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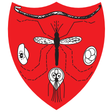

RESEARCH ARTICLE

Detection of enteroviruses during a 2018 hand, foot and mouth disease outbreak in Malaysia

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ABSTRACT

Hand, foot, and mouth disease (HFMD) is a common childhood disease caused by enteroviruses. In 2018, a HFMD outbreak in Malaysia affected over 76,000 children. In this study, we used RT-qPCR and CODEHOP PCR to detect the causative agents in 89 clinically diagnosed HFMD patients in Kuala Lumpur and Selangor. Most (62.9%) of the children were below 3 years old. PCR with either assay detected enteroviruses in 84.2% (75/89) and CODEHOP PCR successfully typed 66.7% (50/75) of the enteroviruses. Sequencing of CODEHOP amplicons showed co-circulation of multiple enteroviruses with coxsackievirus A6 (CV-A6) and A16 as the predominant serotypes, but not the neurovirulent enterovirus A71. CV-A6 infection was more common in children less than 12 months old (p=0.01) and was more likely to cause vesicles in the gluteal area (p=0.01) compared to other enteroviruses. Establishing a robust identification method during HFMD outbreaks is important for patient management and public health responses.

Keywords: hand, foot and mouth disease; HFMD; enterovirus; coxsackievirus A6; Malaysia.

INTRODUCTION

Hand, foot and mouth disease (HFMD) is the second most common infectious diseases in Malaysia (MOH, 2019). In Malaysia, enterovirus 71 (EV-A71), coxsackievirus A16 (CV-A16) and CV-A6 have been reported as causative agents of HFMD outbreaks (Chan *et al.,* 2012; Aw-Yong *et al.,* 2017). However, the disease is caused by a group of enteroviruses with over 100 serotypes. Since virus surveillance is limited, the enteroviruses that cause yearly HFMD epidemics remain unknown.

Within the genus of enteroviruses, EV-A71 and some echoviruses cause neurological complications aside from polioviruses (Solomon *et al.,* 2010; Bubba *et al.,* 2020). Quick identification of these neurotropic enteroviruses is important for patient management. CV-A16 and EV-A71 are the major causative agents of HFMD in China and have been endemic in Southeast Asia and the Pacific region for two decades (Van Tu *et al.,* 2007; Iwai *et al.,* 2009). However since 2011, outbreaks of other enterovirus strains such as CV-A6 and CV-A10 have been reported in China (He *et al.,* 2017), Finland (Blomqvist *et al.,* 2010), France (Mirand *et al.,* 2016), Japan (Fujimoto *et al.,* 2012), United Kingdom (Gaunt *et al.,* 2015) and Taiwan (Wei *et al.,* 2011). An unusual CV-A2 circulation was also recently documented in China (Yang *et al.,* 2018). With co-circulation of many enteroviruses at any one time, the lack of viral surveillance for HFMD in Malaysia hampers the understanding of HFMD epidemiology, which in turn impacts appropriate public health and resource management, including the development of multivalent vaccines and introduction of EV-A71 vaccines.

Routine identification is usually based upon virus isolation followed by immunofluorescence. These are time-consuming and labour-intensive, requiring 7-14 days, and hence do not provide a rapid laboratory diagnosis. Molecular diagnosis based on real-time PCR is time-saving and sensitive (Nijhuis *et al.,* 2002; Robinson *et al.,* 2002). The COnsensus-DEgenerate Hybrid Oligonucleotide Primer (CODEHOP) strategy was developed to detect and identify distant-related pathogens (Rose *et al.,* 2003). When applied to enteroviruses, CODEHOP PCR uses highly degenerate primers targeting VP1 to identify different serotypes compared to traditional specific primers (Nix *et al.,* 2006; Chiang *et al.,* 2012). Therefore, the aim of this study is to describe the use of both real-time PCR and CODEHOP PCR to detect and identify the serotypes of enteroviruses in HFMD patients during an outbreak in Malaysia in 2018.

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MATERIALS AND METHODS

Patients presenting with HFMD at the University Malaya Medical Centre (UMMC), a teaching hospital in Kuala Lumpur from June and July 2018 were included in this study. These patients presented with clinical features consistent with HFMD which include acute febrile illness accompanied by vesicular and tender rash over, but not limited to, the palms/ soles with or without intraoral ulcers. Clinical data and patient demographics were obtained. Throat swabs were collected and transported in virus transport medium (VTM).

VTM received were filtered with a 0.45 µm syringe filter. RNA was extracted from 280 µl VTM using QIAamp Viral RNA mini kit (QIAGEN, Germany) according to the manufacturer's instructions. Primer and probe sequences were from Thanh *et al*. (2015) (Table 1). RT-qPCR reaction consisted of 4× TaqMan Fast Virus 1-Step Mastermix (Thermo Fisher Scientific, USA), 0.5 µM of each primer (ENT-F and ENT-R), 0.25 µM of ENT probe and 5 µl of RNA template. The reaction mixes were subjected to 50°C for 5 minutes for cDNA synthesis, 95°C for 20s, followed by 40 cycles of 95°C for 3s and 60°C for 30s. RT-qPCRs were performed using StepOne Plus Real-Time PCR (Applied Biosystems, USA) and analyzed using StepOne Plus Software version 2.3.

For CODEHOP PCR (Nix *et al.,* 2006), synthesis of cDNA was carried out in a 10 µl mixture containing 4.5 µl of RNA, 100 µM deoxynucleoside triphosphate (dNTP), 5X first-strand buffer, 0.01 M dithiothreitol (DTT), 0.5 µM cDNA primer mixture (AN32, AN33, AN34, AN35), 20U of RNaseOUT and 100U of SuperScript III reverse-transcriptase (Invitrogen, USA). Following incubation at 22°C for 10 min, 42°C for 45 min, 95°C for 5 min, 5 µl of the RT reaction mixture was then used in the first PCR (PCR1), consisting of 2X MyTaq reaction buffer, 5U of MyTaq DNA polymerase (Bioline, UK), 400 nM each of primers 222 and 224, with 40 cycles of amplification (95°C for 15s, 42°C for 15 s, 72°C for 15s). One microliter of the first PCR was added to a second PCR (PCR2) for semi-nested amplification. PCR2 contained primers AN88 and AN89, and the reaction mixture was the same as PCR1, with 40 cycles (95°C for 15s, 60°C for 15s, 72°C for 15s). The amplicons were separated and visualized in 1.5% agarose gel containing GelRed and were gel purified using DNA Clean & Concentrator (Zymo, USA) prior to Sanger sequencing (Apical Scientific Sdn. Bhd). Alignment of the sequences was performed using Geneious Prime 2020 (Biomatters Inc., New Zealand). VP1 nucleotide sequences were checked against the NCBI database by Blast search to determine the enterovirus serotype with the highest identity.

To compare the sensitivity of RT-qPCR and CODEHOP PCR, both assays were performed as described above with enterovirus control RNA from *in vitro* transcribed EV-A71 (Tan *et al.,* 2016). Ten-fold serially diluted RNA copy numbers were assayed with 6 technical replicates with both RT-qPCR and CODEHOP PCR.

The associations of enterovirus PCR positivity with age groups, gender and clinical symptoms were determined by Chi-square test with SPSS version 23.0 (IBM, USA), and $p < 0.05$ was regarded as statistically significant. This study was approved by the UMMC Medical Ethics Committee (reference number: 932.17). Our institution does not require informed consent for retrospective studies of anonymised samples.

RESULTS AND DISCUSSION

During 2018, a major HFMD outbreak occurred across Malaysia with over 76,000 infected (MOH, 2019). We tested 89 samples collected from UMMC during the outbreak period. The median

Table 1. Primers and probes used for molecular detection in CODEHOP and RT-qPCR

Primers	Sequences (5'-3')	Target region	Polarity
CODEHOP		AN32	GTYTGCCA
VP1	antisense		
AN33	GAYTGCCA	VP1	antisense
AN34	CCRTCRTA	VP1	antisense
AN35	RCTYTGCCA	VP1	antisense
222	CICCIGGIGGIAYRWACAT	VP1	antisense
224	GCIATGYTIGGIACICAYRT	VP3	sense
AN88	CCAGCACTGACAGCAGYNGARAYNGG	VP1	antisense
AN89	TACTGGACCACCTGGNGGNAYRWACAT	VP1	sense
RT-qPCR			
ENT-F	CCCTGAATGCGGCTAAT	5' UTR	sense
ENT-R	ATTGTCACCATAAGCAGCC	5' UTR	antisense
ENT probe	ATTO550N-ACCCAAAGTAGTCGGTTCCG-IAbRQSp		

Primer and probe sequences for CODEHOP and RT-qPCR were from Nix *et al.* (2006) and Thanh *et al.* (2015), respectively.

ATTO 550N is the 5' fluorophore and IAbRQSp (Iowa Black RQ) is the 3' quencher.

age of the patients was 27 months (range 4 months–10.6 years). The suspected HFMD patients comprised of 45 males (50.6%) and 44 females (49.4%). The RT-qPCR based on the 5'-untranslated regions detected enteroviruses in 83.1% (74/89) of the samples. By utilising a quick and sensitive RT-qPCR, patients with suspected HFMD could be diagnosed in two hours. CODEHOP PCR amplicons which require sequencing will take at least an additional day. Early and timely detection could aid in the control of the spread of HFMD among susceptible children especially those attending preschools.

By using the CODEHOP PCR, we detected a total of 56.2% enteroviruses (50/89). Of these, 55.1% (49/89) were positive with both assays, and one sample was detected only with CODEHOP but not RT-qPCR. Therefore, a total of 75/89 (84.3%) of samples were positive with either or both PCR assays. We found that the limits of detection for RT-qPCR and CODEHOP were 10^2 RNA copies per reaction and 10^3 RNA copies per reaction respectively, which explained the lower sensitivity of CODEHOP PCR compared to RT-qPCR. Despite its lower sensitivity, the 350-400 bp amplicon from CODEHOP PCR can be sequenced to type the enterovirus species. In total, 44% CV-A6 (22/50), 40% CV-A16 (20/50), 10% EV-A71 (5/50), 2% CV-A10 (1/50) and 4% CV-B3 (2/50) were sequenced from the CODEHOP PCR amplicons. Nine samples positive for CODEHOP PCR remained undetermined as the sequences had similarity to human genes, which could be due to the degeneracy properties of the CODEHOP primers. A positive CODEHOP amplicon therefore should be sequenced for confirmation of enterovirus species. The National Public Health Laboratory collected 2236 samples for the same duration of this 2018 HFMD outbreak and showed 32.96% enterovirus positive (737/2238); with 30.53% EV-A71 (225/737), 32.29% CV-A16 (238/737) and 37.18% other enteroviruses (273/737)) (personal communication). Similar to the current study, more robust typing will be required to type the enteroviruses to determine the presence of other enteroviruses.

Univariate analysis was performed to determine the correlation between age, gender and clinical symptoms with PCR-positivity for enteroviruses (Table 2). Children $\leq$12 months were significantly more likely to be infected by CV-A6 (47.6%) than by CVA16 (10.5%) or other EV (16.7%) (Table 2). Patients

Table 2. Comparison of demographic and clinical features of patients with CV-A6, CV-A16 and other enterovirus infections

	CVA6 (n=22)	CVA16 (n=20)	Other EV* (n=33)	p-value#
Age (months)				
≤ 12 months	10 (47.6%)	2 (10.5%)	5 (16.7%)	
> 12 months	11 (52.4%)	17 (89.5%)	25 (83.3%)	**0.01**
Unknown	1 (4.5%)	1 (5.0%)	3 (0.9%)	
Sex				
Male	9 (40.9%)	9 (45.0%)	17 (51.5%)	0.73
Female	13 (59.1%)	11 (55.0%)	16 (48.5%)	
Signs and symptoms				
Fever	18 (81.8%)	10 (50.0%)	21 (63.6%)	0.09
Mouth ulcers	18 (81.8%)	15 (75.0%)	22 (66.7%)	0.45
Vesicles on hand	16 (72.7%)	14 (70.0%)	18 (54.5%)	0.31
Vesicles on feet	16 (72.7%)	16 (80.0%)	20 (60.6%)	0.31
Vesicles on buttocks	12 (54.5%)	7 (35.0%)	5 (15.2%)	**0.01**

*Other enteroviruses include EV-A71 (5), CV-A10 (1), CV-B3 (2) and non-typable enteroviruses (25).
#Chi-square test was used. Significant p-values (p<0.05) are indicated in bold.

with CV-A6 infection showed significantly more vesicles on the gluteal area than other enterovirus infections (p=0.01), which supports previous reports (Mirand *et al.,* 2016; Uppala *et al.,* 2018).

The number of CV-A6 detected in Malaysia has been increasing since 2012 (Aw-Yong *et al.,* 2017). An increase in CV-A6 infections have been reported both within and outside the Asia Pacific region (Fujimoto *et al.,* 2012; Puenpa *et al.,* 2014). The main circulating viral causes of HFMD in Malaysia had been EV-A71 for many years since the first major outbreak in the Asia Pacific region in 1997 (Chan *et al.,* 2000) and CV-A16, but since 2012 CV-A6 has become a major contributor (Aw-Yong *et al.,* 2017). There has been a corresponding decline of EV-A71 in Asia since the last fatal outbreak in Cambodia in 2012 (Duong *et al.,* 2016), although it still contributes to neurological cases in Europe (Bubba *et al.,* 2020). Therefore, it is essential to type other enteroviruses with tools such as CODEHOP PCR.

In conclusion, our findings showed that CV-A6 has emerged and co-circulated with other enteroviruses during the 2018 outbreak in Malaysia. The co-circulation of various enteroviruses highlights the dire need for a multivalent HFMD vaccine. The usage of RT-qPCR and CODEHOP PCR in this study are efficient methods for detection and surveillance of emerging enteroviruses, in order to monitor and initiate early control for the disease. The early and timely detection and identification of the circulating enterovirus will greatly assist national policy and decision makers on deciding the most suitable HFMD vaccine when is available in the near future.

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Conflict of interest
The authors declare that they have no conflict of interest.

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