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Molecular Detection of Human Enterovirus 71 Causing Hand, Foot and Mouth Disease in Klang Valley, Malaysia

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Abstract: Hand, Foot and Mouth Disease (HFMD) is a childhood infection caused by Enterovirus 71 (EV71) or Coxsackievirus A16 (CA16). Occasionally, Enterovirus 71 becomes fatal and causes encephalitis. The objective of the present study was to determine HFMD caused by Enterovirus 71 if prevalent in the Klang Valley, Malaysia. 184 specimens were collected from the patients reported to University Kebangsaan Malaysia Medical Centre (UKMMC). All the specimens were subjected to culture in rhabdomyosarcoma cell line and confirmed by Reverse transcription polymerase chain reaction (RT-PCR). The positive RT-PCR products were then sequenced to determine the viral genotype. Out of the 184 specimens, 89 showed cytopathic effects (CPE), indicating the presence of viral infections. Out of 89 positive CPE specimens 18 were positive with RT-PCR. Of the 18 positive specimens, 6 were Enterovirus 71, 3 Coxsackievirus A16, 8 Coxsackievirus A10, and 1 was vaccine-associated poliovirus 2. All the patients identified with strain Enterovirus 71 infection presented hand, foot and mouth disease and one of them had signs of paralysis as well. Collected Enterovirus 71 strains were classified under genotype C1 by phylogenetic analysis. This study proved that Enterovirus 71, genotype C1 prevalent in the study area and it did not cause serious outbreak in the Klang Valley, Malaysia. This prevalent strain could be used to choose for the development of a future vaccine candidate against HFMD.

Key words: Cell culture, enterovirus 71, hand foot and mouth disease, RT-PCR, sequencing

INTRODUCTION

Hand Foot and Mouth Disease is an Enterovirus causes common illness of infants and children, characterized by fever, sores in the mouth and a rash with blister (Alexander *et al*., 1994). The virus belongs to genus Enterovirus in the family Picornaviridae. The virus caused fatal encephalitis in children previously in Malaysia (Abubakar *et al*., 1999a) and in Taiwan (Ho *et al*., 1999). Enterovirus 71 (HFMD), is a virus like poliovirus invades the central nervous system to give rise to aseptic meningitis, encephalitis, or myelitis. (Murray *et al*., 1988). HFMD is also caused by coxsackie virus A16. Coxsackie virus A16 infection causes mild disease and nearly all patients recover within 7 to 10 days without medical treatment. But for Enterovirus 71, infection may cause viral meningitis and more serious disease, such as encephalitis or a poliomyelitis-like paralysis (Melnick 1985). In mid 2000, a large outbreak of EV71 occurred in Sarawak, Malaysia as well as in Taiwan but the case fatality rate was lower than in the 1997 and 1998 outbreaks, respectively (Cardosa *et al*., 2003). However, a number of highly publicized deaths associated with EV71 infection occurred in Singapore in late 2000 leading to worries about the frequency with EV71 outbreaks. World health organization updated the repot of HFMD on august 4, 2011 and mentioned that during 1-19 weeks of 2011 a total number of 1296 HFMD cases recorded without any death report(Western Pacific Regional Office of the World Health Organization WPRO Hand, Foot and Mouth Disease Situation Update, 4 Aug 2011)

With the forecast eradication of poliomyelitis virus in the near future, it is likely that EV71 will fill the niche left by polio and it is important that there would be effort to develop a vaccine against EV71 since it has been shown to have great epidemic potential. Baseline data of genotype EV71 in the Malaysia is important. This collection is a valuable resource, which can be used to choose strain for the development and the testing of a future vaccine candidate against HFMD. Cell culture remains the "gold standard" for laboratory detection of the enterovirus despite its significant limitations, which include the failure of certain EV strain to replicate in culture. (Melnick, 1985). To detect Enterovirus with molecular technique, reverse transcription polymerase chain reaction (RT-PCR) using Enterovirus 71 specific primers sets is important and was performed previously (Zoll *et al*., 1992). The present study was aimed at to detect HFMD virus and related viral agents by molecular methods to confirm their prevalence and identify their genotypes in the Klang Valley, Malaysia.

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MATERIALS AND METHODS

Specimen collection: Specimens were collected from University Kebangsaan Malaysia Medical Centre and Klang Valley Hospital, Malaysia from the patient presented with clinical symptom of HFMD, meningitis, encephalitis, myocarditis, herpangina, and acute paralysis. Specimens collected from throat, rectum, vesicle and mouth ulcer were kept in virus transport medium (VTM). All the specimens were labeled and kept frozen at -70ºC until analysis. The samples of the study were collected in different times from 2003 to 2006 and some of them processed immediately and rest processed later on which continued till 2010.

Specimen processing: The specimens were processed in the Department of Medical Microbiology and Immunology, UKMMC, Kuala Lumpur, Malaysia. Frozen specimen were thawed completely and vortexed to ensure the material present in cotton bud was dispersed into the VTM. Specimens from each swab was filtered with 2 mL screw-capped labelled tubes through 0.22 μL syringe filter and then put into and frozen it at -70ºC until analysis.

Virus isolation: Filtarate specimens were inoculated for virus isolation in 24 well plates containing 0.5 mL/well of rabdomyosarcoma cell suspension (2-3×105 cells/mL) in RPMI with antibiotics containing 5% heat inactivated foetal calf serum. Upon observation of more than 80% CPE contents of the wells were harvested and stored at -70ºC until analysis by RT-PCR. Culture showing CPE were aliquoted for extraction of viral nucleic acids.

Viral nucleic acids extraction: Viral nucleic acid was extracted by tri-reagent LS. 750 μL tri-reagent LS was added into 1.5 mL micro centrifuze tube containing 250 μL of cell suspension of culture with CPE. Then vortexed and incubated at room temperature for 5 min. Subsequently, 200 μL of chloroform was added and vortexed vigorously before leaving on rotor wheel for 15 min. After centrifuge at 12000 g for 15 min at 4ºC, the aqueous phase was carefully transferred into a new microcentrifuze tube. Five hundred μL of isopropanol was then added and mixed by inverting tube a few times and left incubated at room temperature for 10 min. It was then centrifuged at 12000 g for 15 min at 4ºC. 1 ml of 75% ethanol was added and centrifuged at 12000 g for 15 min at 4ºC. Supernatant was removed and pellet dried by leaving in speed vac for 5 min at low heat. Pellet was finally resuspended in sterile sterile 20 μL UHQH_2O and stored at -70ºC with RNase inhibitor.

Reverse trtranscriptase polymersae chain reaction (RT-PCR):

Primer: Primer upstream EVP2 (5'-CCT CCG GCC CCT GAA TGC GGC TAA-3') (Chu *et al*., 2001). Primer downstream OL68-1 (5'-GGT AA(C/T) TTC CAC CAC CA(A/T/G/C) CC-3') (Ishiko *et al*., 2002).

Reverse transcriptase step: Six μL of template (i.e., extracted viral nucleic acids) +1 μL of downstream primer (OL68-1 of 20 ρmol/uL). Incubated at 70ºC for 10 min, and then placed on ice immediately to prevent reforming of secondary structure.

RT master mix:

Reagents final conc. Volume/ μL
M-MLV RT 5X 1X 2.0
10 mM dNTP 0.5 mM 0.5
M-MLV transkriptase 100 U 0.5

Then, 3 μL of RT master mix is added, flicked tube to mix and spinned down. Therefore total reaction volume for reverse transcription was 10 μL. After 60 min of incubation at 42ºC, reaction was stopped by heating at 70ºC for 10 min, then immediately put on ice.

PCR step:
PCR master mix:

Reagents final conc.	Volume/ μL	
UHQ H_2O steril		11.7
10X buffer dengan $(NH_4)_2SO_4$	1X	2.0
25 mM $MgCI_2$	1.5 mM	1.2
Primer VP2 (upstream)	20 ρmol	1.0
Primer OL68-1 (downstream)	20 ρmol	1.0
10 mM dNTP (MBI Fermentas, Cat. No.R0191)	0.3 Mm	0.6
Taq DNA polimerase (MBI Fermentas, Cat. No.EP0402)	2.5 U	0.5

Eighten μL of PCR master mix was added to each tube of 2 μL RT product, mixed and spin down.

PCR conditions:

Steps	Temp (ºC)	Time
Initial denaturing	95	5 min
Denaturing	95	45 sec
Annealing	55	45 sec 35
Extension	72	90 sec
Final Extension	72	5 min

PCR product (750 bp) was detected by 1.8% (w/v) agarose gel electrophoresis with 1X TBE buffer. Gene Ruler 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania. Cat. No. SM 0241) was used as molecular weight marker. The lanes were labeled with 100 bp DNA ladder, specimen number and control. A confirmed EV71 isolate was used as positive control for the RT-PCR.

DNA extraction: DNA was extracted from agarose gel by Geneclean III kit as per the procedure to determine the weight of the gel slice in micrograms, added 0.5 volume TBE Modifier and 4.5 volumes NaI solution per 1 volume of agarose. Then incubated at 55ºC to melt gel for 5 min and adding EZ-GLASSMILK®. After 2 times washed with NEW Wash, adding a volume of TE or water to elute DNA. Finally centrifuged for 30 sec and removed supernatant containing DNA.

Sequencing: Sequencing was done by using the ABI PRISM 377 DNA Sequencer following the procedure of Cardosa *et al*. (2003) and Ishiko *et al*. (2002). The ABI PRISM 377 DNA Sequencer is an automated instrument designed for analyzing fluorescently-labeled DNA fragments by gel electrophoresis. It is used to separate DNA fragments by size. Complete nucleotide sequence was obtain from sequencer and analyzed with the strains in Genebank.

Statistical analysis: Data were analyzed using program SPSS 11.5 with 0.05% level of significance.

RESULTS

A total number of 184 were examined and out of which 89 showed cytopathic effects (CPE), indicating the presence of viral infections (Fig. 1).Of the 89 positive CPE specimens 18 were positive with RT-PCR (Fig. 2).Of the 18 positive specimens, 6 were positive Enterovirus 71 (Table 1) 3 Coxsackievirus A16, 8 Coxsackievirus A10, and 1 was vaccine-associated poliovirus 2. All the patients identified with strain Enterovirus 71 infection presented hand, foot and mouth disease and one of them had signs of paralysis as well.Collected Enterovirus 71 strains were classified under genotype C1 by phylogenetic analysis.

Among the patients, 67 (64%) were male and 38 (36%) were female. Community based analysis showed that 48 (46%) Malay, 48 (46%) Chinese and only 9 (8%) were Indian and others. Age of patient was between one month to 42.8 months. 25 patients presented clinical menifestations of Hand, Foot and Mouth disease. Three patients shown HFMD and herpangina. Only one patient exhibited both HFMD and acute paralysis symptoms. Type of speciments analysed were : 49 (27%)CSF, 45 24%) rectal swab, 36 (20%) Nasal swab, 26 (14%) mouth ulcer swab, 18 (10%) vesicle swab, 9 (5%) specimen faeses and one (1%) nasopharyngeal aspirate.

PCR product 750 bp size Enterovirus 71. DNA ladder DNA 100 bp. G1/5, G2/4, U33/3, U34/8, U35/3, H69/3 and G9/4 are Enterovirus positive.

(Product RT-PCR was sequenced after extraction and sequenced data was submitted to a BLAST search of Genebank (http://www.ncbi.nlm. nih.gov/BLAST/) and

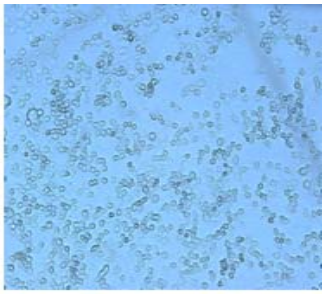

Fig. 1: Rabdomyosarcoma cell with cytopatic effect by

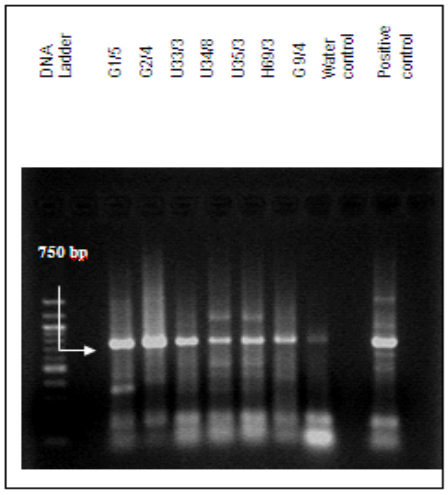

Fig. 2: Gel image for RT-PCR for enterovirus detection legends

the result returned with match of EV71 gene sequences available in the Genbank. This confirmed that these sequence is of Enterovirus genotype C1(Fig. 3).

DISCUSSION

A total of 184 specimens collected from 105 patients with Enterovirus infections. Enterovirus isolated by cultivation in cell culture, and then amplified by RT-PCR. This was followed by sequencing to confirm the serotype and genotype Enterovirus 71.

The result from the study showed that a total of 20 from 29 HFMD patient were recorded from one month to three years of age. Out of 20, fourteen (70%) confirmed Enterovirus 71 infection. A study condcuted in USA reported that the children were the high risk for outbreak of Enterovirus (Rotbart *et al*., 1998). A 2 to 3-year cyclical epidemic of HFMD was noted in the United Kingdom (Communicable Disease Report, 1980). It is postulated that this cyclical pattern could have been due to the accumulation of immunologically naive preschool children between epidemics until a critical threshold level is breached.(Podin *et al*., 2006).

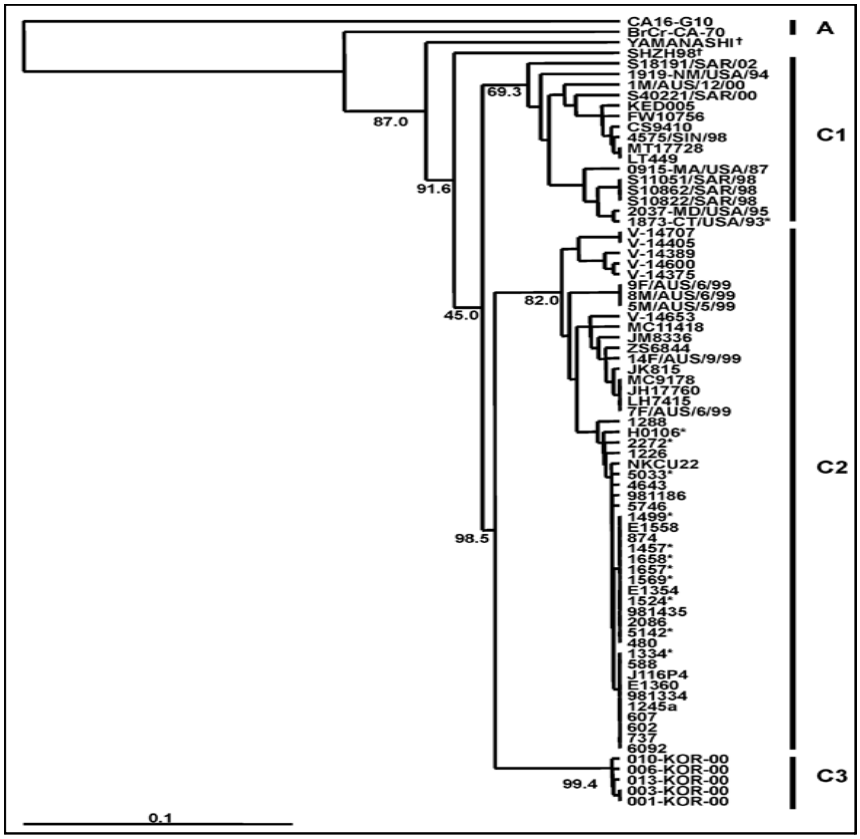

Fig. 3: Results of the phylogenetic analysis revealed that enterovirus 71 isolated from HFMD cases were under enterovirus genotype C1

CHI-square test showed that there were no significant different between HFMD Enterovirus infection with gender ($p>0.05$). Besides, CHI-square test also showed that there were no significant different between HFMD Enterovirus infection with races ($p>0.05$). RT-PCR detected Enterovirus and Enterovirus 71 infection. A total of 11 from 29 HFMD patient (21%) infected by Enterovirus whereby five (17%) of them caused by Enterovirus 71. Finally it was obseved that all the patient with Enterovirus 71 positive were HFMD patient. It is normally associated with epidemics of Hand, Foot and Mouth Disease (HFMD) with typical symptoms including lesions on the palms, soles and oral mucosa (Minor *et al*., 1995).

RT-PCR test indicated 2/45 (4%) rectal swab, 1/36 (3%) nasal swab, 1/18(6%) vesicle swab, 1/26 (4%) mouth ulcer swab and 1/9 (11%) feces were Enterovirus 71-positive. The best specimens for isolation of virus were, in order of preference: stool specimens or rectal swabs, throat swabs or washings, and cerebrospinal fluid. Throat swabs or washings and CSF were most likely to yield virus isolates if they were obtained early in the acute phase of the illness. (Schmidt and Emmons, 1989).

We could efficiently identified the viruses realting to HFMD by RT-PCR. Molecular technique was proved to be important for dignosis and to trace the specific genome and determine the genotype of the virus(Chapman *et al*., 1990).

This study proved that Enterovirus 71, genotype C1 prevalent in the study area and it did not cause serious outbreak in the Klang Valley, Malaysia

The result of phylogenetic analysis of all thirty-seven EV71 strains based on alignment of the complete (891 nts) VP1 gene sequences together with the complete VP1

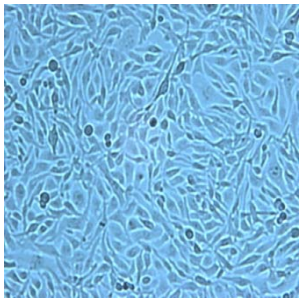

Fig. 4: Normal rabdomyosarcoma cells

Table 1: Enterovirus 71 genotype isolated from different specimenns of HFMD

No of specimen	Specimen	Clinical presentation	Gene bank accession number	Enterovirus 71 genotype
G0010/5	Vesicle swab	HFMD	E71300758	CS9410/99
G0010/6	Mouth ulcer swab	HFMD	E71300758	CS9410/99
G0027/4	Nasal sawab	HFMD	E71300758	CS9410/99
P0033/3	Rectal swab	HFMD	E71300758	CS9410/99
P0034/8	Faeces	HFMD	E71300758	CS9410/99
P0035/3	Rectal swab	HFMD	E71300758	CS9410/99

gene sequences of representative EV71 strains belonging to the respective previously known subgenogroups has been presented in Fig. 3. This study identified 4 subgenogroups (C1, C2, B3, and B4) cocirculated in peninsular Malaysia. In the 2005 outbreak, besides the subgenogroup C1, EV71 isolates belonged to a distinct cluster which is designated B5cv were isolated. The distinction of this cluster variant within subgenogroup B5 from previously described classical subgenogroup B5 strains isolated in 2003 was supported by a strong bootstrapped value of 100 (Fig. 4). All the four Sarawak EV71 strains belonged to subgenogroup B5 but clustered within the cluster variant B5cv which also contained the strains isolated in peninsular Malaysian as early as May

2005. The aligned 891 nucleotides of EV71-VP1 gene of a Sarawak strain and two representative peninsular Malaysia strains isolated in 2005. The Sarawak strain shared a higher nucleotide identity with the peninsular Malaysia strain isolated in the later part of 2005 (2005P888) than the strain (2005P588) that was isolated in the earlier part of the year. It was interesting to note that EV71 strains which belonged to the subgenogroup C1 were isolated in all three outbreaks that spanned over a period of 9 years (Chua *et al*., 2007).

Another report on HFMD mentioned that from 2001 to 2009, four genetic lineages of HEV71 have been found to be prevalent in Peninsular Malaysia and Sabah, the authors ponted that the predominant circulating strain was subgenogroup B4 in 2001 and this was later followed by subgenogroup B5 in 2003. The subgenogroup B5 was dominant between 2005 and 2009. Viruses belonging to subgenogroups C1 and C4 were also detected. (Apandi *et al*., 2011).Our study correlated with the findings of Apandi *et al*. (2011)

In the year 2011, in Vietnam HFMD was reported to have claimed 98 lives, 75% of whom were children under 3 years old (http:// en. wikipedia. org/wiki/ Hand,_foot_ and_mouth_disease).

The World Health Organization reporting between January to October of 2011 (1,340,259) states the number of cases in China has dropped by approx, 300,000 from 2010's (1,654, 866) cases. With new cases peaking in June. 437 deaths down from 2010 (537 deaths) (http://english.peopledaily.com.cn/90001/90782/90880/ 7039439.html)

From the present study it may be concluded that out of the total 184 specimens processed 18 showed positive results by RT-PCR, of the 18 positive specimens, 6 were Enterovirus 71 (Table 1) 3 Coxsackievirus A16, 8 Coxsackievirus A10, and 1 was vaccine-associated poliovirus 2. The patients those were identified with strain Enterovirus 71 presented hand, foot and mouth disease and one of them had signs of paralysis as well.The present study also confirmed that the isolated Enterovirus 71 is under genotype C1. This result is a valuable resource and can be used to choose strain for the development of future vaccine candidate against HFMD

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