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Molecular epidemiology of enterovirus 71 in peninsular Malaysia, 1997–2000

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Summary. Human enterovirus 71 (EV71) (genus *Enterovirus*, family *Picornaviridae*) has been responsible for sporadic cases and outbreaks of hand-foot-and-mouth disease (HFMD), aseptic meningitis, encephalitis and poliomyelitis-like disease in Europe, the U.S.A., Australia and Asia. Recently, there has been an increase in EV71 activity in the Asia-Pacific region, with many outbreaks of HFMD associated with brainstem encephalitis manifesting as neurogenic pulmonary oedema with a high case fatality rate. In 1997, and again in 2000, EV71 outbreaks occurred in peninsular Malaysia. Variations in VP1 gene sequences have been shown to divide all known EV71 field isolates into three distinct genogroups (A, B and C). Consequently we examined the VP1 gene sequences of 43 EV71 strains isolated in peninsular Malaysia between 1997 and 2000 in order to determine the genogroup prevalence over the period. In this study we show that four subgenogroups (B3, B4, C1 and C2) of EV71 circulated in peninsular Malaysia between 1997 and 2000. Subgenogroups B3, B4 and C1 have been identified as the primary cause of the outbreaks of EV71 in peninsular Malaysia. Subgenogroup C1 also displayed endemic circulation from 1997 to 2000 and subgenogroup C2 was present at a low level during the 1997 outbreak.

Introduction

Human enterovirus 71 (EV71) is a member of the genus *Enterovirus* and belongs to the species *Human enterovirus A*. This species contains a total of 12 serotypes

formerly classified as "Coxsackie A viruses" [3, 19] including Human coxsackievirus A2, A5 and A10 as well as Human coxsackievirus A16 which, along with EV71, is the major causative agent of hand-foot-and-mouth disease (HFMD). Since its discovery in 1970 [30] EV71 has been responsible for sporadic cases and outbreaks of HFMD, aseptic meningitis, encephalitis and poliomyelitis-like disease throughout the world [1, 6, 10, 12, 14, 16, 17, 20, 21, 23, 24, 29, 36]. Despite this wide variation in clinical presentation, the epidemiology of this virus has not been extensively researched and little is known about the molecular basis for observed differences in virulence.

EV71 was first isolated from a child with aseptic meningitis in California in 1969, and by 1974, the virus had been described as a new serotype of the genus *Enterovirus* [30]. In the years following the initial isolation of EV71, outbreaks occurred in the U.S.A. [23], Australia [17], Sweden [2] and Japan [13, 16]. In 1975 EV71 gained global attention when it was responsible for an outbreak in Bulgaria that resulted in 705 cases of poliomyelitis-like disease and the deaths of 44 people; 93% of the poliomyelitis-like disease cases occurred in children under the age of five [10]. A similar outbreak occurred in Hungary in 1978, which also involved many cases of poliomyelitis-like disease and 47 deaths [24]. Since this time outbreaks of EV71 have continued to occur throughout the world.

More recently there has been an increase in EV71 activity in the Asia-Pacific region with several epidemics of HFMD being reported, including multiple cases associated with brain stem encephalitis and pulmonary oedema. The first such epidemic occurred in Sarawak (Malaysian Borneo) in 1997 [7] followed by smaller outbreaks in peninsular Malaysia [20] and Japan [32]. In 1998 outbreaks continued in Singapore [34] and Taiwan [8, 9, 31]. The outbreak that occurred in Taiwan is the largest recorded, with greater than 100,000 cases of HFMD. Of these, 400 children were admitted to hospital with central nervous system (CNS) involvement and 78 died of brainstem encephalitis with neurogenic pulmonary oedema [8]. In 1999, a large HFMD outbreak occurred in Perth, Western Australia [22] and in 2000, EV71 was the cause of epidemics in Korea [5], Japan [33], Singapore [6, 21], Taiwan [37] and peninsular Malaysia, resulting in a range of clinical presentations including HFMD, aseptic meningitis, encephalitis and poliomyelitis-like disease [34].

Three studies of EV71 evolution from 1970 to the present, based on the complete VP1 gene sequence, have demonstrated the evolution of three genogroups – A, B and C [3, 5, 21]. Genogroup A consists of a single member, which is the EV71 prototype strain BrCr-USA-70. This virus was responsible for the first case of enterovirus infection in California [30]. Genogroup B is divided into four subgenogroups – 1, 2, [3, 21] 3 and 4 [21] and genogroup C is divided into three subgenogroups – 1, 2 [3, 21] and the newly identified C3 [5].

To date, little data is available on the epidemiology of EV71 in peninsular Malaysia, with the majority of analyses concentrating on other areas of the Asia-Pacific region (particularly Taiwan, Australia, Sarawak, Singapore and Japan), this is despite several large EV71 epidemics being reported in peninsular Malaysia. In this study we report on the molecular epidemiology of EV71 in peninsular Malaysia during the period 1997 to 2000. Forty-three strains of EV71 isolated

between 1997 and 2000 were analysed by comparison of their complete VP1 gene sequences and through phylogenetic inference analysis.

Materials and methods

Virus isolation

The forty-three EV71 strains analysed in this study are listed in Table 1, with year of isolation, source of virus isolate, associated clinical manifestations and GenBank accession numbers. Virus isolation was undertaken in cell culture using both Vero (ATCC CCL81) and rhabdomyosarcoma (RD) (ATCC CCL136) cell lines and all isolates were identified by immunofluorescence using an EV71 monoclonal antibody (Light Diagnostic, Chemicon International Inc., CA, U.S.A., Cat No. 3324) in addition to neutralisation assays using EV71-specific polyclonal rabbit antisera (R385JS) [17].

RNA extraction and RT-PCR

Viral RNA was extracted from cell culture supernatants using either the QIAamp® Viral RNA Mini Kit (QIAGEN®) or the High Pure Viral RNA Kit (Boehringer Mannheim, U.S.A.) according to the manufacturer's instructions. The VP1 gene was amplified using two approaches, the first being a two-step process of RT followed by PCR and the second as a one-tube RT-PCR reaction.

In the first approach the VP1 gene was reverse transcribed using either Superscript™ II (GIBCO BRL) or M-MLV™ (Promega) reverse transcriptase under the following conditions. RNA (5 μL), NP1A primer (100 pmol) [3] and ddH_2O to a total volume of 20 μL were heated at 70 °C for 3 min. After quenching on ice for 5 min, 10 μL 5 × Buffer (appropriate to the reverse transcription enzyme), 0.01 M DTT, 0.5 mM dNTPs, 80 u RNasin (Promega) and ddH_2O were added to a final volume of 50 μL and heated at 42 °C for 2 min. The reverse transcriptase enzyme was added (400 u) and the reaction mixture subjected to one cycle of: 42 °C for 90 min, 70 °C for 15 min with the resultant cDNA RT mix being stored at −80 °C. Amplification of the VP1 gene was achieved using 7 μL of the RT mix, 0.2 mM dNTPs, 50 pmol primer 159, 50 pmol primer NP1A (see Table 2 for primer information), 10 μL of Elongase™ buffer B and ddH_2O, which were combined in a final volume of 50 μL and heated at 94 °C for 2 min. Elongase™ (1 u) was then added and the reaction mixture subjected to 35 cycles of denaturing at 94 °C for 1 min, primer annealing at 50 °C for 45 sec and extension at 68 °C for 3.5 min.

The one-tube RT-PCR reaction was performed with each VP1 gene being amplified in either two or three fragments, using the Access RT-PCR Kit (Promega, U.S.A.) in a 50 μL reaction, according to the manufacturer's instructions, with 20 pmol of each primer (either primer pairs 159/162b and 161a/NP1Ab or primer pairs 001a/001b, 002a/002b and 003a/003b, see Table 2 for primer information) and 2 μL of extracted RNA. Each reaction was subjected to reverse transcription at 42 °C for 60 min, reverse transcriptase inactivation at 98 °C for 5 min, followed by 30 cycles of denaturing at 98 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 1 min.

cDNA purification and sequencing

The resultant VP1 gene cDNA was purified using the QIAquick® PCR Purification Kit (QIAGEN®) and gel extracted using the MinElute™ Gel Extraction Kit (QIAGEN®) or the QIAquick Gel Purification Kit (QIAGEN®), following the manufacturer's instructions.

Table 1. EV71 strains from peninsular Malaysia, 1997–2000

ID number[a]	Clinical association[b]	Specimen type[c]	GenBank accession number
03907-MAA-97	HFMD	V/S	AY207611
03784-MAA-97	HFMD	V/S	AY207612
03300-MAA-97	HFMD	V/S	AY207613
0091-MAA-97	Myocarditis	Left brain	AY207649
0128-MAA-97	HFMD	STL	AY207647
0870-MAA-97	HFMD	O/S	AY207614
03750-MAA-97	HFMD	V/S	AY207615
0473-MAA-97	HFMD	STL	AY207616
0414-MAA-97	Encephalitis	Brainstem	AY207646
0897-MAA-97	HFMD	STL	AY207644
0898-MAA-97	Encephalitis	Spinal cord	AY207643
0899-MAA-97	Encephalitis	Cerebellum	AY207642
0903-MAA-97	Encephalitis	Spinal cord	AY207648
0884-MAA-97	HFMD	STL	AY207645
0036-MAA-97	HFMD	O/S	AY207641
0175-MAA-97	HFMD	R/S	AY207640
0245-MAA-97	Paralysis	T/S	AY207639
0283-MAA-97	HFMD	T/S	AY207638
0343-MAA-97	HFMD	T/S	AY207637
04716-MAA-97	HFMD	T/S	AY207636
0557-MAA-98	HFMD	R/S	AY207631
0808-MAA-98	Meningitis	T/S	AY207630
0838-MAA-99	HFMD	T/S	AY207629
0749-MAA-99	HFMD and Meningitis	V/S	AY207653
0615-MAA-99	Myocarditis	R/S	AY207652
0627-MAA-99	Oral ulceration	STL	AY207651
0919-MAA-99	HFMD	R/S	AY207650
05716-MAA-00	HFMD	U/S	AY207635
0778-MAA-00	HFMD	T/S	AY207617
0774-MAA-00	HFMD	T/S	AY207634
0807-MAA-00	HFMD	R/S	AY207618
0815-MAA-00	HFMD	V/S	AY207633
0832-MAA-00	HFMD	T/S	AY207632
0836-MAA-00	HFMD	V/S	AY207619
0915-MAA-00	HFMD	T/S	AY207620
0937-MAA-00	HFMD	T/S	AY207621
0948-MAA-00	HFMD	R/S	AY207622
0042-MAA-00	HFMD	R/S	AY207623
0066-MAA-00	HFMD	T/S	AY207624
0113-MAA-00	HFMD	T/S	AY207625
0389-MAA-00	HFMD	R/S	AY207626
0431-MAA-00	HFMD	V/S	AY207627
0467-MAA-00	HFMD	T/S	AY207628

[a]ID number: identification number, all isolates in this study were typed by immunofluorescence. The ID number contains a unique number, country of isolation (in this case MAA = peninsular Malaysia) and year of isolation (1997–2000); [b]HFMD: Hand foot and mouth disease; [c]STL: Stool, R/S: Rectal Swab, V/S: Vesicle Swab, T/S: Throat Swab, O/S: Oral Swab, U/S: Ulcer Swab

Table 2. Primers used to amplify and sequence the EV71 VP1 gene

Primer[a]	Direction	Sequence[b]	Length (nu)	Position[c]	G+C%	(Ref)
159	Forward	ACYATGAAAYTGTGCAAGG	19	2385–2403	37%	[3]
NP1A	Reverse	GCICCICAYTGITGICCRAA	20	3355–3336	45%	[3]
162b	Reverse	CCRGTAGGICGTRCACGCRAC	21	2869–2850	57%	Modified from Brown et al., 1999 [3]
161a	Forward	TTGGGACTAGAYATAACWGG	21	2766–2785	40%	Modified from Brown et al., 1999 [3]
NP1Ab	Reverse	GCICCIGAYTGITGICCRAA	20	3355–3336	50%	Modified from Brown et al., 1999 [3]
001a	Forward	CACCCTTGTAATACCATGGATCAG	24	2210–2233	46%	
001b	Reverse	GTGAATTAAGAACRCAYCGTGTYT	24	2658–2635	39%	
002a	Forward	TCGTCAAATRCTAGTGATGAGAGT	24	2604–2627	39%	
002b	Reverse	AACCAYTGRTARGCGCTCGCRGGT	24	3053–3030	56%	
003a	Forward	GCCACWAAYCCCTCAGTTTTG	21	2962–2982	50%	
003b	Reverse	GTCGCGGGAGCTRTCTTCCCA	21	3449–3429	64%	

[a]Primers in bold were designed by aligning the complete nucleotide sequences of a random selection of 7 EV71 isolates; [b]R: (A or G), Y: (C or T), W: (A or T), I: inosine, primer sequences are shown from 5′ to 3′; [c]Position relative to the genome of EV71 strain 7423-MS-87 [4]

Sequencing was performed using the dideoxy chain terminator sequencing principle analysed with an ABI Prism® 3700 DNA Analyzer. Sequencing was performed using the same primers as was used in PCR. Analysis of DNA chromatograms was performed using Chromas™ software (Technelysium Pty Ltd., Aus).

Phylogenetic analysis

Alignment of the complete VP1 sequences was undertaken using ClustalW software [35]. A phylogenetic tree was constructed by neighbour joining using the Kimura two-parameter distance method [18] with the Eneighbor program [11] and viewed using the program TreeView [26]. The robustness of the tree was then tested by bootstrap analysis using 1,000

Table 3. Virus isolates used in reconstruction of the EV71 dendrograms

Identification number[a]	Specimen type[b]	Clinical association[c]	GenBank accession no.	(Ref)
CA16-G10-SAF-51	Stool	HFMD	NC001612	[27]
BrCr-USA-70	NA	Meningitis	U22521	[4]
2609-AUS-74	NA	Meningitis	AF135886	[3]
258-Bulgaria-75	Brain	Poliomyelitis-like disease	AB059814	[10]
Hungary-78	Brain	Poliomyelitis-like disease	AB059815	[24]
2258-USA-79	NA	Tremors	AF135880	[3]
7673-USA-87	NA	NA	AF009535	[3]
2222-USA-88	NA	Fever	AF009540	[3]
0926-USA-91	NA	Tremors	AF009548	[3]
2644-AUS-95	NA	NA	AF135949	[3]
0756-MAA-97	NA	NA	AF135935	[3]
MY104-9-SAR-97	Throat swab	Acute cardiogenic shock	AF376072	[21]
MY821-3-SAR-97	Vesicle swab	Meningitis	AF376077	[21]
18-SIN-97	Stool	Paralysis	AF251359	[34]
2286-USA-97	NA	NA	AF135941	[3]
2289-MAA-97	NA	NA	AF135914	*
TW-1465-98	NA	NA	AF116814	*
NCKU9822-TW-98	NA	Encephalitis	AF136379	[38]
1567-TW-98	NA	NA	AF116810	*
2086-TW-98	Vesicle swab	HFMD	AF119796	[31]
S11051-SAR-98	Throat swab	HFMD	AF376081	[21]
S10862-SAR-98	Stool	HFMD	AF376080	[21]
4F-AUS-4-99	Stool	Guillian-Barre Syndrome	AF367105	[21]
2M-AUS-3-99	Stool	Myelitis	AF376103	[21]
1M-AUS-12-00	Vesicle swab	HFMD	AF376098	[21]
5511-SIN-00	Stool	HFMD	AF376121	[21]
2864-SAR-00	Throat swab	Poliomyelitis-like disease	AF376066	[21]
001-KOR-00	Throat swab	NA	AY125966	[5]
013-KOR-00	Throat swab	NA	AY125976	[5]
2027-SIN-01	Brain	Encephalitis	AF376111	[21]

[a]Identification number = unique number, abbreviation of country of isolation, year of isolation. Abbreviations are as follows, SAF: South Africa, USA: United States of America, AUS: Australia, SAR: Sarawak, TW: Taiwan, SIN: Singapore, KOR: Korea; [b]NA: Not Available, [c]HFMD: Hand foot and mouth disease, NA: Not available; *Published in GenBank only

pseudo-replicates generated by the program Eseqboot [11]. A consensus tree was subsequently produced using the program Econsense [11]. All programs were obtained from WebAngis (http://www.angis.org.au). Previously published VP1 gene sequences used to reconstruct the genogroup and subgenogroup divisions of EV71 are listed in Table 3. As established previously by Brown et al. [3] and McMinn et al. [21], the nucleotide sequence homologies that dictate genogroup and subgenogroup allocation in the construction of the dendrograms are at least 80% and at least 91% respectively.

Results

Virus isolates

The forty-three EV71 strains represent 34% (43/128) of the total number of EV71 isolates from peninsular Malaysia during the period 1997–2001. In turn the 128 isolates of EV71 represented 75% (128/171) of all enteroviruses isolated in peninsular Malaysia over the same time period [15].

Nucleotide sequence analysis

Of the forty-three samples that were analysed, nine belonged to subgenogroup B3, sixteen to subgenogroup B4, a further sixteen to subgenogroup C1 and two to subgenogroup C2. The nine samples belonging to subgenogroup B3 displayed nucleotide sequence identity in the range of 98–99%, with a mean of 98.1%. The sixteen samples belonging to subgenogroup B4 displayed nucleotide sequence identity in the range of 96–99%, with a mean of 97.5%. The sixteen samples belonging to subgenogroup C1 displayed nucleotide sequence identity in the range of 97–98%, with a mean of 97.6% and the two samples in subgenogroup C2 displayed 99.4% nucleotide sequence identity. Percentage nucleotide sequence identities of isolates belonging to each subgenogroup are presented in Table 4.

Phylogenetic analysis

Phylogenetic analysis of all forty-three samples was based on alignment of the complete (891 nucleotide) VP1 gene sequences and construction of dendrograms using the neighbour-joining method; the results are shown in Fig. 1. Phylogenetic analysis using the maximum-parsimony and maximum-likelihood methods resulted in dendrograms with identical genogroup and subgenogroup divisions to the neighbour-joining method (data not shown). Previously published VP1 sequences (Table 3) were used to reconstruct the three genogroups and their subgenogroups (Fig. 1A).

This study has identified four subgenogroups that circulated in peninsular Malaysia between 1997 and 2000: B3, B4, C1 and C2. As indicated previously, the genetic boundaries dictating genogroup and subgenogroup allocation are 80% and 91% nucleotide sequence homology, respectively. A dendrogram showing the phylogenetic relationships of genogroup B viruses is presented in Figure 1B. Subgenogroup B3 viruses (9 strains) were isolated in peninsular Malaysia only during 1997, and appeared to be closely related to strains circulating in Sarawak

Table 4. Percentage nucleotide homology between EV71 subgenogroups

EV71 subgenogroup[a]	Comparison[a]	% Homology range	Mean % homology
Within B3	–	98–99	98.1
Within B4	–	96–99	97.5
Within C1	–	97–98	97.6
Within C2	–	–	99.4
B3	B4	94–95	94.2
B3	C1	81–82	81.9
B3	C2	82	82
B4	C1	81–82	81.9
B4	C2	82	82
C1	C2	91	91
B3	A	81–82	81.7
B4	A	80–82	81.2
C1	A	81–82	81.25
C2	A	83	83
B3	B1	91	91
B4	B1	91	91
C1	B1	83	83
C2	B1	82–83	82.5
B3	B2	92–93	92.7
B4	B2	91–93	92
C1	B2	82	82
C2	B2	82	82

[a]Refers to the subgenogroup identified in this study, except for genogroup A and subgenogroups B1 and B2, for which the reference strains are BrCr-USA-70 [4], 2609-AUS-74 [3] and 7423-MS-87 [4], respectively

during the same period. In contrast, subgenogroup B4 viruses (16 strains) were isolated over several years (1997; 7 strains, 1999; 3 strains and 2000; 6 strains) and are related to strains circulating in both Singapore and Sarawak during the same period.

A dendrogram showing the phylogenetic relationships of genogroup C viruses is presented in Fig. 1C. Subgenogroup C1 viruses (16 strains) were isolated in every year from 1997–2000 (2 in 1997, 3 in 1998, 1 in 1999 and 10 in 2000), whilst subgenogroup C2 viruses (two strains) were isolated only in 1997.

Based on the above data, viruses belonging to subgenogroups B3 and B4 were identified as the primary cause of the EV71 epidemic in peninsular Malaysia in 1997, and C1 and B4 were the primary cause of the EV71 epidemic in 2000. Viruses from subgenogroup C1 also displayed endemic circulation between 1997 and 1999. Two isolates each from subgenogroups C1 and C2 were identified during the 1997 outbreak, however B3 and B4 appeared to be the most prevalent subgenogroups in the 1997 epidemic representing 16 of the 20 isolates identified

in this year (ratio of B3/B4 is 9:7). These 20 viruses resulted in a range of clinical manifestations(Table 1), including encephalitis (4 cases), poliomyelitis-like disease (1 case), myocarditis (1 case) and HFMD (14 cases). By contrast, only viruses from subgenogroups B4 and C1 were identified during the 2000 outbreak, (ratio 6:10) and were isolated from only cases of HFMD and aseptic meningitis (however, little specific clinical information is available). In this study, viruses from subgenogroups B3 and B4 were associated with both mild clinical

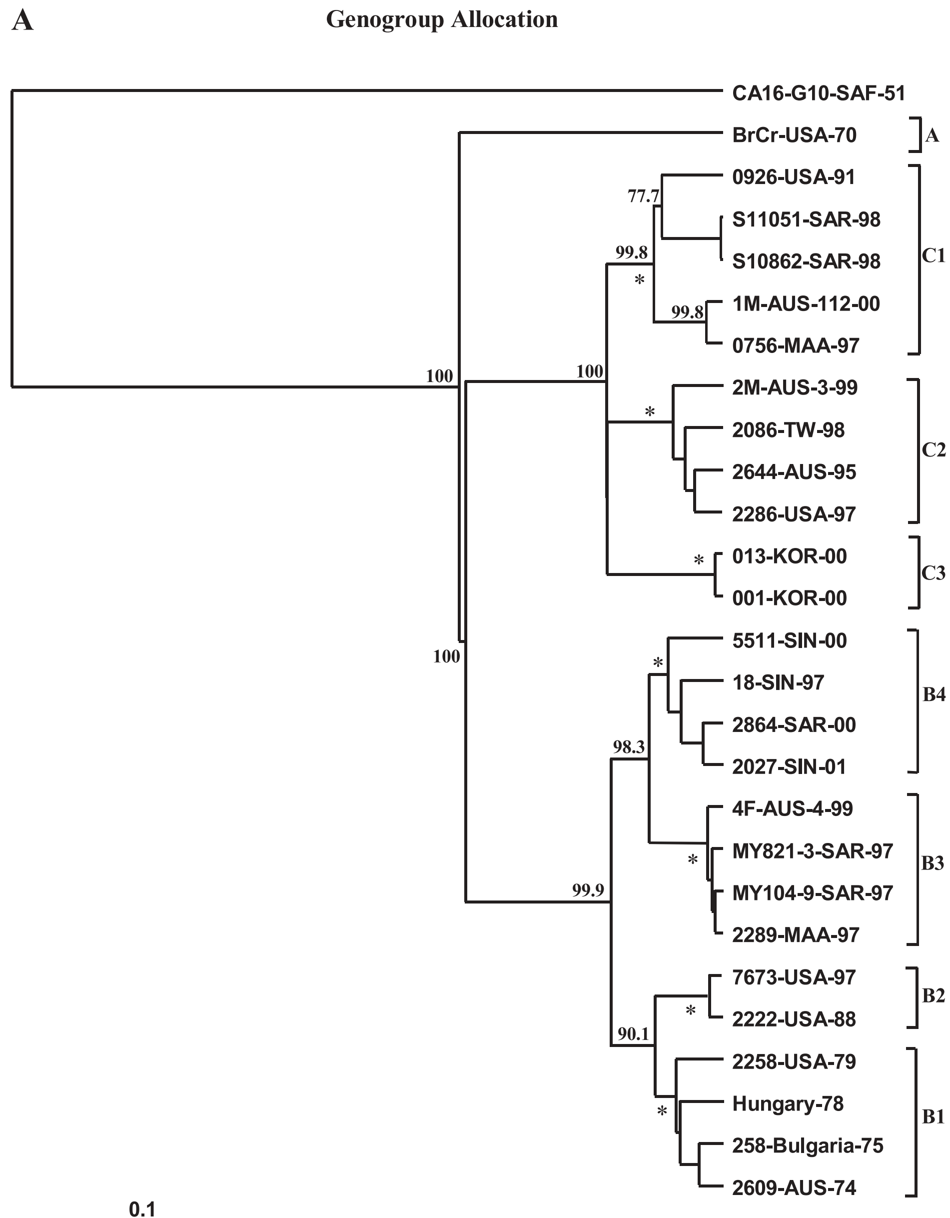

Fig. 1 (*continued*)

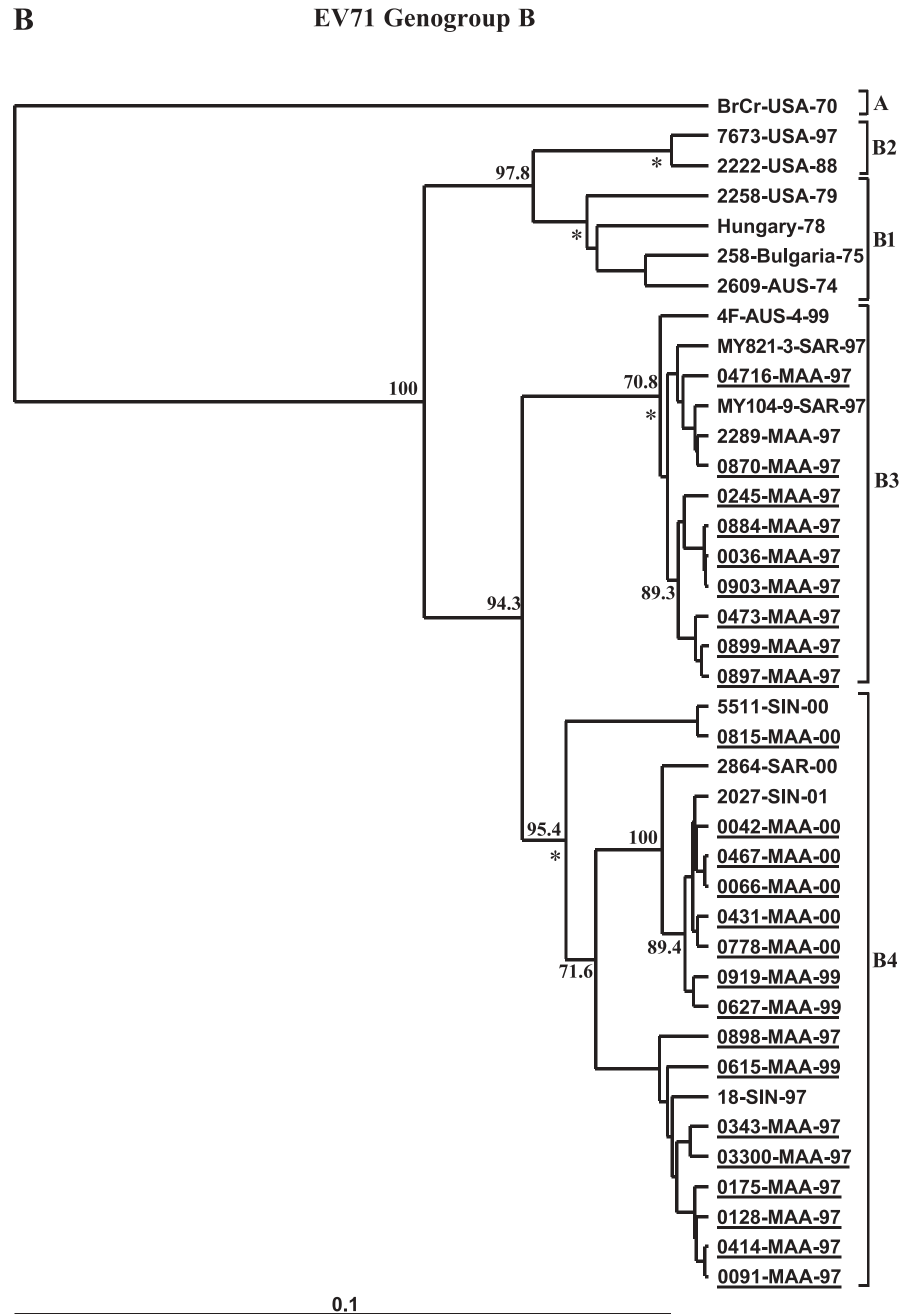

Fig. 1. Dendrogram constructed using the neighbour-joining method [18] showing the genetic relationships among forty-three EV71 isolates from peninsular Malaysia, based on the alignment of the complete VP1 gene sequence. Branch lengths are proportional to the number of nucleotide differences. The bootstrap values in 1,000 pseudo-replicates for major lineages within the tree are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. Strain names indicate a unique number-abbreviation of country where isolated-and year of isolation. Strains analysed during the course of the study are underlined and identified by a unique number-MAA-and year of isolation. Asterisks (*) indicate dendrogram nodes that distinguish between genogroups. The outgroup for (**A**) is the

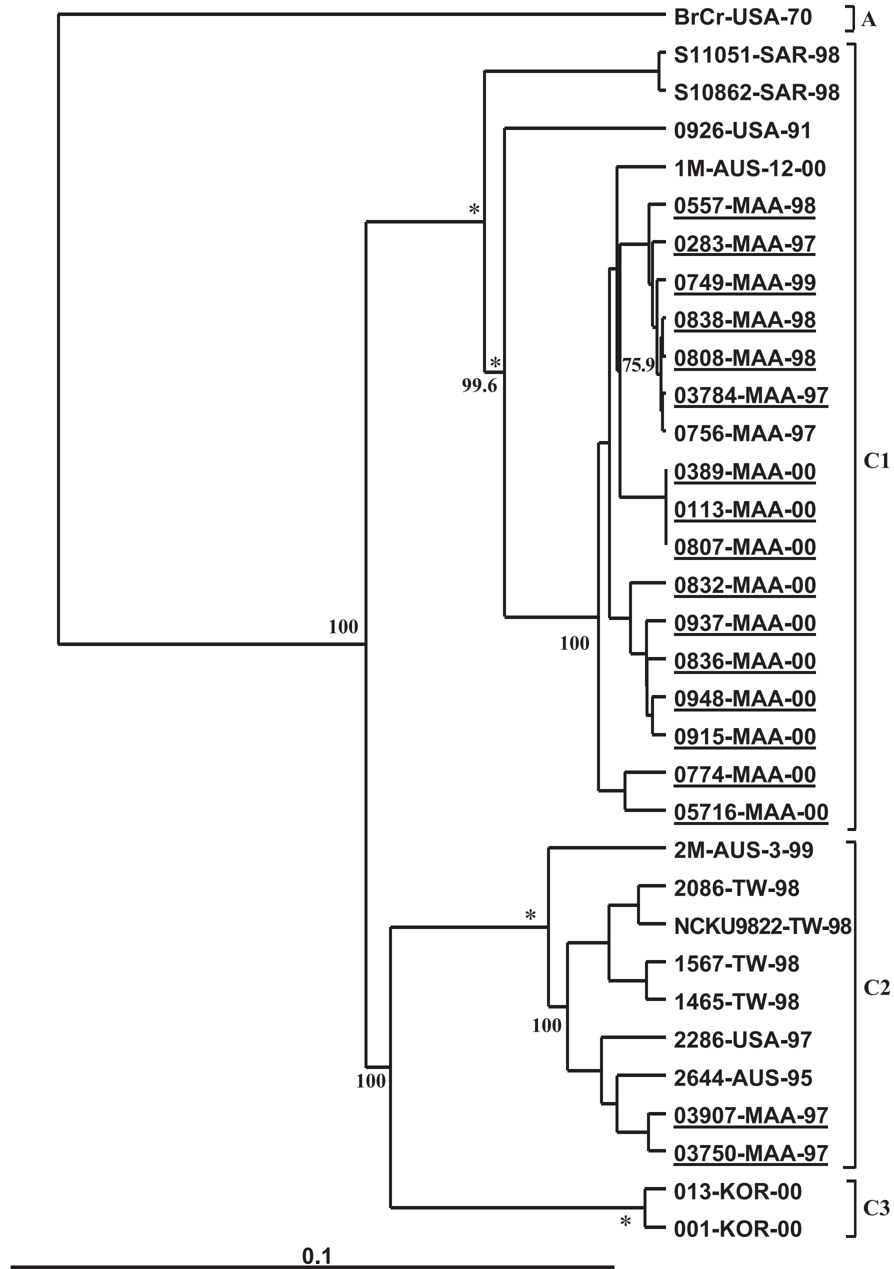

Fig. 1 (*continued*)
prototype strain of Coxsackievirus A16 (CA16-G10-SAF-51) [27]. The outgroup for both (**B**) and (**C**) is the EV71 prototype strain (BrCr-USA-70) [4]. **A** Dendrogram showing genogroups A, B and C as identified by Brown et al., 1999 [3], Cardosa et al. [5] and McMinn et al., 2001 [21]. **B** Dendrogram of strains belonging to genogroup B. **C** Dendrogram of strains belonging to genogroup C

Table 5. Consensus amino acid alignments of EV71 subgenogroups

Subgenogroup[a]	Strain[a]	VP1 amino acid position[b]							
		43	58	164	184	240	249	262	292
A	BrCr-USA-70	K	A	D	S	S	I	I	T
B1	2609-AUS-74	E	T	E	T	S	I	I	N
B2	7423-MS-87	E	T	E	T	S	V	I	N
B3	–	E	T	E	T	S	V	I	N/T/K
B4	–	E	T	E	T	S	V	I	T
C1	–	K	A	D	S	T	I	V	T
C2	–	K	A	D	S	T	I	I	T

[a]Refers to the subgenogroup identified in this study, except for genogroup A and subgenogroups B1 and B2, for which the reference strains are BrCr-USA-70 [4], 2609-AUS-74 [3] and 7423-MS-87 [4], respectively. Differences observed in other amino acid positions appeared to be random and did not correlate with either clinical presentation, genogroup or subgenogroup allocations

[b]K: Lysine, A: Alanine, E: Glutamic Acid, T: Threonine, S: Serine, I: Isoleucine, N: Asparagine, D: Aspartic acid, V: Valine

presentations (HFMD) and more serious manifestations (encephalitis, paralysis and myocarditis), whereas viruses from subgenogroups C1 and C2 were appeared to be associated with less severe clinical presentations (HFMD and aseptic meningitis, see Table 1), however the small number of isolates studied does not allow a clear interpretation of disease associations.

Amino acid alignment

The amino acid sequences of the forty-three isolates were aligned in an attempt to correlate both clinical presentation and genogroup allocation with specific amino acid changes. No obvious amino acid changes could be found to correlate with differences observed in clinical presentation, however, consensus changes were found to correlate with specific genogroups at positions 43, 58, 164, 184, 240, 249, 262 and 292 (Table 5).

Discussion

In this study, we have endeavored to determine the origin of the EV71 outbreaks that occurred in peninsular Malaysia between 1997 and 2000. We identified four subgenogroups (B3, B4, C1 and C2) of EV71 that circulated in peninsular Malaysia between 1997 and 2000. B3 and B4 were the prevalent subgenogroups in the 1997 epidemic with a small contribution from subgenogroups C1 and C2. Viruses from subgenogroups C1 and B4 were identified as the primary cause of the 2000 outbreak.

There have been numerous molecular epidemiological studies of EV71 reported in the last few years [3, 9, 21, 31, 32, 34]. The data presented in this

study is consistent with those presented by Brown et al. [3], Cardosa et al. [5], McMinn et al. [21], Shih et al. [31] and Wang et al. [36], in which the complete VP1 gene (891 nucleotides) was used for the nucleotide sequence analysis (with the exception of Wang et al. [36] who used only 841 nucleotides of VP1 in their analysis).

Despite differences in nomenclature, the dendrograms presented in this study are also relatively consistent with those presented by both Chu et al. [9] and Shimizu et al. [32]. Both studies presented dendrograms based on small regions of the VP4 gene (207 nucleotides of the VP4 gene and a 420 nucleotide region of the VP4-VP2 junction respectively). The similarities, although difficult to identify due to differences in genogroup nomenclature, become evident upon analysis of strains analysed both in this study and by Chu et al. [9] and Shimizu et al. [32]. It is likely that the subgenogroup C3 identified by Chu et al. [9] (distinct from the VP1-based subgenogroup C3 which consists of Korean strains and is unequivocally a new lineage) [5], is equivalent to the VP1-based subgenogroup C2 [21]. Similarly genogroups A and B, identified by Shimizu et al. [32] correlate to VP1 based genogroups B and C, respectively [3, 31]. Hence the division of EV71 into genogroups A, B and C, as suggested by Brown et al. [3], is supported by both studies.

It is likely that any minor variations observed by Chu et al. [9] and Shimizu et al. [32] result from the analysis of a different region of EV71 and also on the reliance on shorter genome sequences (207 and 420 nucleotides respectively). Furthermore, the value of the VP4 gene as an indicator of EV71 evolutionary history is unclear, because this gene does not encode an external capsid protein and is therefore not subject to immune pressure [28].

For ease of data comparison, future studies should attempt to use a universal system of genogroup and subgenogroup allocation, preferably using sequence data based on the entire VP1 gene (891 nucleotides) and the highly supported genogroup and subgenogroup divisions suggested by Brown et al. [3], Cardosa et al. [5] and McMinn et al. [21]. The VP1 gene is highly suitable for this purpose because it is the most external and immunodominant of the capsid proteins [25] and is therefore targeted by the immune system. As a result, most of the neutralisation sites are located on VP1, meaning that VP1-based genotyping correlates well with serotype designations [25]. Furthermore, there has been no evidence of recombination occurring in this region to date, thus permitting an uncomplicated analysis of virus evolution. Most importantly, there is a greater availability of data in public databases for this region of the genome. Such conformity would limit confusion and place isolates in context with the majority of other studies on EV71 molecular epidemiology.

Four subgenogroups of EV71 (B3, B4, C1 and C2) were identified as having circulated in peninsular Malaysia between 1997 and 2000. The co-circulation of more than two subgenogroups over the same time period has not been observed in any other country within the Asia-Pacific region to date. Therefore, the molecular epidemiology of EV71 in peninsular Malaysia appears to be more complex than that of other countries in the Asia-Pacific region. However, it should be noted

that it is possible that viruses belonging to subgenogroups circulating at low prevalence may not be detected even in the presence of sensitive virological surveillance.

In peninsular Malaysia, viruses from three subgenogroups (B3, B4 and C1) have been responsible for the recent epidemics in 1997 and 2000. Subgenogroups B3 and B4 predominated in 1997 (with a minor contribution from subgenogroups C1 and C2) and subgenogroups C1 and B4 predominated in 2000. Based on the phylogenetic data presented here, in addition to McMinn et al. [21], subgenogroup B3 viruses appeared to be prevalent in peninsular Malaysia, Sarawak and Singapore during 1997–98, spread to Perth in 1999, and have not been isolated since [21]. Subgenogroup B4 viruses were also prevalent in peninsular Malaysia in 1997 at a time when subgenogroup B3 viruses were predominant elsewhere in the Asia-Pacific region. Subgenogroup B4 viruses have since appeared to spread throughout the Asia-Pacific region, causing epidemics in Sarawak, Singapore and Taiwan [5]. With the exception of one isolate in Singapore (a country with active surveillance), viruses belonging to subgenogroup B4 were not isolated in any other country in the Asia-Pacific region during 1997. Hence, the predominance of this subgenogroup in peninsular Malaysia during the 1997 outbreak suggests that this region may have served as a source of virus for the epidemics that followed in the neighboring countries during 2000–01. As has been observed for the rest of the Asia-Pacific region, subgenogroup C1 viruses underwent low-level circulation in peninsular Malaysia from 1997 to 1999 (demonstrated by consistent isolation over this time period). However, subgenogroup C1 viruses were isolated with much higher frequency during the 2000 outbreak. This has not been observed in any other outbreak to date and suggests that subgenogroup C1 may have developed epidemic potential.

In summary, four subgenogroups of EV71 co-circulated in peninsular Malaysia between 1997–2000: B3, B4, C1 and C2. Subgenogroups B4 and C1 are of particular interest. Subgenogroup B4 viruses appear to have circulated solely in peninsular Malaysia for three years before spreading to Singapore, Sarawak and Taiwan in 2001, resulting in large HFMD epidemics. Viruses belonging to subgenogroup C1, although identified at low level throughout the Asia-Pacific region during 1997–2000, appeared to become epidemic in peninsular Malaysia during 2000. This suggests that C1 may be responsible for future epidemics in the Asia-Pacific region. Both these observations are unique to peninsular Malaysia and suggest that peninsular Malaysia may have served as a source of virus for the rest of the Asia-Pacific region.

We propose that future studies on the molecular epidemiology of EV71 should use the complete VP1 gene sequence in order to generate data comparable with the majority of the current data. The focus of such studies should be to identify the current activity of EV71 genogroups and subgenogroups in the entire Asia-Pacific region, including areas such as Indonesia and The Philippines, which, due to their large populations, have the potential to serve as endemic foci of EV71. This information will improve our understanding of the major sources of EV71, which is particularly important for ongoing surveillance within the Asia-Pacific region,

the purpose of which is to predict impending epidemics and identify endemic sources of virus.

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