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Human enterovirus A71 (EV-A71) isolated from acute flaccid paralysis patients without symptoms of hand foot and mouth disease

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ABSTRACT

Background: Human enterovirus A-71 (EV-A71) is one of the causative agents for hand, foot, and mouth disease (HFMD). It is also reported to cause acute flaccid paralysis (AFP) during HFMD outbreaks and very rare to isolate them from non-HFMD cases. **Materials and Methods:** A total of 2809 stool samples from AFP cases were cultured on RD and L20B cells. Positive on L20B was screened for poliovirus and those only positive on RD were subjected to non-polio EV (NPEV) screening by real-time polymerase chain reaction. Phylogenetic analysis using VP1 and VP4 gene was constructed. **Results:** A total of 74 EVs were isolated, 61 were NPEV and 13 were poliovirus Sabin like. The NPEVs were identified as coxsackieviruses, echoviruses, and EV-A71. Phylogenetic analysis showed that this EV-A71 belong to subgenogroup B5. **Conclusions:** The discovery of EV-A71 which caused AFP in patients but not related or associated with HFMD outbreaks showed that this virus could circulate independently. It should be investigated thoroughly as their ability to cause paralysis on the eradication of polio.

1. INTRODUCTION

Human enterovirus 71 (EV-A71) is classified under the human EV A species, a positive-sense RNA virus from EV genus in the family Picornaviridae. It is one of the common EVs associated with hand, foot, and mouth disease (HFMD) and typical symptoms include lesions on the palms, soles, and oral mucosa [1]. Since 1990s, together with Coxsackie A16 and Coxsackie A8, EV-A71 has been involved in major HFMD outbreaks worldwide such as outbreak in Sarawak, Malaysia in 1997 [2], Taiwan in 1998 [3], and China [4,5]. Many other countries including Japan, Singapore, Vietnam, and Peninsular Malaysia have experienced cyclical epidemics that occur every 2–3 years [6,7].

Lately, this virus has been reported to cause several HFMD outbreaks with cases not only presented with the typical HFMD syndromes but also with neurological diseases such as aseptic meningitis, encephalitis, and meningoencephalitis [8-12]. EV-A71 was also known to cause common non-polio EV (NPEV) associated with poliomyelitis-like paralysis in HFMD cases [13,14].

In the polio eradication program initiated by the World Health Organization, countries in the Western Pacific Region including Malaysia have been declared free from poliovirus since 2000 [15]. Despite the elimination of wild poliovirus associated with poliomyelitis in Malaysia, active surveillance of acute flaccid paralysis (AFP) cases is still ongoing intensively until global eradication is achieved. AFP is a clinical manifestation characterized by weakness or paralysis and reduced muscle tone excluding trauma in a child below 15 years involving one or more limbs [16]. In the road to achieve total eradication of poliomyelitis, viruses other than polioviruses have been reported to cause AFP. These include viruses such as echoviruses, coxsackieviruses, and EV-A71 [17].

Previously, EV-A71 is the most important etiology of AFP in patients during HFMD outbreaks [3,5,18]. These were also observed in the HFMD outbreaks in Sarawak, Taiwan, and China. It is very rare to have EV-A71 that caused AFP, isolated from non-HFMD cases. Recently, with the discovery of an isolate from India [19], which caused AFP in patients, but not related or associated with any HFMD showed that this virus could circulate independently and not associated with HFMD outbreaks. Later, [20] in Iran, [21,22] in Central Africa Republic and Madagascar, and [23] in India had also reported EV-A71 in non-HFMD patients. All of these isolates were detected during the AFP surveillance in their national eradication program. These findings suggested that EV-A71 could cause AFP not only in HFMD patients but also in children not related to HFMD outbreaks.

Molecular epidemiology showed that EV-A71 was clustered into three genogroups namely A, B, and C as proposed by Brown *et al*. [24]. These were based on the isolates from HFMD cases only. Subsequently, sequencing of isolates that caused AFP from non-HFMD cases was very diverse and not clustered into these genogroups. EV-A71 isolated from AFP cases in India formed a new genogroup D [19], followed by

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genogroup E from Central Africa Republic [21], genogroup F from Madagascar [22], and genogroup G from India [23]. Therefore, so far, we have seven genogroups of EV-A71 instead of five as initially proposed. In this study, we isolated two EV-A71 viruses from AFP cases without HFMD symptoms during the surveillance of AFP in polio eradication program.

This paper discussed molecular characteristic of two EV-A71 viruses isolated from AFP cases without HFMD symptoms.

2. MATERIALS AND METHODS

2.1. Samples

Virology Unit at the Institute for Medical Research (IMR) has been accredited as the National Polio Laboratory for Malaysia in 1992. Since then, it received all samples for AFP cases from all hospitals in Malaysia. For the past 10 years from 2004 to 2014, a total of 2809 stool samples from 1506 reported AFP cases were received (Table 1). The specimens were accompanied by an AFP notification forms with details of patient personal and clinical history.

2.2. Virus Isolation

All stool samples were processed with chloroform using the WHO standard protocol [25], before inoculation into rhabdomyosarcoma cells (RD) and a genetically engineered mouse cell line (L20B) cells using minimum essential medium (GIBCO, Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Invitrogen, USA), 50 U/ml benzylpenicillin, and 50 mg/ml streptomycin sulfate (Sigma, St Louis, USA). Cultures were observed daily for cytopathic effect (CPE) and harvested when more than 90% of the cell monolayer showed CPE.

2.3. Screening for NPEV

Since L20B cells are specific for poliovirus only, samples positive on L20B cells were screened for poliovirus using the WHO standard protocol [26]. Those that are only positive on RD cells and negative on L20B cells were screened for NPEV by amplifying the VP4 gene, a short DNA fragment for serotyping [7]. Sequence data were then subjected to analysis by Blast in GenBank to determine the serotypes. Those NPEV that were found to be EV-A71 were then selected for full VP1 gene amplification which is 891 nucleotides for genotyping.

2.4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Viral RNAs were extracted using the QIAamp® Viral RNA Mini Kit from Qiagen (Hilden, Germany). Briefly, samples were added to buffer AVL carrier RNA in microcentrifuge tubes and incubated at room temperature for 10 min. Then, ethanol was added and mixtures were transferred to QIAamp spin column for centrifugation. Buffer AW1 and AW2 were used to wash viral RNA in the spin column, and finally, buffer AVE was added to elute viral RNA in the clean microcentrifuge tubes.

Subsequently, two protocols were used to amplify the VP4 and VP1 gene of the virus. The VP4 gene was amplified using forward primer EVP2 (5'-CCT CCG GCC CCT GAATGC GGC TAA-3') [27] and reverse primer OL68-1(5'-GGT AAY TTC CAC CAC CAN CC-3') [28] in a one tube reaction (50µl) containing 5µl of RNA, 20 µM of each primer, 2.5 U of AMV reverse transcriptase and Promega Access Quick RT-PCR Kit (Cat. No: A1703). Reverse transcription was carried out at 48°C for 45 min followed by 10 min at 70°C to stop the reaction to get the first strand cDNA synthesis. Samples were then subjected to 35 PCR cycles with the following parameters: Denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 60 s.

The VP1 gene was also amplified using the Promega access quick RT-PCT Kit with primers VP1F2 (5'-ATA ATA GCA YTRGCG GCA GCC CA-3')-VP1R1 (5'-TGR GCR GTG GTA GAY GAYAC-3') as described by [6]. PCR cycling conditions were set up at 51°C for 30 min for reverse transcription followed by 35 cycles of 92°C for 30 s, 51°C for 45 s, and 72°C for 1 min. PCR products (1.1 kb) were examined by gel electrophoresis. QIAquick Gel Extraction Kits (QIAGEN Inc, Valencia, CA) were used to extract the PCR amplicons from the gel using the kit's standard protocol.

2.5. Nucleotide Sequencing of HEV-A71 VP1 Gene

The VP1 gene amplicons were sequenced by PCR primers and in-house internal VP1 primers; VP1 Int F (5'-TTC ACY TAY ATG CGY TTT GA-3') and VP1 Int R (5'-ACA AAC ATA TAY TGR AGY AAT TG-3'). Sequencing was performed using the Big Dye Cycle Sequencing Kit version 3.0 and an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, USA). The SeqMan software module in the Lasergene suite of programs (DNASTAR, Madison, USA) was used to format the nucleotide sequences. Reference EV-A71 sequences data

Table 1: Number of isolates from AFP cases between 2004 and 2014

Year	Number AFP cases	Number of stool specimens	Number of NPEV isolates	Number of polio isolates	Total isolates
2004	128	194	9	0	9
2005	146	283	5	4	9
2006	118	240	4	4	8
2007	110	220	6	0	6
2008	131	229	1	5	6
2009	106	119	1	0	1
2010	142	250	3	0	3
2011	143	252	7	0	7
2012	158	314	6	0	6
2013	159	316	12*	0	12
2014	165	312	7	0	7
Total	1506	2809	61	13	74

*Indicates the year where two HEV71 were isolates, AFP: Acute flaccid paralysis, NPEV: Non-polio enterovirus

were obtained from Genbank. The VP4 gene and VP1 gene sequences of EV-A71 strains from different genogroups were obtained from Genbank for generating dendrograms.

2.6. Phylogenetic Analysis

VP1 sequences were aligned using the Megalign software module in Lasergene suite of programs (DNASTAR, Madison, USA). Phylogenetic trees were constructed using the neighbor-joining method from the Software MEGA4. The CA16 strain G10 was used as an outgroup for phylogenetic analysis of the VP1 sequences data.

3. RESULTS

From 2004 to 2014, a total of 2809 stool samples from 1506 AFP cases were screened for poliovirus and NPEV by isolation in RD and L20B cells (Table 1). 74 EV were isolated in which 61 were NPEV and the remaining 13 were poliovirus and confirmed as Sabin-like strains by rRT-PCR [29]. The NPEVs were coxsackieviruses, echoviruses, and EV 71.

A phylogenetic tree based on VP1 gene and VP4 gene [Figures 1 and 2] showed that these EV-A71 isolates were closely related to genogroup B and clustered in subgenogroup B5 with sequence similarity of more than 85%. There were no significant changes in amino acid sequence in both genes compared to isolates from genogroup B, even with isolate from fatal cases.

4. DISCUSSION

Previously, EV-A71 isolates were classified into three genogroups A to C [24]. With the new findings of Indian isolate as genogroup D [19], genogroup E from sub-Saharan Africa and F from Madagascar [21,22], and genogroup G from India [23], there are now seven genogroups ranging from A to G. The first EV-A71 isolated in the world was from California, USA. This strain was known as prototype BrCr-CA-70 strain and the only member of the genogroup A [30,31]. It has been never reported since then until the recent outbreak in Anhui province of Central China in 2008 [32], where five isolates were claimed to be the reemergence of genogroup A. However, the sequence results of the complete VP1 revealed very little divergence between these isolates and the prototype. The second genogroup B has evolved over the years from two subgroups described by Brown *et al*. [24] into five subgenogroups; B1, B2, B3, B4, and B5 while genogroup C has been grouped into C1, C2, C3, C4, and C5.

In every outbreak of HFMD in any part of the world, EV-A71 was found to be cocirculating with other EV, especially CA16, CA10, and CA8. In Malaysia, the landmark of EV-A71 involvement in HFMD was the Sarawak outbreak in 1997. Report of child deaths [2,33] and patients presented with acute myocardial dysfunction and AFP gave a major concern about the role of EV-A71 infections. Toward the final stage of polio eradication program, probably less poliovirus will be found circulating due to change of vaccine from OPV to IPV in many countries including Malaysia. Therefore, viruses other than polioviruses have been found associated with AFP cases [34], among them were echoviruses, coxsackieviruses, and EV-A71 [17].

With the emergence of genogroup D [19], E and F [21,22], and G [23] indicated wide genetic diversity of EV-A71 isolated from AFP cases in patients not presenting the typical HFMD. What

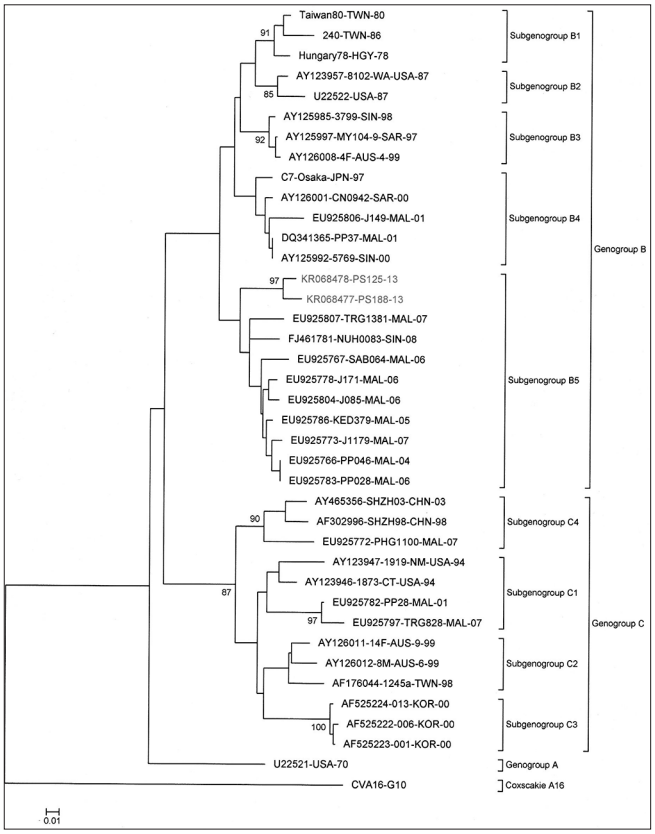

Figure 1: Phylogenetic tree of EV-A71 based on the complete VP4 gene. Genogroups and subgenogroups are indicated by the square brackets with CA16-G10 as an outgroup

is more worrying is the ability of EV-A71 to cause AFP on the eradication of polio. This was as shown by EV-A71 in genogroup D to G, which were isolated from AFP cases but not associated with HFMD outbreaks. During the screening of EVs that are associated as possible cause of AFP in Malaysia for the past 10 years, we found in additional to poliovirus, other EVs such as ECHO and coxsackieviruses were isolated from the stool samples of patients presenting with AFP cases. Interestingly, EV-A71 was isolated from two patients aged 5 years (PS188/13) and 7 years (PS125/13) old with AFP without HFMD. These findings indicated that EV-A71, which were normally isolated during HFMD outbreak worldwide now could be isolated in AFP cases. The similar findings were reported in India and Madagascar. Therefore, toward the final stages of polio eradication program, surveillance of AFP must be continued due to that EV-A71 possibly to cause neurological complications in some children which included AFP and brainstem encephalitis [2] as occurred in 1997 and 2000.

The phylogenetic trees either based on either VP4 (Accession no: KR068477 for isolate PS188/13; KR068478 for isolate PS125/13) gene or VP1 gene (Accession no: KR068479 for isolate PS188/13; KR068480 for isolate PS125/13) showed these two isolates were clustered in genogroup B and closely to the subgenogroup B5 which were prominent circulating in Malaysia since 2012 [35]. Analyzing the VP4 gene with 69 amino acid, there was similar amino acid at all positions compared to isolates from genogroup B1 to B5 regardless either from HFMD cases or from fatal cases such as isolate MY 104-9/SAR-97. This indicates there were no significant substitutions of

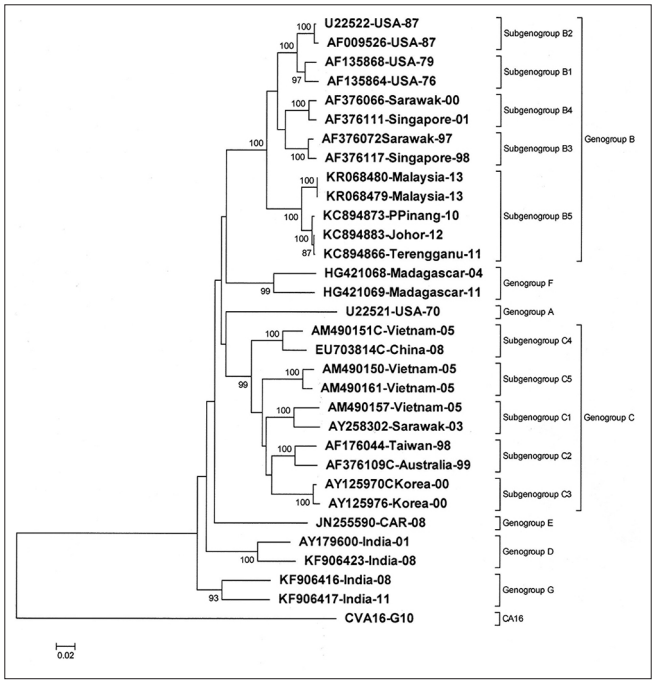

Figure 1: Phylogenetic tree of EV-A71 based on the complete VP1 gene. Genogroups and subgenogroups are indicated by the square brackets with CA16-G10 as an outgroup

amino acid that determined the severity or the disease and involved in fatality. Amino acid sequences in the VP1 gene also showed similar pattern, noted no differences among isolates in genogroup B. It was different from isolates in Australia in 2013, where they were clustered in subgenogroup C4A [36]. The Australian isolates which also caused AFP had some amino acid substitutions in the VP1 gene. This showed that other than genogroup D, E, F, and G, the genogroup B5 and C4A also involved in AFP, indicating the severity of EV-A71 across the region.

Therefore, the EV-A71 that was isolated from AFP cases, but non-HFMD had played an important role in neurological diseases. It was recognized as a neurotropic virus, associated with a diverse range of neurological diseases such as aseptic meningitis, brainstem and/or cerebellar encephalitis, and AFP [18,37-39]. AFP is one of the most common neurological symptoms in children with suspected infection of polio, even though most children are asymptomatic. Now EV-A71 considered as one of the leading causes of AFP since the wild poliovirus has been nearly eradicated [11,17]. This was proven in animal model [18,40] by orally administered of EV-A71 in neonatal mice. Radiological evidence showed induced paralysis through infection and destruction of anterior horn motor neurons of the spinal cord [14,39], a process identical to that of poliovirus [41] and recently D68 virus [42]. Hence, it is very important to sustain good surveillance of AFP in the polio eradication program to trace the emergence of EV-A71 that caused AFP.

5. CONCLUSION

A high level of laboratory surveillance has to be maintained in the National Polio Laboratory and play an integral role in the AFP surveillance and is committed toward the WHOs goal of polio eradication. EV-A71 isolated from the AFP cases should be investigated thoroughly as their ability to cause paralysis.

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