



Molecular epidemiology of human enterovirus71 (HEV71) strains isolated in Peninsular Malaysia and Sabah from year 2001 to 2009

Mohd Apani Yusof*, Fazilah Rais, Maizatul Akma Abdullah, Liyana Ahmad Zamri, Hariyati Md Ali, Fauziah Md Kassim and Zainah Saat

Virology Unit, Infectious Disease Research Centre, Institute for Medical Research, Kuala Lumpur, Malaysia.

Abstract

Human enterovirus71 (HEV71) together with other enteroviruses such as Coxsackie A16 and Coxsackie A10 is known to be responsible for hand, foot and mouth disease. Several of the hand, foot and mouth disease (HFMD) outbreaks caused by HEV71 were associated with neurological manifestations and deaths. In Peninsular Malaysia and Sabah, even though huge outbreaks of HFMD have never been reported; HEV71 strains however, were isolated periodically from HFMD cases throughout the year. From 2001 to 2009, four genetic lineages of HEV71 have been found to be prevalent in Peninsular Malaysia and Sabah. 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.

Keywords: Human enterovirus, neurological diseases, molecular epidemiology, gene sequences.

INTRODUCTION

Human enterovirus71 (HEV71) is a positive ssRNA virus belonging to the genus enterovirus in the family *Picornaviridae*. 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). Since its isolation in 1969 in California, USA from an encephalitis case (Schmidt et al., 1974), this virus has been reported to cause several outbreaks around the world with cases which do not only present with the typical HFMD syndromes but also with neurological diseases such as aseptic meningitis, encephalitis and meningioencephalitis (Blomberg et al., 1974; Shindarov et al., 1979; Lum et al., 1998; McMinn et al., 2001; Kehle et al., 2003). HEV71 is also the most common non-polio enterovirus associated with poliomyelitis-like paralysis (Melnick et al., 1984) due to its affinity to anterior horn cell (Chumakov et al., 1979).

In Malaysia, the 1997 cases of HFMD in Sarawak presented with acute myocarditis dysfunction and acute flaccid paralysis, and resulted in 34 paediatric deaths (Cardosa et al., 2003) aside the typical symptoms of HFMD. HEV71 was isolated in several of these cases

(Chan et al., 2000). This outbreak became a landmark in molecular epidemiology of HEV71 and the study of HEV71 strains in association with disease severity. Numerous reports on the molecular epidemiology of HEV71 strains from the Asia-Pacific region have been published (AbuBakar et al., 1999a; Brown et al., 1999; Shimizu et al., 1999; Wang et al., 1999; Shih et al., 2000; Singh et al., 2000; McMinn et al., 2001; Chu et al., 2001; Wang et al., 2002; Cardosa et al., 2003; Herrero et al., 2003; Shimizu et al., 2004; Li et al., 2005) and all have indicated the existence of three genogroups. Genogroup A represented by prototype BrCr-CA-70 which was isolated in California, USA in 1970 (Schmidt et al., 1974; Brown and Pallanch, 1995) has not been reported elsewhere. Genogroup B has 5 genetic lineages namely subgenogroup B1, B2, B3, B4, B5 and Genogroup C has 5 lineages; C1, C2, C3, C4 and C5.

HEV71 strains isolated from the state of Sarawak in East Malaysia have been described by many researchers involving huge number of HEV71 isolates (McMinn et al., 2001; Cardosa et al., 2003; Podin et al., 2006; Ooi et al., 2007). However, only a few data is available on the

molecular epidemiology of HEV71 strains from Peninsular Malaysia and Sabah despite the fact that several HEV71 outbreaks have been reported by AbuBakar et al. (1999b), Singh et al. (2000) and Herrero et al. (2003). Work done by AbuBakar et al. (1999b) only involved nine HEV71 isolated in 1997. Singh et al. (2000) reported on four HEV71 isolates in 1997 and 1998; while Herrero et al. (2003) reported on molecular epidemiological data of 43 HEV71 isolates from 1997 to 2000. Even though there was no report on huge HFMD outbreaks in these two regions, HEV71 was constantly isolated from patients with HFMD without CNS infection throughout 2001 to 2009. Therefore, the main objective of this study was to analyze and characterise all HEV71 that have been circulating in both Peninsular Malaysia and Sabah for the past 10 years.

MATERIALS AND METHODS

Samples

Samples such as vesicle swabs, throat swab and rectal swabs were collected from patients showing signs and symptoms of HFMD. All state hospitals in Peninsular Malaysia and Sabah were involved in this study and acted as a specimen collection centre. All samples were cultured in rhabdomyosarcoma cells (RD cells) using Minimum Essential Medium (GIBCO, Invitrogen, USA) supplemented with 10% heat inactivated fetal bovine serum (GIBCO, Invitrogen, USA), 50 U/ml benzyl penicillin and 50 µg/ml streptomycin sulphate (Sigma, St Louis, USA). Cultures were observed daily for cytopathic effect (CPE) and harvested when more than 90% of the cell monolayer showed CPE.

Viruses

A total of 70 HEV71 strains were isolated. There were 4 isolates in 2001, 12 in 2003, 1 in 2004, 7 in 2005, 15 in 2006, 4 in 2007, 23 in 2008 and 4 in 2009. All the HEV71 isolates including information of accession number, type of specimen, age, gender, clinical diagnosis and year of isolation are shown in Tables 1 and 2.

HEV71 screening assays

Cell cultures showing CPE were screened for HEV71 using HEV71-specific RT-PCR assays as described by Perera et al. (2004).

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 a microcentrifuge tubes and incubated at room temperature for 10 min. Later 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. The VP4 gene was amplified using forward primer EVP2 (5'-CCT CCG GCC CCT GAA TGC GGC TAA-3') (Chua et al., 2001) and reverse primer OL68-1 (5'-GGT AAY TTC CAC CAC CAN CC-3') (Ishiko et al., 2002) in a

one tube reaction (50µl) containing 5µl of RNA, 20 µM of each primer, 2.5U 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 35PCR cycles, 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-PCR Kit with primers VP1F2 (5'-ATA ATA GCA YTR GCG GCA GCC CA-3')-VP1R1 (5'-TGR GCR GTG GTA GAY GAY AC-3') as described by Tu et al. (2007). Polymerase Chain Reaction (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) was used to extract the DNA from the gel.

Nucleotide sequencing of HEV71 VP4 and VP1 gene

Whereas HEV71 VP4 gene amplicons were sequenced on both strands by using PCR primers, 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 by using the Big Dye Cycle Sequencing kit version 3.0 and an ABI377 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.

HEV71 Sequence data obtained from genbank

The VP4 gene and VP1 gene sequences of HEV71 strains from different genogroups were obtained from genbank for the purpose of generating dendograms.

Phylogenetic analysis

Alignment of the VP1 and VP4 gene sequences was undertaken by using the Megalign software module in Lasergene suite of programs (DNASTAR, Madison, USA). Phylogenetic trees were constructed by using the neighbor-joining method from the Software MEGA4. The CA16 strain G10 was used as an outgroup for phylogenetic analysis of both the VP4 and VP1 sequence data.

RESULTS

From year 2001 to 2007 a total of 43 HEV71 strains were isolated from patients suspected of having HFMD in Malaysia. Phylogenetic tree constructed based on the complete VP4 gene (207) nucleotides, shown in Figure 1, revealed that 4 HEV71 strains isolated in 2001, 2 isolates belonged to subgenogroup C1 and another 2 to subgenogroup B4. Of the 12 HEV71 strains isolated in 2003, 11 isolates belonged to subgenogroup C1 and 1 isolate in subgenogroup B5. All the isolates in 2004 and 2005 were in the subgenogroup B5 whereas in 2006, about 13 isolates were in B5 and 2 were in C1. The scenario was different in 2007 in which for the first time the subgenogroup C4 was isolated in Malaysia and this

Table 1. HEV71 used in the study to sequence VP4 gene.

Isolate	Accession No	Sample type	Sex/Age	Diagnosis	Isolation year
PP046-MAL-04	EU925766	R/S	M/3	HFMD	2004
SAB064-MAL-06	EU925767	V/S	M/1	HFMD	2006
PHG065-MAL-06	EU925768	V/S	M/2	HFMD	2006
SAB070-MAL-06	EU925769	T/S	F/0.5	HFMD	2006
PHG084-MAL-06	EU925770	V/S	M/10	HFMD	2006
J092-MAL-06	EU925771	R/S	M/5	HFMD	2006
PHG1100-MAL-07	EU925772	V/S	M/4	HFMD	2007
J1179-MAL-07	EU925773	T/S	F/4	HFMD	2007
J142-MAL-03	EU925774	T/S	M/1	HFMD	2003
KL161-MAL-06	EU925775	T/S	M/2	HFMD	2006
PP166-MAL-03	EU925776	T/S	M/3	HFMD	2003
PP168-MAL-03	EU925777	T/S	F/3	HFMD	2003
J171-MAL-06	EU925778	R/S	M/6	HFMD	2006
J183-MAL-03	EU925779	V/S, T/S	F/3	HFMD	2003
NS213-MAL-03	EU925780	V/S, R/S	M/3	HFMD	2003
PTJ218-MAL-06	EU925781	T/S	M/3	HFMD	2006
PP28-MAL-01	EU925782	R/S	F/4	HFMD	2001
PP028-MAL-06	EU925783	R/S	F/4	HFMD	2006
PP307-MAL-06	EU925784	T/S	F/2	HFMD	2006
J317-MAL-06	EU925785	T/S	F/1	HFMD	2006
KED379-MAL-05	EU925786	T/S	F/2	HFMD	2005
KED393-MAL-05	EU925787	T/S	M/2	HFMD	2005
KED394-MAL-05	EU925788	T/S	F/4.5	HFMD	2005
J514-MAL-06	EU925789	V/S	F/1	HFMD	2006
PP525-MAL-05	EU925790	R/S	F/4	HFMD	2005
PP533-MAL-05	EU925791	T/S	M/2	HFMD	2005
PP550-MAL-05	EU925792	T/S	F/2	HFMD	2005
PP576-MAL-05	EU925793	R/S	F/1	HFMD	2005
PP648-MAL-06	EU925794	V/S	M/2	HFMD	2006
J78-MAL-03	EU925795	T/S	M/1.5	HFMD	2003
J80-MAL-03	EU925796	R/S	F/2.5	HFMD	2003
TRG828-MAL-07	EU925797	V/S	M/2	HFMD	2007
PP85-MAL-03	EU925798	T/S	M/2	HFMD	2003
PP96-MAL-03	EU925799	T/S	F/2	HFMD	2003
J98-MAL-03	EU925800	V/S	F/3	HFMD	2003
J05-MAL-01	EU925801	R/S	M/2	HFMD	2001
J145-MAL-01	EU925802	T/S	M/3	HFMD	2001
PP76-MAL-03	EU925803	T/S	M/5	HFMD	2003
J085-MAL-06	EU925804	V/S	F/5	HFMD	2006
PP72-MAL-03	EU925805	T/S	M/3	HFMD	2003
J149-MAL-01	EU925806	V/S,T/S,R/S	M/2	HFMD	2001
TRG1381-MAL-07	EU925807	V/S	F/4	HFMD	2007
PHG53-MAL-06	EU925808	R/S	M/3	HFMD	2006

T/S = throat swab, R/S = rectal swab, V/S = vesicle swab, M = Male; F = Female; age in year.

was closely related to the strains from China. Two of the isolates from 2007 were from subgenogroup B5 and one was from C1.

Twenty-seven of HEV71 isolated in 2008 and 2009 were sequenced for the complete VP1 gene which

consists of 891 nucleotides. The phylogenetic tree was constructed together with the complete VP1 gene derived from the Genbank (Figure 2). All the HEV71 strains isolated in 2008 and 2009 were from the subgenogroup B5.

Table 2. HEV71 used in the study to sequence VP1 gene.

Isolate	Accession No	Sample type	Sex/Age	Diagnosis	Isolation year
EV1075-Pahang-08	HM358809	V/S	M/7	HFMD	2008
EV0336-Sabah-08	HM358810	V/S	M/3	HFMD	2008
EV0372-Sabah-08	HM358811	T/S	F/2	HFMD	2008
EV0408-Penang-08	HM358812	R/S	F/2	HFMD	2008
EV0466-Johor-08	HM358813	T/S	M/4	HFMD	2008
EV0482-Sabah-08	HM358814	T/S	F/4	HFMD	2008
EV0562-Penang-08	HM358815	T/S, R/S	M/4	HFMD	2008
EV0577-Pahang-08	HM358816	T/S	M/1.5	HFMD	2008
EV0758-Sabah-08	HM358817	V/S	M/6	HFMD	2008
EV0764-Johor-08	HM358818	V/S	M/1	HFMD	2008
EV0811-Penang-08	HM358819	R/S	M/1	HFMD	2008
EV0879-Bintulu-08	HM358820	T/S	M/2	HFMD	2008
EV0884-Johor-08	HM358821	V/S	M/1	HFMD	2008
EV0891-Johor-08	HM358822	V/S	M/6	HFMD	2008
EV0911-Kedah-08	HM358823	V/S	M/7	HFMD	2008
EV0943-Johor-08	HM358824	T/S	F/2	HFMD	2008
EV0972-Johor-08	HM358825	V/S	M/4	HFMD	2008
EV1019-Penang-08	HM358826	T/S	F/5	HFMD	2008
EV1025-Penang-08	HM358827	T/S	M/1	HFMD	2008
EV1035-Pahang-08	HM358828	T/S	M/1	HFMD	2008
EV1078-Johor-08	HM358829	T/S	F/5	HFMD	2008
EV1094-Johor-08	HM358830	T/S	M/1	HFMD	2008
EV0338-Sabah-08	HM358831	V/S	F/3	HFMD	2008
EV0031-Johor-09	HM358832	V/S	M/1	HFMD	2009
EV0076-KLumpur-09	HM358833	R/S	F/1	HFMD	2009
EV1705-Johor-09	HM358834	T/S	M/1	HFMD	2009
EV1945-Kuching-09	HM358835	T/S	F/2	HFMD	2009

T/S = throat swab, R'S = rectal swab, V/S = vesicle swab, M = Male; F = Female; age in year.

DISCUSSION

Before the HFMD outbreak in Sarawak in 1997, HEV71 was considered as an etiological agent of HFMD without any impact together with other enteroviruses especially CA16 and CA10. However, the report of child deaths (Chan et al., 2000; Cardosa et al., 2003) in which HFMD presented with not only the typical symptoms of HFMD but also with acute myocardial dysfunction and acute flaccid paralysis resulted in a major impact in government policy towards the public health including monitoring and control of HFMD cases. The Malaysian government made a ruling that all HFMD cases either with or without neurological manifestations become a notifiable disease in 2007 (MOH, 2007) and since then a surveillance system for monitoring HFMD has been established.

Analyzing the phylogenetic relationship of HEV71 strains in order to determine whether there is some relationship between their genotypes and pathogenic properties has been carried out by many researchers (Shimizu et al., 1999; Brown et al., 1999; Shih et al., 2000; Wang et al., 2002; Munemura et al., 2003). It was

initially thought that the new virulent form of HEV71 was responsible for the outbreaks and deaths among HFMD cases. However, until now, a distinct association of certain genogroups with particular neurovirulence has not been identified from molecular analysis (Shimizu et al., 2004). No significant nucleotide difference has been found between the fatal and non fatal cases by sequence comparison regardless of whether those findings are based on the phylogenetic analysis of VP4 (Shimizu et al., 1999), VP1 (Brown et al., 1999) or 5'UTR (Abubakar et al., 1999b). Thus, it was suggested that virulence might not be determined by a single viral gene (Chua et al., 2001).

In this study, phylogenetic trees were performed using the complete VP4 gene for strains isolated from 2001 to 2007 and VP1 gene for HEV71 in 2008 to 2009. Although, different regions were used, identical clustering was shown regardless of the regions examined (Cardosa et al., 2003 and Shimizu et al., 2004). The VP4 was normally used for molecular typing of human enterovirus (Ishiko et al., 2002) and also for detailed molecular epidemiological analysis (Cardosa et al., 2003). However,

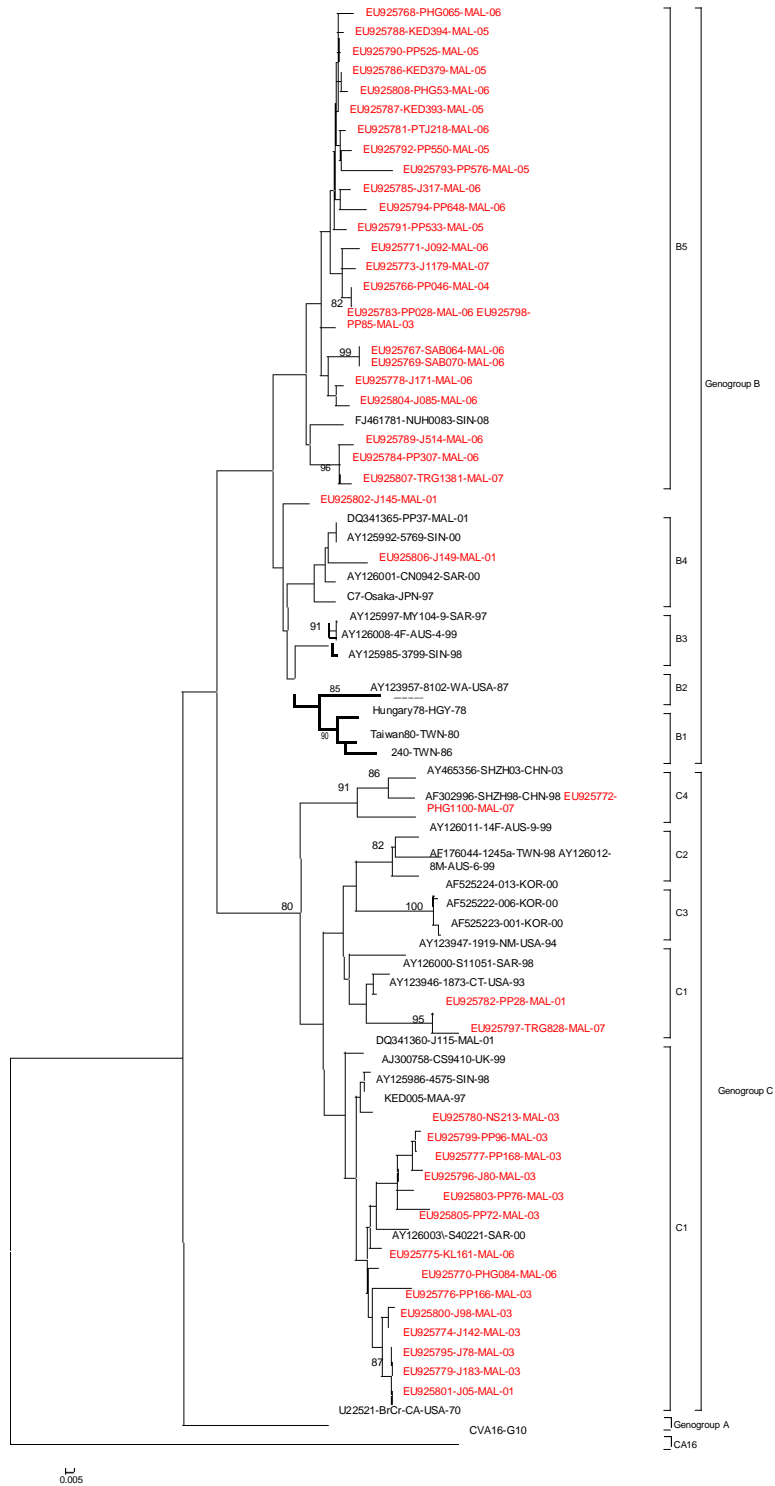


Figure 1. Phylogenetic tree of complete VP4 gene (207) of HEV71 inferred using the Neighbor-Joining method from the software MEGA 4. The evolutionary distances were computed using the Maximum Composite Likelihood method. Genogroups and subgenogroups are indicated by square brackets with CA16-G10 virus as an outgroup. 43 HEV71 isolates in 2001-2007 are indicated in red and underline words. Representative strains of each genogroup obtained from GenBank are labeled using the following format: 'Accession number'- 'isolate'-'Country of origin'- 'Year isolation'. Bootstrap values (>80%) for 1,000 pseudoreplicate dataset are indicated at branch nodes.

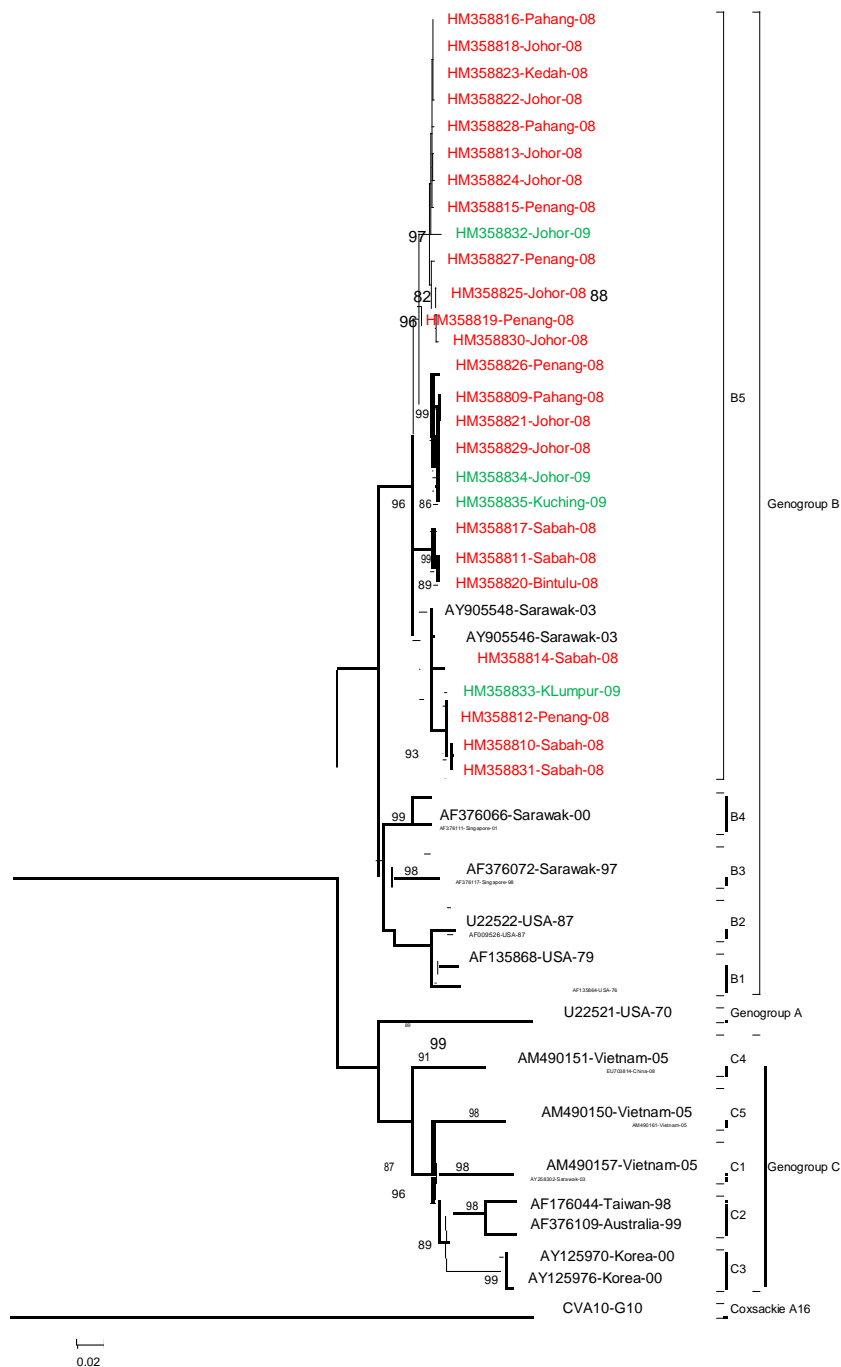


Figure 2. Phylogenetic tree of complete VP1 gene (891) of HEV71 inferred using the Neighbor-Joining method from the software MEGA 4. The evolutionary distances were computed using the Maximum Composite Likelihood method. Genogroups and subgenogroups are indicated by square brackets with CA16- G10 virus as an out group. 27 HEV71 isolates in 2008 and 2009 are indicated in red and green underline words. Representative strains of each genogroup obtained from GenBank are labeled using the following format: 'Accession number'-'Country of origin'- 'Year isolation'. Bootstrap values (>80%) for 1,000 pseudoreplicate dataset are indicated at branch nodes.

VP1 based phylogenetic tree provided more reliable genogrouping of HEV71 because of longer nucleotides

and possible correlation of the genogroups with viral antigenicity (McMinn et al., 2001; Cardosa et al., 2003 and

Herrero et al., 2003). Currently there are three genogroups related to HEV71. The first HEV71 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 (Schmidt et al., 1974; Brown and Pallanch, 1995). It has not been reported since then. The genogroup B has evolved over the years from 2 subgroups described by Brown et al. (1999) into 5 subgenogroups; B1, B2, B3, B4 and B5 while genogroup C has been grouped into C1, C2, C3, C4 and C5.

Molecular epidemiological analysis in this study showed that only 4 subgenogroups namely B4, B5, C1 and C4 were circulating in Peninsular Malaysia and Sabah between 2001 and 2009. The subgenogroup B4 was isolated in 2001 however, analysis 43 HEV71 isolated from 1997 to 2000 in peninsular Malaysia by Herrero et al. (2003) showed that the B4 subgenogroup was the most prevalent subgenogroups in 1997, 1999 and 2000. HEV71 belonging to genogroup B4 were also identified in several HFMD cases in Singapore in 1997 (McMinn et al., 2001), Peninsular Malaysia in 1997 to 1998 (Herrero et al., 2003), Taiwan in 1998 (Shih, et al., 2000) and Sarawak in 2000 (Cardosa et al., 2003). Thus, indicating that this genogroup was widespread, although not predominant, throughout the Asia-Pacific region until it became the focus of large epidemic activities in Peninsular Malaysia and Sarawak, and Singapore in 2000 (Cardosa et al., 2003), apparently replacing subgenogroup B3 viruses. The subgenogroup B3 was reported as the predominant strain in 1997 especially in Sarawak (Cardosa et al., 2003), but none was found to be circulating in Peninsular Malaysia and Sabah from 2001 to 2009. The subgenogroup B5, which emerged after the subgenogroup B4, was the predominant strain between 2003 and 2009. Thus, indicating that viruses in the genogroup B appeared to be evolving over the years from 2 subgroups described by Brown et al. (1999). To date, 5 subgenogroups have been identified viz. B1, B2, B3, B4 and B5.

The subgenogroup B1 viruses were circulating from 1974 to 1979 in Bulgaria, Hungary, USA and Australia and also in Taiwan in the early eighties. This was followed by subgenogroup B2, which was isolated mostly in the USA from 1987 to 1988. However, B2 has not been isolated in the USA since 1988 (Brown et al., 1999). A decade later in 1998, B2 was isolated from meningitis cases in Germany (Kehle et al., 2003). Between 1997 and 1999, the subgenogroup B3 viruses were the predominant strains in Southeast Asia and were identified as the major cause of epidemics in Sarawak in 1997 (Cardosa et al., 2003). The subgenogroup B3 viruses might be associated with cases of severe encephalitis resulting in fatalities in some cases in Sarawak from 1997 to 1999 (McMinn et al., 2001; Cardosa et al., 2003; Herrero et al., 2003), Singapore in 1998 and Western Australia in 1999 (McMinn et al., 2001). Later, the subgenogroup B4 viruses were isolated in several HFMD

cases in Singapore in 1997 (McMinn et al., 2001), Peninsular Malaysia from 1997 to 1998 (Herrero et al., 2003), Taiwan in 1998 (Shih et al., 2000) and Sarawak in 2000 (Cardosa et al., 2003). In 2003, the subgenogroup B5 was also isolated from Sarawak during a large HEV71 outbreak that started in February 2003 (Cardosa, unpublished data).

Two subgenogroup C were also isolated in this study. The subgenogroup C1 was isolated annually isolated from 2001 to 2007 but none in 2008 and 2009. This finding was parallel to the report by Brown et al. (1999) where the subgenogroup C1, previously prevalent in North America and Eastern Australia, appears to have undergone low-level endemic circulation within Southeast Asia and Western Australia between 1997 and 2003 and were isolated sporadically throughout 1997 to 2003 either from Sarawak, Malaysia, Singapore, Australia, United Kingdom and USA. However, subgenogroup C1 has not been reported as the cause of large outbreaks. Viruses belonging to the subgenogroup C4 were isolated in China in year 2000 (Shimizu et al., 2004; Li et al., 2005) even though no outbreaks were recorded. In Taiwan the subgenogroup C4 emerged and became predominant in 2004 (Lin et al., 2006). However, only one HEV71 in subgenogroup C4 was isolated in the east coast state of Peninsular Malaysia in 2004. No subgenogroups C2, C3 and C5 were isolated in this study.

The subgenogroup C2 was first reported in Malaysia in 1997 (Abubakar et al., 1999) from HFMD cases and this subgenogroup was found mostly during the large Taiwan outbreaks in 1998 (Shih et al., 2000; Wang et al., 2002) which was known to be associated with the emergence of fatal encephalitis. It was also occasionally isolated in Japan in 1997, Australia in 1999 and UK from 1998 to 1999. The subgenogroups C3 and C5, so far have never been reported in Malaysia (AbuBakar et al., 1999b; Cardosa et al., 2003; Herero et al., 2003). In the Asia-pacific region, the subgenogroup C3 was isolated only during the major outbreaks in Korea in 2000. A new subgenogroup C5 has been found circulated widely in Southern Vietnam throughout 2005 and became the predominant virus strain identified during the second half of the year in Vietnam (Tu et al., 2007).

Hand foot and mouth disease and HEV71 infections are still a major problem in Malaysia. However, a good surveillance system for HFMD and monitoring of severe encephalitis due to enteroviruses especially HEV71 has been established in Malaysia resulting in an easier and prompt action in order to stop spread of infection. This system has assisted the government in containing HEV71 outbreaks and has benefited young Malaysians from the disease.

Conclusion

Both genogroups B and C were found to be circulating in

Peninsular Malaysia and Sabah from 2000 to 2009. In the genogroup B, B4 was isolated in 2001 and was replaced by the B5 in 2003 which later became the most predominant HEV71 strain circulating between 2005 and 2009. In the genogroup C, C1 was periodically isolated through 2001 to 2007 but C4 was only isolated in 2007 in Peninsular Malaysia. These four strains were closely related to the strains that caused outbreaks of HFMD in several Asia-Pacific countries; however, no major outbreak has been reported in Peninsular Malaysia and Sabah so far.

ACKNOWLEDGEMENT

The authors would like to thank the Director General of Health and the Director of the Institute for Medical Research for permission to publish this paper. The authors would also like to thank and acknowledge Professor Dr Jane Cardosa and Dr David Perera from UNIMAS for their assistance in sequencing the VP1 gene of the 2008 HEV71 isolates.

REFERENCES

- AbuBakar S, Chee HY, Shafee N, Chua KB, Lam SK (1999a). Molecular detection of enterovirus from an outbreak of hand, foot and mouth disease in Malaysia in 1997. *Scan. J. Infect. Dis.*, 31: 331-335.
- AbuBakar S, Chee HY, Al-Kobaisi MF, Xiaoshan J, Chua KB, Lam SK (1999b). Identification of enterovirus 71 isolates from an outbreak of hand, foot and mouth disease with fatal cases of encephalomyelitis in Malaysia. *Virus Res.*, 61: 1-9.
- Blomberg J, Lycke E, Ahlfors K, Johnson T, Wolontis S, von Ziepel G (1974). New enterovirus type associated with epidemic of aseptic meningitis and /or hand, foot, and mouth disease [letter]. *Lancet*, 2: 112.
- Brown BA, Pallansch MA (1995). Complete nucleotide sequence of enterovirus 71 is distinct from poliovirus. *Virus Res.*, 39: 195-205.
- Brown BA, Oberste MS, Alexander JP, Kennett ML, Pallansch MA (1999). Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. *J. Virol.*, 73: 9969-9975.
- Cardosa MJ, Perera D, Brown BA, Cheon D, Chan HM, Chan KP, Cho H, McMinn PC (2003). Molecular Epidemiology of Human Enterovirus 71 Strains and Recent Outbreaks in the Asia-Pacific Region: Comparative Analysis of the VP1 and VP4 Genes. *Emerging Infect. Dis.*, 9(4): 461-468.
- Chan LG, Parashar UD, Lye MS, Ong FGL, Zaki SR, Alexander JP, Ho KK, Han LL, Pallansch MA, Suleiman AB, Jegathesan M, Anderson LJ (2000). Deaths of children during an outbreak of hand, foot and mouth disease in Sarawak, Malaysia. *Clin. Pathol. Characteristics Dis. Clin. Infect. Dis.*, 31: 678-683.
- Chu PY, Lin KH, Hwang KP, Chou LC, Wang CF, Shih SR, Wang JR, Shimada Y, Ishiko H (2001). Molecular epidemiology of enterovirus 71 in Taiwan. *Arch. Virol.*, 146: 589-600.
- Chua BH, McMinn PC, Lam SK, Chua KB (2001). Comparison of complete nucleotide sequences of echovirus 7 strains UMMC and the prototype (Wallace) strain demonstrates significant genetic drift over time. *J. Gen. Virol.*, 82: 2629-2639.
- Chumakov M, Voroshilova M, Shindarov L, Lavrova I, Gracheva L, Koroleva G, Vasilenko S, Brodvarova I, Nikolova M, Gyurova S, Gacheva M, Mitov G, Ninov N, Tsyilka E, Robinson I, Frolova M, Bashkirtsev V, Martiyanova L, Rodin V (1979). Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. *Arch. Virol.*, 60: 329-340.
- Herrero LJ, Lee CS, Hurrelbrink RJ, Chua BH, Chua KB, McMinn PC (2003). Molecular epidemiology of enterovirus 71 in peninsular Malaysia, 1997-2000. *Arch. Virol.*, 148(7): 1369-1385.
- Ishiko H, Shimada Y, Yonaha M, Hashimoto O, Hayashi A, Sakae K, Takeda N (2002). Molecular diagnosis of human enteroviruses by phylogeny-based classification by use of the VP4 sequence. *J. Infect. Dis.*, 185: 744-754.
- Kehle J, Roth B, Metzger C, Pfitzner A, Enders G (2003). Molecular characterization of an enterovirus 71 causing neurological disease in Germany. *J. Neurovirol.*, 9: 126-128.
- Li L, He Y, Yang H, Zhu J, Xu X, Dong J, Zhu Y, Jin Q (2005). Genetic Characteristics of Human Enterovirus 71 and Coxsackievirus A16 Circulating from 1999 to 2004 in Shenzhen, People's Republic of China. *J. Clin. Microbiol.*, 3835-3839.
- Lin KH, Hwang KP, Ke GM, Wang CF, Ke LY, Hsu YT, Tung YC, Chu PY, Chen BH, Chen HL, Kao CL, Wang JR, Eng HL, Wang SY, Hsu LC, Chen HY (2006). Evolution of EV71 genogroup in Taiwan from 1998 to 2005: An emerging of subgenogroup C4 of EV71. *J. Med. Virol.*, 78(2): 254-262.
- Lum LCS, Wong KT, Lam SK, Chua KB, Goh AYT (1998). Neurogenic pulmonary oedema and enterovirus 71 encephalomyelitis. *Lancet*, 352: 1391.
- McMinn PC, Lindsay K, Perera D, Chan HM, Chan KP, Cardosa MJ (2001). Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore and Western Austr. *J. Virol.*, 75(16): 7732-7738.
- Melnick JL (1984). Enterovirus type 71 infections: a varied clinical pattern sometimes mimicking paralytic poliomyelitis. *Rev. Infect. Dis.*, 6(2): S387-S390.
- Ministry of Health, Malaysia (MHM) (2007). Prevention and Control of infectious diseases 9amendment of first schedule0 order 2007.
- Minor PD, Brown F, Domingo E, Hoey E, King A, Knowles N, Lemon S, Palmenberg A, Rueckert RR, Stanway G, Wimmer E, Yin-Murphy M (1995). The Picornaviridae. In virus taxonomy. Sixth Report of The International Committee on Taxonomy of Viruses, Edited by FA Murphy, C.M. Fauquet, D.H.L. Bishop, S.A. Chabrial, A.W. Jarvis, G.P. Martelli, M.A. Mayo and M.D. Summer. Vienna & New York: Springer-Verlag, pp. 268-274.
- Munemura T, Saikusa M, Kawakami C, Shimizu H, Oseto M, Hagiwara A, Kimura H, Miyamura T (2003). Genetic diversity of enterovirus 71 isolated from cases of hand, foot and mouth disease in Yokohama City between 1982 and 2000. *Arch. Virol.*, 148: 253-263.
- Ooi MH, Wong SC, Podin Y, Akin W, Syvia del S, Mohan A, Chieng CH, Perera D, Clear D, Wong D, Blake E, Cardosa J, Solomon T (2007). Human Enterovirus 71 Disease in Sarawak, Malaysia: A Prospective Clinical, Virological, and Molecular Epidemiological Study. *Clin. Infect. Dis.*, 44: 646-656.
- Perera D, Podin Y, Winnie A, Tan CS, Cardosa MJ (2004). Incorrect identification of recent Asian strains of Coxsackievirus A16 as human enterovirus 71: Improved primers for the specific detection of human enterovirus 71 by RT-PCR. *BMC Infect. Dis.*, 4: 1-11.
- Podin Y, Edna LMG, Ong F, Leong YW, Yee SF, Apani Y, Perera D, Teo B, Wee TY, Yao SK, Kiyu A, Arif MT, Cardosa MJ (2006). Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. *BMC Pub. Health*, 6: 180.
- Schmidt NJ, Lennette EH, Ho HH (1974). An apparently new enterovirus isolated from patients with disease of the central nervous system. *J. Infect. Dis.*, 129: 304-309.
- Shih SR, Ho MS, Lin KH, Wu SL, Chen YT, Wu CN (2000). Genetic analysis of enterovirus 71 isolated from fatal and non-fatal cases of hand, foot and mouth disease during an epidemic in Taiwan, 1998. *Virus Res.*, 68: 127-136.
- Shimizu H, Utama A, Yoshii K, Yoshida H, Yoneyama T, Sinniah M (1999). Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997-1998. *Jpn. J. Infect.*, 52: 12-15.
- Shimizu H, Utama A, Onnimala N, Li C, Li-Bi Z, Yu-Jie M, Pongsuwana Y, Miyamura T (2004). Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region. *Paediatr. Int.*, 46: 1-5.
- Shindarov LM, Chumakov MP, Voroshilova MK, Bojinov S, Vasilenko SM, Iordanov I (1979). Epidemiological, clinical and pathomorphological characteristics of epidemic poliomyelitis-like disease caused by enterovirus 71. *J. Hyg. Epidemiol. Microbiol.*

- Immunol., 23: 284-295.
- Singh S, Chow VTK, Chan KP, Ling AE, Poh CL (2000). RT-PCR, nucleotide, amino-acid and phylogenetic analyses of enterovirus type 71 from Asia. *J. Virol. Meth.*, 88: 193-204.
- Tu PV, Thao NTT, Perera D, Huu TK, Tien NTK, Thuong TC, Ooi MH, Cardosa MJ, McMinn PC (2007). Epidemiologic and virologic investigation of hand, foot, and mouth disease, Southern Vietnam, 2005. *Emerg. Infect. Dis.*, 13(11): 1733-1741.
- Wang SM, Liu CC, Tseng HW, Wang JR, Huang CC, Chen YJ, Yang YJ, Lin SJ, Yeh TF (1999). Clinical spectrum of enterovirus 71 infection in children in southern Taiwan, with emphasis on neurological complications. *J. Clin. Infect. Dis.*, 29: 184-190.
- Wang JR, Tuan YC, Hsai HP, Yan JJ, Liu CC, Su IJ (2002). Change of major genotype of enterovirus 71 in outbreaks of hand foot and mouth disease in Taiwan between 1998 and 2000. *J. Clin. Virol.*, 40: 10-15.