A16 were the main causative agents of HFMD in Malaysia (Chan *et al.*, 2012). In our study, we found that CV-A6 has recently emerged to be an important virus co-circulating with EV-A71 and CV-A16 in Malaysia, a similar trend seen in unusual HFMD outbreaks in Europe (Mirand *et al.*, 2012; Osterback *et al.*, 2009) and Asia (Fujimoto *et al.*, 2012; Lu *et al.*, 2012; Wu *et al.*, 2010). With the co-circulation of various enteroviruses and the potential for viral co-infection and recombination, enteroviruses other than EV-A71 and CV-A16 also have the propensity to cause HFMD outbreaks (Chan and AbuBakar, 2004; Yoke-Fun and AbuBakar, 2006).

We found no significant differences in clinical manifestations between CV-A6 and EV-A71 patients. A few studies have reported that CV-A6 was more commonly associated with herpangina than HFMD (Chen *et al.*, 2012; Lo *et al.*, 2011; Mirand *et al.*, 2012). CV-A6 is reported to cause more severe and widespread skin manifestations, involving sites such as knees, elbows, trunk and neck, which are not usually involved in EV-A71 and CV-A16 (Kobayashi *et al.*, 2013; Puenpa *et al.*, 2013). Onychomadesis, or nail shedding, has been reported as a hallmark for CV-A6-infected HFMD patients 1-2 months after the disease (Fujimoto *et al.*, 2012; Kobayashi *et al.*, 2013; Osterback *et al.*, 2009; Wei *et al.*, 2011). This observation could not be confirmed in the present study as no patient follow-up was performed. However, we found that CV-A6 patients were younger than EV-A71 patients, similar to other studies (He *et al.*, 2013; Lu *et al.*, 2012).

EV-A71 subgenotype B5 and CV-A16 subgenotype B2 have been circulating in Malaysia since 2000 and 2003, respectively (Chan *et al.*, 2012), and continue to persist in this and other Malaysian studies (Chua & Kasri, 2011; Ling *et al.*, 2014). Our CV-A6

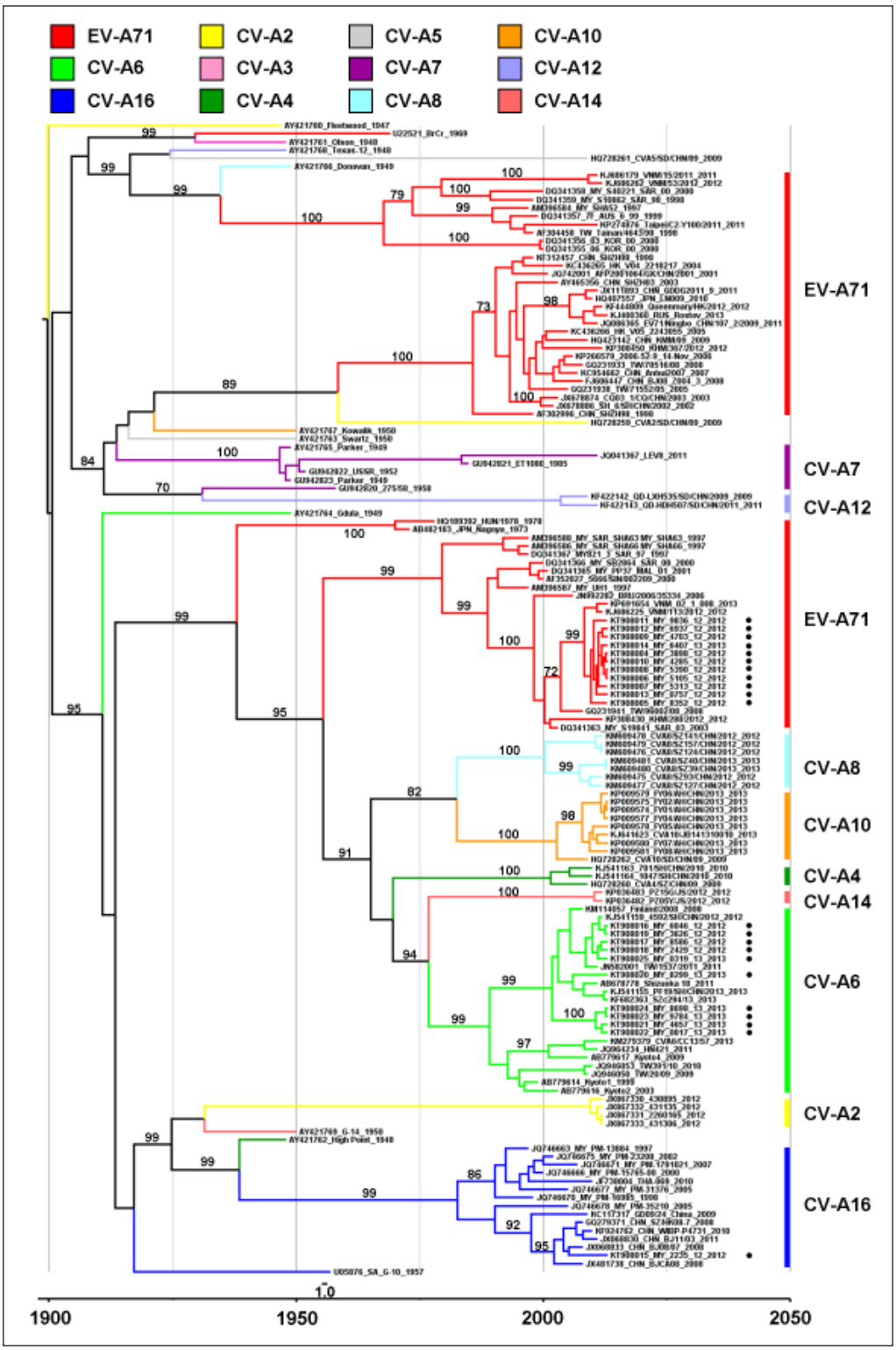

Figure 1. Phylogenetic analysis of enteroviruses based on partial 5' UTR gene sequences (434 bp). The phylogenetic tree was constructed using the Bayesian MCMC method implemented in BEAST with a TIM2EF+I+G nucleotide substitution model. Only bootstrap values over 70% are shown. The dots (•) indicate sequences from the present study.

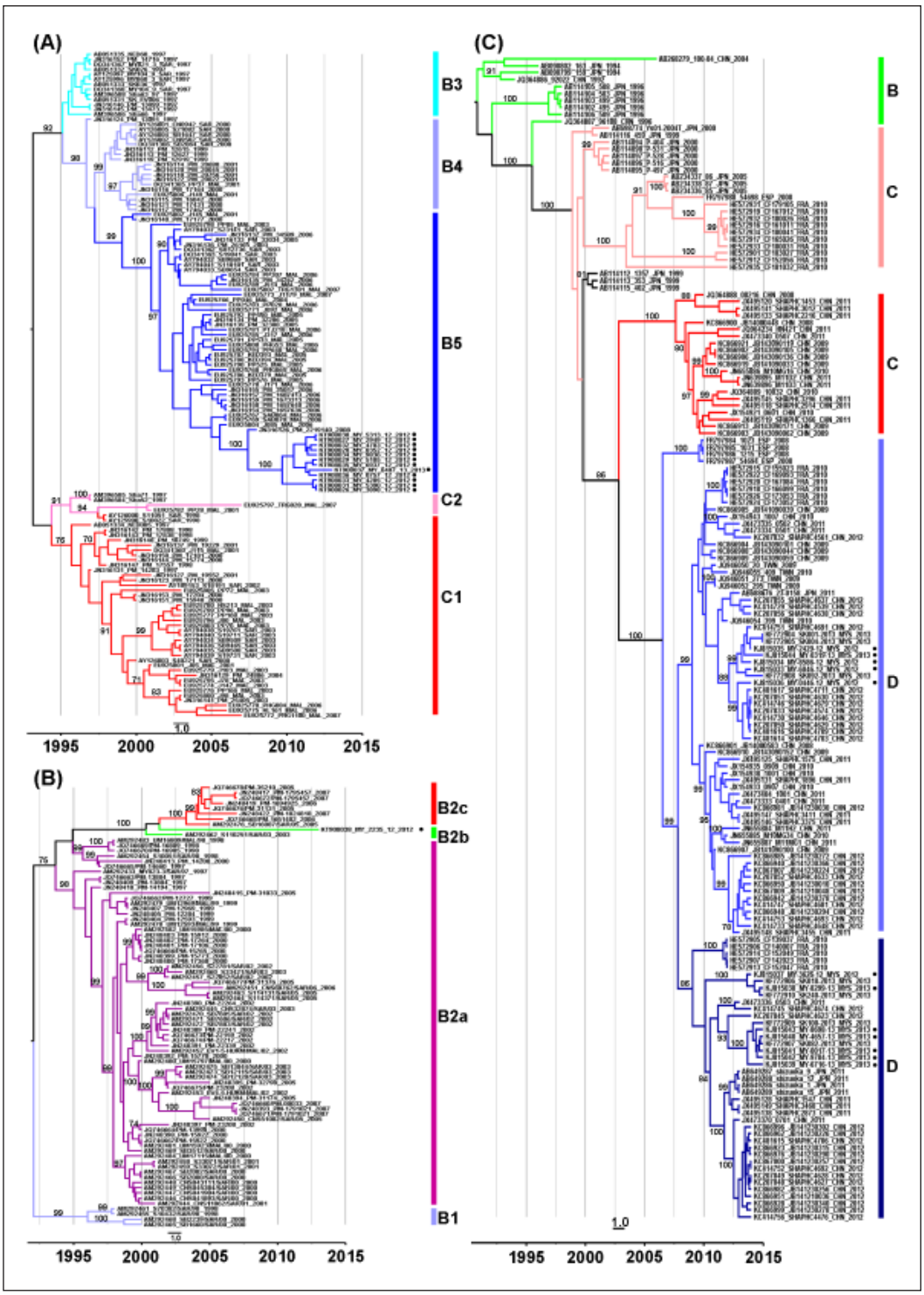

Figure 2. Phylogenetic analysis of EV-A71, CV-A16 and CV-A6. Phylogenetic analysis of (A) EV-A71 based on VP4 gene sequences (207 bp), (B) CV-A16 based on VP1 gene sequences (891 bp), and (C) CV-A6 based on partial VP1 gene sequences (657 bp). The phylogenetic trees were constructed using the Bayesian MCMC method implemented in BEAST with HKY+G, TIM2+I+G and K80+G nucleotide substitution models, respectively. Only bootstrap values over 70% are shown. The dots (•) indicate sequences from the present study.

isolates were clustered into genotypes D1 and D2, and were closely related to isolates from Malaysia and China.

In summary, EV-A71, CV-A16 and CV-A6 were detected from patients clinically diagnosed with HFMD in Kuala Lumpur, Malaysia. As we demonstrated that enteroviruses were difficult to differentiate clinically, the highly conserved 5' UTR is useful for broad-range detection of enteroviruses during HFMD outbreaks, as co-circulation of various enteroviruses occurs frequently (AbuBakar *et al.*, 1999; Hyypia *et al.*, 1989; Zhou *et al.*, 2011; Zhou *et al.*, 2014). As 5' UTR is a hotspot for recombination, specific capsid genes such as VP1 and VP4/VP2 are required to further confirm genotypes. Early confirmation of EV-A71 as the main causative agent of HFMD is critical for prompt public health measures to control the spread of virus and minimizes deaths seen in young children.

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