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Genetic diversity of enterovirus 71 isolated from cases of hand, foot and mouth disease in the 1997, 2000 and 2005 outbreaks, Peninsular Malaysia

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Abstract

All known field isolates of enterovirus 71 (EV71) can be divided into three distinct genogroups (A, B, C) and 10 subgenogroups (A, B1-5, C1-4) based on VP1 gene sequences. We examined VP1 gene sequences of 10, 12 and 11 EV71 strains isolated in peninsular Malaysia during the outbreaks of hand, foot and mouth disease in 1997, 2000 and 2005 respectively. Four EV71 strains isolated in the hand, foot and mouth disease outbreak of 2006 in Sarawak (Malaysian Borneo) were included to describe their genetic relationship.

Four subgenogroups (C1, C2, B3 and B4) of EV71 co-circulated and caused the outbreak of hand, foot and mouth disease in peninsular Malaysia in 1997. Two subgenogroups (C1 and B4) were noted to cause the outbreak in 2000. In the 2005 outbreak, besides EV71 strains of subgenogroup C1, EV71 strains belonged to subgenogroup B5 were isolated but formed a cluster which was distinct from EV71 strains of the subgenogroup B5 isolated in 2003. The four EV71 strains isolated from clinical specimens of patients with hand, foot and mouth disease in the Sarawak outbreak in early 2006 also belonged to subgenogroup B5. Phylogenetic analysis of the VP1 gene sequences showed that the four Sarawak EV71 isolates belonged to the same cluster as the EV71 strains that were isolated in peninsular Malaysia as early as May 2005. The data suggested that the EV71 strains causing the outbreak in Sarawak could have originated from peninsular Malaysia.

Key words: enterovirus 71, genetic diversity, Malaysia

INTRODUCTION

Human enterovirus 71 (EV71) belongs to the species *Human enterovirus A* under the genus *Enterovirus* within the family *Picornaviridae*. EV71 has been associated with an array of clinical diseases including hand, foot and mouth disease (HFMD), aseptic meningitis, encephalitis and poliomyelitis-like paralysis.^{1,2} The virus was first isolated from a child with aseptic meningitis in California, USA and subsequently characterized as a new serotype of the genus *Enterovirus*.³ In the years following its initial isolation, outbreaks of HFMD with complications due to the virus were described in various parts of the world.⁴⁻⁷

The neurovirulence of EV71 gained global attention in an outbreak in Bulgaria which caused

705 cases of poliomyelitis-like disease with 44 deaths in 1975.^{8,9} A similar outbreak followed in Hungary in 1978 resulted in many cases of poliomyelitis-like disease and 47 deaths.¹⁰ Subsequently, several milder epidemics of CNS disease associated with EV71 have been reported in New York, Hong Kong, Australia and Philadelphia.¹¹⁻¹⁴ In Japan two epidemics of EV71 occurred with most cases characterized by HFMD and a low incidence of CNS disease.¹⁵⁻¹⁷ In 1997, a large outbreak of HFMD due to highly neurovirulent EV71 emerged in Malaysia and caused 48 deaths.^{18,19} The largest outbreak recorded thus far occurred in Taiwan in 1998 with more than 100,000 cases of HFMD and 78 died of acute brainstem encephalomyelitis with neurogenic cardiac failure and pulmonary

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oedema.²⁰⁻²³

In late 2000, a recurrence of an outbreak of HFMD with fatalities occurred in peninsular Malaysia and subsequently spread to the neighbouring country.²⁴ A similar outbreak of HFMD with 2 recorded deaths in young children occurred in peninsular Malaysia in 2005 (unpublished data) and this was followed by a larger outbreak in Sarawak (Malaysian Borneo) in the early part of 2006. In order to understand the strain variation and genetic relation over time of EV71 strains isolated from cases of HFMD since its first emergence in the 1997 outbreak, we conducted a molecular and phylogenetic analysis of the VP1 gene sequences of representative isolates of EV71 isolated from the 1997, 2000, 2005 outbreaks together with a couple of EV71 isolates from Bintulu, Sarawak.

MATERIALS AND METHODS

Thirty-three isolates of enterovirus 71 (EV71) isolated from the throat secretions, stool or vesicle fluid of patients with HFMD were chosen for this study. All the isolates of EV71 were isolated from clinical samples using Vero cells (ATCC, CCL-81) and typed using the commercial typing monoclonal antibodies 3323 and 3324 (Chemicon Inc., USA). The isolates of low passage number (3 or less) were plaque purified once according to the method described.²⁵ A 25 cm² culture flask containing confluent monolayer Vero cells was respectively infected with each EV71 isolate at a multiplicity of infection (m.o.i) of 2×10^1 plaque forming unit (pfu) of virus per cell. At 3-plus cytopathic effect (CPE), the supernatant was clarified by centrifugation at $1,000 \times g$ for 10 minutes and aliquoted into 10 microfuge tubes at 200 μ l each for storage at -80°C until further processing.

Total RNA was extracted from the 200 μ l supernatant using High Pure Viral RNA Kit (Boehringer Mannheim, USA). Briefly, lysis of EV71 in the supernatant was accomplished by incubation of the sample (200 μ l) in 400 μ l of special lysis/binding buffer for 10 minutes. The lysate was transferred into a filter tube and passed through the glass fleece at the base of the tube by centrifugation at $8,000 \times g$ for 15 seconds. Residual impurities in the tube were removed by a wash step using the supplied wash buffer and 30 μ l of elution buffer was added. After allowing the elution buffer to equilibrate for a minute at room temperature, the RNA is eluted into a sterile RNase-free microfuge tube by centrifugation at $8,000 \times g$ for 15 seconds.

The complete nucleotide sequences of EV71 VP1 gene were obtained by two approaches. Firstly, two pairs of oligonucleotide primers based on the published work of Brown *et al.* with modification were used to amplify the nucleotide sequences of EV71 VP1 gene.²⁶ In this approach, a genetic fragment of approximately 950 nts encompassing 3' end of VP3, all of VP1 and the 5' end of 2A was amplified using primer pair (5'-ACYATGAAAYTGTGCAAGG-3', position 2,385-2,403; 5'-CCRGTAGGICGTRCACGCRAC-3', position 2,869-2,850) for the proximal fragment and primer pair (5'-TTGGGACTAGAYATAACWGG-3', position 2,766-2,785; 5'-GCICCIGAYTGITGICCRAA-3', position 3,355-3,336) for the distal fragment. In the second approach, the complete nucleotide sequences of 7 EV71 isolates; EV71BrCr (accession no. NC001769), EV71MS (accession no. U22522), EV71Tainan4643/98 (accession no. AF304458), EV71Tainan5746/98 (accession no. AF304457), EV71Tainan6092/98 (accession no. 304459), EV71Sing/000009 (accession no. AF316321) and EV71Sing002209 (accession no. AF352027) deposited in the GenBank, NCBI were obtained and aligned for the region of the genomes extending from the beginning of VP3 gene to the end of 2A gene using Clone Manager 5/Align Plus 4 programme (S&E package, USA). Three pairs of degenerate primers based on their regions of high nucleotide similarity were synthesized by the same company to produce 3 overlapping fragments that covered 3' end of VP3, all of VP1 and 5' end of 2A. Thus a genetic fragment of approximately 1,000 nts was amplified by primer pair (5'-CACCTTGTAATACCATGGATCAG-3', position 2,210-2,233; 5'-GTGAATTAAGAACRCAYCGTGTYT-3', position 2,658-2,635) for the proximal fragment, primer pair (5'-TCGTCAAATRCTAGTGATGAGAGT-3', position 2,604-2,627; 5'-AACCAYTGRTARGCGCTCGCRGGT-3', position 3,053-3,030) for the middle fragment and primer pair (5'-GCCACWAAYCCCTCAGTTTTTG-3', position 2,962-2,982; 5'-GTCGCGRGAGCTGTYTCCCA-3', position 3,449-3,429) for the distal fragment.

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed in a single reaction tube using the Access RT-PCR Kit (Promega, USA). Each fragment was amplified

in a 50 µl reaction mix containing the respective pair of forward and reverse primers of 20 pmol each and 2 µl of the extracted viral RNA as template. Each reaction mix was subjected to a 60-minute of reverse transcription at 42°C, reverse transcriptase inactivation of 98°C for 5 minutes, followed by 30 cycles of amplification at a denaturing temperature of 98°C for 30 seconds, annealing temperature of 50°C for 30 seconds and an extension temperature of 72°C for 1 minute cycle. The amplified products were confirmed by electrophoreses 3 µl of each of the amplified products in 1% agarose gel. The right RT-PCR products were gel purified and extracted using QIAquick Gel Purification Kit (Qiagen, Germany) and sequenced by ABI Prism Big-Dye (Pharmacia, USA) dideoxyl termination cycle sequencing using respective degenerate forward and reverse primers, and analysed on an ABI 377 automatic sequencer (Applied Biosystems, USA). The derived sequences of all segments were assembled using Clone Manager/Align Plus-4 programme. Thus, each nucleotide of the VP1 gene was sequenced at least four times.

The sequence data derived from cDNA fragments generated by RT-PCR were routinely managed using the Clone Manager 5 and Align Plus 4 programme package (S&E Software, USA). Multiple sequence alignments were done using standard linear scoring matrix with the following parameter settings: mismatch penalty of 1, open gap penalty of 4, extended gap penalty of 1 and similarity significance value cut-off of 60%.

Multiple sequence alignments were generated using the ClustalX programme and phylogenetic analysis was conducted using MEGA version 3 programme.²⁷ A phylogenetic tree was constructed by neighbour-joining using Kimura two-parameter distance method and viewed using the MEGA3 Tree Explorer programme. Bootstrap verification of the resulting phylogenetic tree was performed by analysis of 1,050 bootstrapped replicates using the MEGA Bootstrap Test of Phylogeny programme. A boot-strapped consensus tree was subsequently produced. Representative members of previously published EV71-VP1 gene sequences used to re-construct

TABLE 1: Representative enterovirus 71 strains belonging to respective previously known genogroup and subgenogroup used in the reconstruction of the dendrogram and phylogenetic analysis.

Virus Identity	Genogroup/Subgenogroup	GenBank accession no.
BrCr	A	NC001612
2609-AUS-74	B1	AF135886
2610-AUS-74	B1	AF135887
2258-CA-79	B1	AF135880
7628-PA-87	B2	AF009530
2222-IA-88	B2	AF009540
2289-MAA-97	B3	AF135914
MY104-9-SAR-97	B3	AF376072
S110031-SAR-03	B5	AY258307
S19871-SAR-03	B5	AY258309
2419-Yamagata-03	B5	AB213647
2542-Yamagata-03	B5	AB177815
2716-Yamagata-03	B5	AB177816
2933-Yamagata-03	B5	AB213648
2934-Yamagata-03	B5	AB213649
2972-Yamagata-03	B5	AB213650
0756-MAA-97	C1	AF135935
0926-OR-91	C1	AF009548
2623-AUS-86	C1	AF135945
2644-AUS-95	C2	AF135949
2286-TX-94	C2	AF135941
TW-2086-98	C2	AF119796
001-KOR-00	C3	AY125966
013-KOR-00	C3	AY125976

the genogroup and subgenogroup divisions of EV71 are listed in Table I. The previously established nucleotide sequence homologies based on the complete VP1 gene by Brown *et al.*, McMinn *et al.*, Cardosa *et al.*, Mizuta *et al.* and Podin *et al.* [2006] that dictate genogroup (at least 80%) and subgenogroup (at least 91%) allocation in the construction of phylogram were used as the basis for phylogenetic analysis.^{26, 28-31}

RESULTS

Molecular and phylogenetic analysis was carried out on thirty-three strains of EV71 representing 10, 12, and 11 strains isolated during the outbreaks of HFMD that occurred in peninsular Malaysia in 1997, 2000 and 2005 respectively. Four strains of EV71 isolated from 12 clinical samples of patients with HFMD during the outbreak in Bintulu, Sarawak in early 2006 were included in the study. The date, specimen type and source of isolation of each of the EV71 isolate are shown in Table II.

Of the total thirty-seven EV71 strains analysed; fourteen strains (1997B, 1997H, 2000B, 2000C, 2000D, 2000E, 2000F, 2000I, 2000J, 2005P418, 2005P688, 2005P756, 2005P816, 2005P840) belonged to subgenogroup C1, two strains (1997A, 1997E) to subgenogroup C2, three strains (1997D, 1997F, 199J) to subgenogroup B3, eight strains (1997C, 1997G, 1997I, 2000A, 2000G, 2000H, 2000K, 2000L) to subgenogroup B4, and ten strains (2005P588, 2005P734, 2005P789, 2005P793, 2005P796, 2005P888, 2005SB1, 2005SB5, 2005SB9, 2005SB12) to a distinct cluster within the subgenogroup B5 which is designated B5cv in this study. The nucleotide sequence identity of EV71 as a group in comparison with a reference Coxsackievirus A16 (G-10, Genbank accession no. NC001612) as an outlier and comparison among various genogroups and subgenogroups within the EV71 species is shown in Table III. The findings in this study supported previous reports that the required genetic boundaries dictating genogroup and subgenogroup allocation were at least 80% and 91% nucleotide sequence homology respectively. Analysis based on the complete VP1 gene (891 nucleotides) showed that the EV71 strains shared a sequence homology with the CA16 (G-10 strain) ranged from 557 to 588 nucleotides (62.5 % to 66%). The sequence homology between genogroups using EV71-BrCr strain as reference ranged from 717 to 745

nucleotides (80.5% to 83.6%). The subgenogroup C1 had the greatest nucleotide sequence diversity (ranged from 92.4 to 98.1% homology) and this was followed by subgenogroup B4 (ranged from 96.5 to 99.6% homology) in comparison to the other subgenogroups. The cluster variant B5cv, within the subgenogroup B5 had the highest nucleotide divergent from the prototype EV71 strain (BrCr) and highest nucleotide homology to the classical subgenogroup B5 (Table III).

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 gene sequences of representative EV71 strains belonging to the respective previously known subgenogroups is shown in Figure 1. This study identified 4 subgenogroups (C1, C2, B3, and B4) co-circulated in peninsular Malaysia in the 1997 outbreak and 2 subgenogroups (C1 and B4) in the 2000 outbreak. 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 (Figure 1). 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 were shown in Figure 2. 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.

DISCUSSION

The genomic RNA of enteroviruses consists of approximately 7,500 nucleotides (nts), is polyadenylated at the 3' terminus and carries a small viral peptide (VPg) covalently attached to its 5' end.³² The 5' untranslated region (UTR) of approximately 700 nts and the 3'-UTR of approximately 39 nts in length flank the central coding region that encodes the viral proteins. Unlike the 5'-UTR that does not encode any

TABLE 2: Sources and dates of isolation of enterovirus 71 from patients with hand, foot and mouth disease in peninsular Malaysia during the 1997, 2000 and 2005 outbreaks.

Virus Identity	Date of Isolation	Specimen Type	*Source
1997A	4-Jul-1997	Vesicle Swab	Federal Territory
1997B	4-Jul-1997	Vesicle Swab	Federal Territory
1997C	4-Jul-1997	Vesicle Swab	Federal Territory
1997D	13-Jul-1997	Oral Swab	Selangor
1997E	30-Jul-1997	Vesicle Swab	Federal Territory
1997F	18-Aug-1997	Stool	Selangor
1997G	7-Nov-1997	Oral Swab	Selangor
1997H	21-Nov-1997	Throat Swab	Selangor
1997I	1-Dec-1997	Throat Swab	Selangor
1997J	19-Dec-1997	Throat Swab	Selangor
2000A	11-Oct-2000	Throat Swab	Johor
2000B	13-Oct-2000	Rectal Swab	Johor
2000C	14-Oct-2000	Vesicle Swab	Johor
2000D	17-Oct-2000	Throat Swab	Johor
2000E	17-Oct-2000	Throat Swab	Johor
2000F	17-Oct-2000	Rectal Swab	Kedah
2000G	19-Oct-2000	Rectal Swab	Selangor
2000H	20-Oct-2000	Throat Swab	Federal Territory
2000I	24-Oct-2000	Throat Swab	Johor
2000J	7-Nov-2000	Rectal Swab	Johor
2000K	10-Nov-2000	Vesicle Swab	Johor
2000L	14-Nov-2000	Throat Swab	Johor
2005P418	28-Apr-2005	Throat Swab	Pahang
2005P588	27-May-2005	Rectal Swab	Kedah
2005P688	22-Jun-2005	Throat Swab	Johor
2005P734	17-Jul-2005	Throat Swab	Penang
2005P756	25-Jul-2005	Throat Swab	Johor
2005P789	21-Aug-2005	Throat Swab	Melaka
2005P793	28-Aug-2005	Vesicle Swab	Penang
2005P796	28-Aug-2005	Vesicle Swab	Pahang
2005P816	9-Sep-2005	Throat Swab	Pahang
2005P840	19-Sep-2005	Throat Swab	Johor
2005P888	19-Oct-2005	Throat Swab	Johor
2006SB1	31-Jan-2006	Throat Swab	Sarawak
2006SB5	31-Jan-2006	Throat Swab	Sarawak
2006SB9	31-Jan-2006	Throat Swab	Sarawak
2006SB12	31-Jan-2006	Throat Swab	Sarawak

*Source = States of Peninsular Malaysia except Sarawak (Malaysian Borneo)

TABLE 3: Comparison of nucleotide sequence homologies of enterovirus 71 strains with respect to a reference Coxsackievirus A16 as an outlier and among various genogroups and subgenogroups within the EV71 group.

Genogroup/ Subgenogroup comparison	*Reference strain for comparison	Range of nucleotide homology (891 nt)	Percent homology range
EV71 out-group	CA16, G-10	557 - 588	62.5 – 66.0
Genogroup A	EV71-BrCr (A)	717 - 745	80.5 – 83.6
Genogroup B	2609-AUS-74 (B1)	790 - 859	88.7 – 96.4
Genogroup C	2623-AUS-86 (C1)	823 - 874	92.4 – 98.1
Subgenogroup C1	2623-AUS-86 (C1)	823 - 874	92.4 – 98.1
Subgenogroup C2	2286-TX-94 (C2)	861 - 874	96.6 – 98.1
Subgenogroup C3	001-KOR-00 (C3)	885	99.3
Subgenogroup B1	2609-AUS-74 (B1)	851 - 859	95.5 – 96.4
Subgenogroup B2	7628-PA-87 (B2)	878	98.5
Subgenogroup B3	2289-MAA-97 (B3)	876 - 888	98.3 – 99.7
Subgenogroup B4	1997C (B4)	860 - 887	96.5 – 99.6
Subgenogroup B5	2419-Yamagata-03 (B5)	881 - 891	98.9 - 100
Subgenogroup B5cv	EV71-BrCr (A)	725 – 729	81.4 – 81.8
Subgenogroup B5cv	2623-AUS-86 (C1)	735 – 743	82.5 – 83.4
Subgenogroup B5cv	2286-TX-94 (C2)	732 – 740	82.2 – 83.1
Subgenogroup B5cv	001-KOR-00 (C3)	737 - 740	82.7 – 83.1
Subgenogroup B5cv	2609-AUS-74 (B1)	790 - 794	88.7 – 89.1
Subgenogroup B5cv	7628-PA-87 (B2)	796 - 803	89.3 – 90.1
Subgenogroup B5cv	2289-MAA-97 (B3)	813 - 821	91.2 – 92.1
Subgenogroup B5cv	1997C (B4)	837 - 841	93.9 – 94.4
Subgenogroup B5cv	2419-Yamagata-03 (B5)	861 - 866	96.6 – 97.2

*Reference strain = Genogroup and subgenogroup comparison of degree of nucleotide homologies with respect to the specified virus strain as the reference for each comparison.

protein, the region of the genome that encodes the viral capsid proteins (P1) is subjected to host immunological pressure. Thus, highest sequence variation is expected to occur in this region among enteroviruses within the same serotype and between different serotype within the genus. VP1 is the major surface accessible protein in the mature picornavirus virion and VP4 is the least accessible being embedded within the viral capsid. VP1 is arrayed around the fivefold axis of symmetry of the icosahedral virion.³³ VP2 and VP3 comprise the remainder of the virion surface. Each of the capsid proteins is composed of conserved elements that form the β -barrel structural elements of the capsid, with variable loops between the β -barrel structures.³² Many of the loops are exposed on the virion surface, and studies of monoclonal antibody-resistant have shown that a number of the loops contribute

to specific antigenic neutralization sites. VP1 contributes to all three of the major neutralization sites that have been identified on the poliovirus surface, whereas VP2 and VP3 contribute to two and one of the sites, respectively. Thus, VP1 is expected to undergo greater genetic drift than the other capsid proteins.

Several previous studies have attempted to describe the molecular epidemiology and phylogenetic relationship of EV71 in the Asia-Pacific region and other parts of the world based on the various regions of the virus genome such as 5' UTR, VP4 and VP1.²⁹ However, a recent study by Cardoso *et. al.* clearly showed that phylogenetic analysis based on the complete VP1 gene sequence is more superior and has a higher discriminating power in differentiating the various EV71 subgenogroups.²⁹ The same study also recommended that the complete VP1 gene

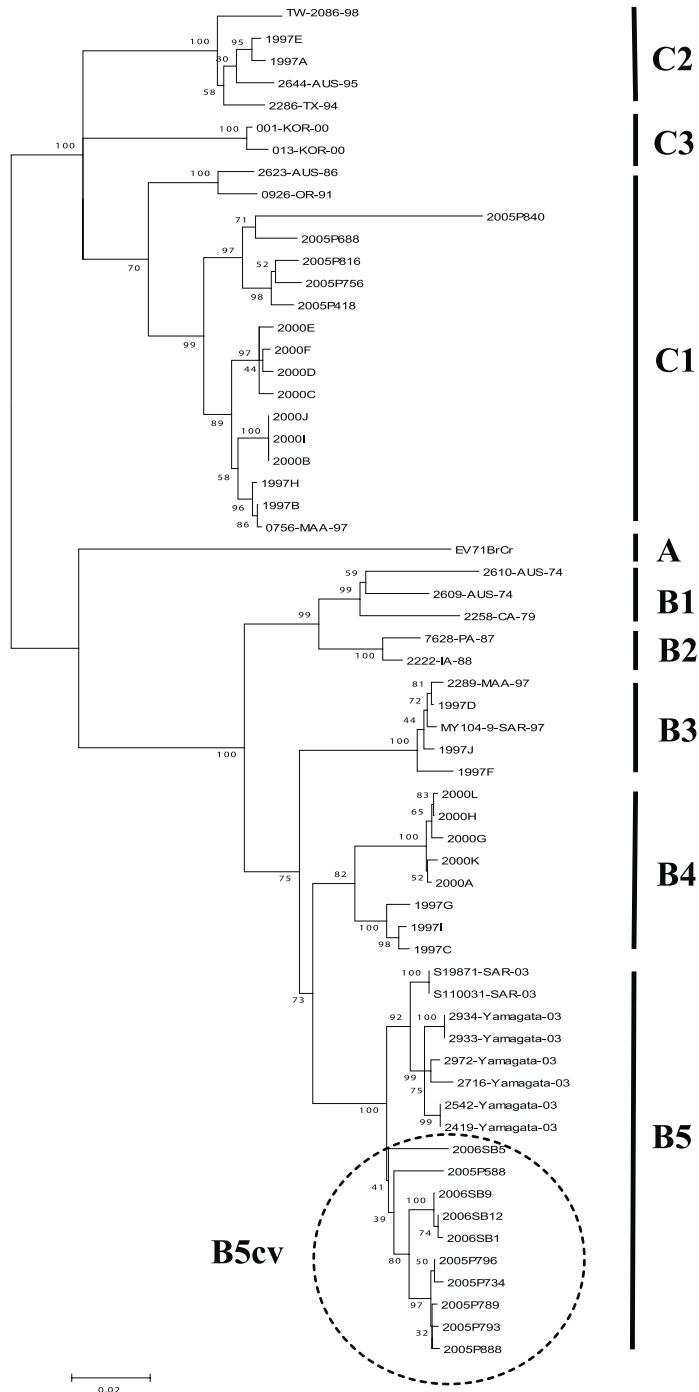


FIG. 1: The bootstrapped consensus phylogenetic tree constructed using the neighbour-joining method of the MEGA 3 programme, showing the genetic relationships among thirty three EV71 strains isolated in peninsular Malaysia and four strains isolated in Sarawak (Malaysian Borneo) with respect to representative EV71 strains of known genogroups and subgenogroups based on the complete VP1 gene (891 nucleotides). The GenBank accession numbers of the representative EV71 strains used in the re-construction of various E71 subgenogroups are shown in Table 1. Branch lengths of the tree are proportional to the number of nucleotide differences. The bootstrap value in 1,050 pseudo-replicates for lineages within the tree are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. The identities of EV71 strains are shown in Tables 1 and 2.

sequences should be considered as the standard for future molecular work on EV71 genogrouping analysis.

Our present study based on the complete VP1 gene sequences showed that more than one subgenogroup of EV71 co-circulated in all three previous outbreaks of HFMD in peninsular Malaysia. The findings of this study are unique to peninsular Malaysia and also supported an earlier study on the molecular epidemiology of EV71 in peninsular Malaysia.³⁴ The data of the earlier study by Herrero *et al.* suggested that peninsular Malaysia may have served as a source of virus for the rest of the Asia-Pacific region in the previous outbreaks of HFMD.³⁴ The present isolation of EV71 strains belonged to this distinct cluster within the subgenogroup B5 in this study is an interesting finding. EV71 strain of this cluster was first isolated in Kedah, a state in the northern part of peninsular Malaysia in May 2005. Since then, it was isolated in other parts of peninsular Malaysia. Though the sample size of this study was relatively small, the data derived from phylogenetic analysis and chronological sequence of isolation of this distinct virus cluster suggests that the EV71 causing the outbreak of HFMD in Sarawak (Malaysian Borneo) could have originated in peninsular Malaysia, especially in the period of intensive movement of people between these two parts of the country during the long school vacation extending from mid-November 2005 to January 2006. It will be of interest to note whether EV71 strains belong to this distinct cluster of subgenogroup B5 will eventually circulate to other parts of the Asia-Pacific region in the near future or will it continue to mutate and evolve into a new B-subgenogroup.

The practical significance of this study demonstrates that different subgenogroups of EV71 could co-circulate and cause the outbreak. Understanding its molecular genetic may shed light on its molecular epidemiology and evolution in the human population.

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