

*Full Length Research Paper*

## Subgenogroup B5 maintains its supremacy over other human Enterovirus71 strains that circulated in Malaysia from 2010 to 2012

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Human enterovirus71 (HEV71) is responsible for hand, foot and mouth diseases (HFMD). Several outbreaks of HFMD were associated with severe neurological disease and deaths. In Malaysia, outbreaks normally occur periodically every two to three years but HEV71 were isolated throughout the year from HFMD cases. From 2010 to 2012, HEV71 strains were isolated from 37 children presented with typical HFMD. All isolates were sequenced and BLAST searched. A phylogenetic tree constructed based on the complete VP1 gene showed that all isolates belonged to subgenogroup B5. This subgenogroup has been found dominant since 2003.

**Key words:** Human enterovirus71, molecular epidemiology, gene sequences, circulating subgenogroup.

### INTRODUCTION

Human enterovirus71 (HEV71) is classified under the human enterovirus A species. It is a positive-sense RNA virus from enterovirus genus in the family picornaviridae. Together with Coxsackie A16 and Coxsackie A6, they cause major outbreaks of hand foot and mouth disease (HFMD) in children. During outbreaks in Sarawak, Malaysia in 1997 (Chan et al., 2000) and Taiwan in 1998 (Wang et al., 2002), HEV71 infections not only presented with typical HFMD but were also associated with neurological disorders such as encephalitis, aseptic meningitis and meningoencephalitis (McMinn et al., 2001; Kehle et al., 2003), and paralysis (Melnick, 1984) due to its affinity to anterior horn cell (Chumakov et al., 1979).

HFMD outbreaks associated with HEV71 had been reported globally. In the Asia Pacific region, large

outbreaks have been recorded in Sarawak and Peninsular Malaysia in 1997, with 41 and 4 deaths, respectively (Chan et al., 2000). The largest outbreak so far, occurred in Taiwan in 1998 where an estimated 1.5 million people were infected with 405 children being hospitalised, of which 78 died (Wang et al., 2002). The recent epidemic in China in 2008, recorded 0.5 million cases with 126 deaths (Zhang et al., 2009, 2010). Many countries including Japan, Singapore, Sarawak, Vietnam and Peninsular Malaysia have experienced cyclical epidemics that occur every 2-3 years (Fujimoto et al., 2002; Podin et al., 2006; Tu et al., 2007; Apandi et al., 2011).

Most of these outbreaks not only caused huge hospital admission but also resulted in fatality. One of the most

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important findings of these outbreaks was the emergence of different strains of HEV71.

Following the 1997 outbreak in Sarawak, the Malaysian Ministry of Health (MOH) made a ruling that all HFMD cases with or without neurological manifestations must be notified. Since then, a surveillance system for monitoring HFMD has been established.

## MATERIALS AND METHODS

Clinical samples such as vesicle swabs, throat swabs, rectal swabs and stool from hospitalized HFMD patients were collected from 15 states in Malaysia. They were screened for enterovirus (EV) by RT-PCR (Perera et al., 2004). Positive samples were cultured in rhabdomyosarcoma (RD) cells and harvested once cytopathic effect was observed.

Viral RNA was extracted using the QIAamp® Viral RNA Mini Kit (Hilden, Germany). RT-PCR with specific HEV71 primers (Tu et al., 2007) was used for complete VP1 gene amplification. All isolates were sequenced using PCR primers as described by Tu et al. (2007) and internal primers VP1 Int F and VP1 Int R (Apandi et al., 2011). Sequencing was performed by using the Big Dye Cycle sequencing kit version 3.0 and an ABI377 automated DNA sequencer (Applied Biosystems, Forster City, USA). The SeqMan and Megalign software modules in the Lasergene Suite of programs (DNASTAR, Madison, WI, USA) were used to format the nucleotide sequences. All sequences were BLAST searched (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and a phylogenetic tree was constructed by using the neighbour-joining method from the MEGA4 software ([www.megasoftware.net](http://www.megasoftware.net)).

## RESULTS

In 2010, Institute for Medical Research (IMR) Kuala Lumpur, Malaysia received specimens from 634 hospitalised HFMD cases and EV was detected from 440 (69%) cases by RT-PCR. Of these, 36 isolates comprised of CA16, HEV71, CA5 and ECHO 4 were isolated.

In 2011, we received specimens from only 268 HFMD cases and 185 (69%) were found positive for EV and 36 isolates of CA16, HEV71, CA10 and ECHO9 were detected. There was an increase in number of cases in 2012, specimens from 583 hospitalised cases were analysed and EV was detected in 407 cases (70%). Eighty five isolates of CA16, HEV71 and CoxB3 were isolated.

Overall, a total of 37 HEV71 strains were isolated with 10 isolates in 2010, 5 in 2011 and 22 in 2012. Details of the isolates are shown in Table 1.

Based on the complete VP1 gene which consists of 891 nucleotides, the phylogenetic tree revealed that all HEV71 circulating in Malaysia from 2010 to 2012 in this study belonged to the HEV71 subgenogroup B5. The tree is shown in Figure 1.

## DISCUSSION

Analyzing the VP1 gene is very important to determine the relationships between the genogroups (Ggs) and

pathogenicity. Based on this, HEV71 has been classified into three genogroups (Ggs) namely GgA, GgB and GgC (Brown et al., 1999). GgA was the prototype strain of HEV71, isolated in California, USA in 1970 (Schmidt et al., 1974) and has never been reported since. Another two Ggs, GgB evolved over the years into 5 subgenogroups: GgB1, GgB2, GgB3, GgB4 and GgB5; while GgC evolved into subgenogroup GgC1, GgC2, GgC3, GgC4 and GgC5. The genetic variation within genogroups was about 12% or was lesser than the variation between genogroups which was about 16.5 to 19.7% (Brown et al., 1999).

In Malaysia, HFMD cases are detected throughout the year. However, outbreaks of HFMD occur periodically nearly every t years. Many researchers have reported different strains of HEV71 in different outbreaks. The huge HFMD outbreak in Sarawak in 1997 (Cardosa et al., 2003) was the starting point of molecular epidemiology study of HEV71 in Malaysia. Several outbreaks in 2000 (8 fatalities) followed by outbreak in 2003 (no fatality) and in 2005 (two fatalities) (Chua and Kasri, 2011), had contributed more HEV71 genomic diversity.

GgB3 isolated in 1997, during the Sarawak outbreak, was documented as the first HEV71 strain circulating and associated with severe encephalitis and fatalities in Malaysia (Cardosa et al., 2003). To date, this strain has never been found circulating in peninsular Malaysia; indicating that GgB3 only appeared in very short period of time and confined to Sarawak State only for Malaysia. However, it has been isolated and reported in Japan at the same time (Fujimoto et al., 2002).

Between 1998 and 1999, HEV71 was still isolated from a few HFMD cases although there were no outbreaks reported. GgC1 was found to be circulating sporadically in 1998 in Sarawak (Cardosa et al., 2003) together with GgC2 in peninsular Malaysia (AbuBakar et al., 1999) from typical HFMD cases. However, GgC2 found circulating during the large HEV71 outbreak in Taiwan in 1998, was associated with fatal encephalitis (Wang et al., 2002).

HEV71 activity appeared low in 1999, and an analysis of 43 HEV71 isolates from peninsular Malaysia from 1997-2000 (Herrero et al., 2003) showed that GgB4 was the only predominant strain circulating in that year. Later in 2000, during the large outbreak of HFMD in peninsular Malaysia and Sarawak, GgB4 together with GgC1 emerged as the dominant strains (Chua and Kasri, 2011; Cardosa et al., 2003; Herrero et al., 2003). These two Ggs were also found widespread in the outbreak in Singapore and Taiwan, and continued to circulate in Malaysia in 2001 (Apandi et al., 2011), replacing GgB3.

GgC1 was found circulating in 2001, 2003, 2006 and 2007, mostly in peninsular Malaysia. From 2001 to 2009, 70 isolates from peninsular Malaysia and Sabah of HEV71 were analysed (Apandi et al., 2011). GgC1 was lastly detected in 2007 and it has neither been documented as the cause of large outbreak nor asso-

**Table 1.** Summary of patients' gender, age, sample type, clinical diagnosis and GenBank accession number.

Isolate	Accession No.	Sample type	Sex/age	Diagnosis	Isolation year
EV1004-Terengganu-11	KC894865	V/S	M/5	HFMD	2011
EV1056-Terengganu-11	KC894866	T/S	M/2	HFMD	2011
EV1268-Pahang-11	KC894867	V/S	F/0.8	HFMD	2011
EV0978-Sarawak-11	KC894868	R/S	F/1.5	HFMD	2011
EV0984-Sarawak-11	KC894869	V/S	M/1.3	HFMD	2011
EV0691-Terengganu-10	KC894872	V/S	M/1	HFMD	2010
EV0733-PPinang-10	KC894873	T/S	F/2	HFMD	2010
EV0744-Johor-10	KC894874	T/S	F/1	HFMD	2010
EV0994-Terengganu-10	KC894875	V/S	M/2	HFMD	2010
EV1233-Kedah-10	KC894876	V/S	F/4	HFMD	2010
EV1297-Melaka-10	KC894877	T/S	M/3	HFMD	2010
EV1299-Melaka-10	KC894878	T/S	M/8	HFMD	2010
EV1301-Melaka-10	KC894879	V/S	M/6	HFMD	2010
EV1312-Johor-10	KC894880	T/S	M/1	HFMD	2010
EV1389-KLumpur-10	KC894881	Stool	M/0.5	HFMD	2010
EV0615-Johor-12	KC894882	T/S	M/1.5	HFMD	2012
EV0616-Johor-12	KC894883	T/S	F/0.5	HFMD	2012
EV0655-Kedah-12	KC894884	V/S	M/4	HFMD	2012
EV0659-Pahang-12	KC894885	V/S, T/S	M/5	HFMD	2012
EV0665-Kelantan-12	KC894886	T/S	F/4	HFMD	2012
EV0673-Johor-12	KC894887	T/S	F/2.1	HFMD	2012
EV0710-Johor-12	KC894888	R/S	M/1.7	HFMD	2012
EV0769-Johor-12	KC894889	T/S	F/2	HFMD	2012
EV0775-Johor-12	KC894890	T/S	F/1.7	HFMD	2012
EV0779-Johor012	KC894891	V/S	F/7	HFMD	2012
EV0791-Johor-12	KC894892	R/S	F/1	HFMD	2012
EV0834-Johor-12	KC894893	T/S	M/2	HFMD	2012
EV0891-Johor-12	KC894894	T/S	F/1.2	HFMD	2012
EV0894-Kedah-12	KC894895	V/S	M/2.7	HFMD	2012
EV0896-Johor-12	KC894896	T/S	F/2	HFMD	2012
EV0953-Johor-12	KC894897	T/S	M/1.6	HFMD	2012
EV0961-Johor-12	KC894898	T/S	F/1.2	HFMD	2012
EV1002-Johor-12	KC894899	R/S	M/5	HFMD	2012
EV1003-Johor-12	KC894900	R/S	M/2.5	HFMD	2012
EV1170-Selangor-12	KC894901	T/S	M/5	HFMD	2012
EV1325-Johor-12	KC894902	T/S	F/1.5	HFMD	2012
EV0997-Pahang-12	KC894903	T/S	M/1.5	HFMD	2012

T/S = throat swab; R/S = rectal swab; V/S = vesicle swab; M = male; F = female; age in years.

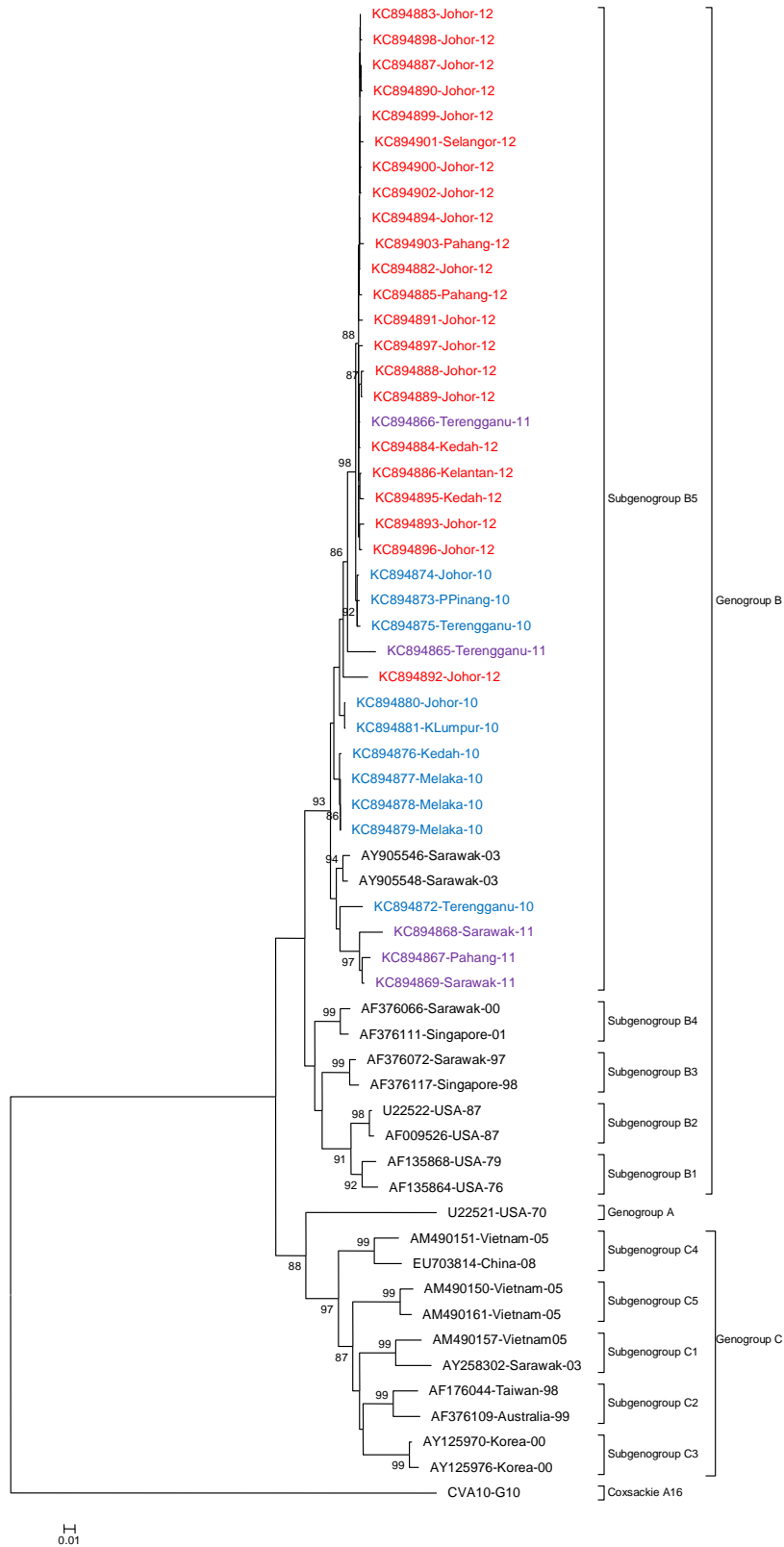
ciated with encephalitis.

A new subgenogroup in GgC, named GgC4, first emerged in China in early 2000. It later became a predominant strain in Taiwan in 2004 (Lin et al., 2006) and was responsible for huge outbreaks of HFMD in Shandong and Fuyang, China in 2007-2008 (Zhang et al., 2009, 2010).

However in Malaysia, only one GgC4 was detected from HFMD cases in 2004 (Apandi et al., 2011) and other strains, GgC3 and GgC5, so far have never been reported (Apandi et al., 2011; AbuBakar et al., 1999;

Herrero et al., 2003). The GgC3 was found only during the major HEV71 outbreak in Korea in 2000 and GgC5 circulated widely in Southern Vietnam in 2005 (Tu et al., 2007).

Over the years, HEV71 in GgB has evolved. GgB3 that emerged in 1997 was displaced by GgB4 in 2000 and 2001. In 2003, the GB4 was displaced by GgB5 and since then, from 2004 to 2009, GgB5 became the only predominant strain in both peninsular Malaysia and Sarawak (Apandi et al., 2011). In this study, GgB5 was still found to be the dominant strain from 2010 to 2012



**Figure 1.** Phylogenetic tree of HEV71 based on the complete VP1 gene. Genogroups and subgenogroups are indicated by square brackets with CA16-G10 as an outgroup. HEV71 isolates in 2010, 2011 and 2012 are indicated in blue, purple and red, respectively, together with representative retrieved from GenBank.

and was the cause of the 2010 HEV71 outbreak in Malaysia.

Hence, for the past 25 years, 6 strains of HEV71, 3 from GgB and 3 from GgC were circulating in Malaysia. In GgB, GgB3 was isolated in 1997 and was followed by GgB4 in 2000-2001 which was later replaced by GgB5 from 2003 till now. In GgC, GgC1 was first detected in 1997 in Sarawak, continued to circulate in peninsular in 2001, 2003, 2006 and 2007, while GgC2 circulated in 1998 and GgC4 in 2007.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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