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Comparative genetic analysis of VP4, VP1 and 3D gene regions of enterovirus 71 and coxsackievirus A16 circulating in Malaysia between 1997-2008

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Abstract. Three genomic regions, VP4 capsid, VP1 capsid and 3D RNA polymerase of human enterovirus 71 (EV-71) and coxsackievirus A16 (CV-A16) were sequenced to understand the evolution of these viruses in Malaysia. A total of 42 EV-71 and 36 CV-A16 isolates from 1997-2008 were sequenced. Despite the presence of many EV-71 subgenotypes worldwide, only subgenotypes B3, B4, B5, C1 and C2 were present in Malaysia. Importation of other subgenotypes such as C3, C4/D and C5 from other countries was infrequent. For CV-A16, the earlier subgenotype B1 was replaced by subgenotypes B2a and the recent B2c. Subgenotype B2a was present throughout the study while B2c only emerged in 2005. No genetic signatures could be attributed to viral virulence suggesting that host factors have a major role in determining the outcome of infection. Only three EV-71 B3 isolates showed non-consistent phylogeny in the 3D RNA polymerase region which indicated occurrence of recombination in EV-71. High genetic diversity was observed in the Malaysian EV-71 but Malaysian CV-A16 showed low genetic diversity in the three genomic regions sequenced. EV-71 showed strong purifying selection, but that occurred to a lesser extent in CV-A16.

INTRODUCTION

Human enterovirus 71 (EV-71) and coxsackievirus A16 (CV-A16) are members of the family of *Picornaviridae*, which also include polioviruses, rhinoviruses and echoviruses. Both viruses belong to the species *Human enterovirus A* (HEV-A), which includes other important human pathogens such as coxsackieviruses A2-A8, A10, A14, and A16. Both viruses are non-enveloped, and their genomes are positive single-stranded RNA of approximately 7400 bp in length. The genome is flanked by untranslated regions in the 5' and 3', and consists of VP4, VP2, VP3 and VP1 as the structural proteins, and 2A, 2B, 2C, 3A, 3B, 3C and 3D as the non-structural proteins.

Both EV-71 and CV-A16 cause outbreaks of hand, foot and mouth disease (HFMD) in children. However, EV-71 can be potentially fatal and cause serious neurological diseases such as meningitis, encephalitis, and acute flaccid paralysis. No severe CV-A16 outbreaks have been reported, and severe disease has only been reported sporadically, including fatalities (Coopers *et al.*, 1989; Wang *et al.*, 2004; Legay *et al.*, 2007) and one case of rhomboencephalitis (Goto *et al.*, 2009). CV-A16 was first isolated in South Africa in 1951 (Pöyry *et al.*, 1994) and EV-71 was first isolated in California in 1969 (Schmidt *et al.*, 1974). Over the last decade, EV-71 epidemics have increased in number and involved large numbers of children in Asia, including Malaysia. Fatalities have been observed in Bulgaria (Chumakov *et al.*, 1979), Hungary (Nagy *et al.*, 1982), Malaysia (Abu Bakar *et al.*, 1999), Taiwan (Huang *et al.*, 1999) and China (Yang

et al., 2009). Many European countries such as Germany, Norway, United Kingdom, Austria, Netherlands, France and Hungary have also reported EV-71 cases (reviewed in Chan *et al.*, 2011). With the near-eradication of poliovirus, EV-71 is the main enterovirus posing a new global health threat in young children.

Understanding of the epidemiology and evolution of EV-71 and CV-A16 are important for vaccine development and public health control and prevention. Sequencing of VP4 and VP1 capsids for EV-71 and CV-A16 have been reported for Malaysia (Abu Bakar *et al.*, 2000; Cardosa *et al.*, 2003; Herrero *et al.*, 2003; Chua *et al.*, 2007; Perera *et al.*, 2007; Yusof *et al.*, 2011). However, very limited data is available for the non-structural gene regions such as 3D. Both viruses can recombine at the non-structural proteins with other HEV-A to form new variants with altered virulence (Chan & Abu Bakar, 2004). It has been reported that recombination is one of the evolutionary driving forces of EV-71 and CV-A16 (Chan & Abu Bakar, 2005; Yoke-Fun & Abu Bakar, 2006). The frequency of recombination in currently circulating EV-71 and CV-A16 viruses is still not known.

Longitudinal epidemiological analyses have only been reported for Taiwan and Japan. There have been a few Malaysian studies which covered peninsular Malaysia and Sarawak, located in the Borneo Island over a short period of time (Cardosa *et al.*, 2003; Herrero *et al.*, 2003; Chua *et al.*, 2007; Perera *et al.*, 2007; Yusof *et al.*, 2011). There are four EV-71 genotypes, A-D (Chan *et al.*, 2010) and two CV-A16 genotypes, A and B (Perera *et al.*, 2007; Zong *et al.*, 2011). EV-71 genotype B can be further divided into B1-B5, and subgenotype C can be divided into C1-C5, and C4 which can also be considered as genotype D (Chan *et al.*, 2010). CV-A16 genotype B can be divided into B1, B2a, B2b and B2c. At present, no Malaysian study has analyzed the circulating genotypes of both EV-71 and CV-A16, the two most important causative agents of HFMD. We report here the longitudinal epidemiological analyses of EV-71 and CV-A16 isolated at our teaching hospital in Kuala Lumpur over the last ten years, and compare VP1, VP4 and 3D gene sequences with other Malaysian isolates. Comparison of genetic changes between EV-71 and CV-A16 may also provide key genetic signatures of virulence differences between these two viruses.

MATERIALS AND METHODS

Clinical specimens

Clinical specimens were received by the diagnostic virology laboratory in University Malaya Medical Centre (UMMC) from 1997 to 2008. Specimens were collected from patients with hand, foot and mouth disease and neurological complications suspected to be of enteroviral origin. The clinical specimens included throat swabs, vesicle swabs, stool, nasopharyngeal secretions and cerebrospinal fluid. Virus isolation was performed in Vero cells (ATCC CCL-81). When cytopathic effects were observed, immunofluorescence was performed with Pan-Enterovirus Blend (Chemicon International, Inc., Temecula, USA), and confirmed using EV-71 and EV-71/CV-A16 specific monoclonal antibodies (Chemicon International, Inc., Temecula, USA).

RNA extraction, reverse-transcription PCR and sequencing

Viral RNA was extracted from 140 µl of viral culture supernatants using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), and cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. PCR was performed using GoTaq Flexi DNA polymerase (Promega, Madison, USA) with 2 µl of cDNA. Each reaction was subjected to 3 min of initial denaturation, followed by 30 cycles of denaturing at 95°C for 1 min, annealing between 42-60°C for 1 min (depending on primer sets), and extension at 72°C for 1 min, and final extension of 72°C for 5 min. Different primers were used to amplify VP4, VP1 and 3D of EV-71 and CV-A16 (Table 1). The amplicons were purified with QIAquick gel purification kit (Qiagen,

Table 1. Primer sequences to amplify VP4, VP1 and 3D of EV-71 and CV-A16

Virus	Gene region	Primers name	Nucleotide sequence (5'-3')	Nucleotide position*	Product size (bp)	Annealing temp (°C)	Polarity	References
EV-71	VP4	EntabF	TCCTCCGGCCCCTGAATGCGGCTAAT	411-1030	620	55	Sense	This study
		VP4/2R	GCCACTCACCATAGCCAACT				Antisense	
		EntabF	TCCTCCGGCCCCTGAATGCGGCTAAT	411-1229	837	55	Sense	Yoke-Fun & Abu Bakar, 2006
		EV1R	TGMACRTGRATGCARAACC				Antisense	
	VP1	EVVP1F	AGGGAGATAGGGTGGCAGAT	2402-3440	1039	55	Sense	Yoke-Fun & Abu Bakar, 2006
		EVVP1R	ACGAGCAATCGTGTCACAAC				Antisense	
		159	ACYATGAAAYTGTGCAAGG	2358-3310	953	42	Sense	Brown *et al.*, 1999
		NP1A	GCICCICAYTGICCRAA				Antisense	
		EV3F	GGRGCRCCYAAYACWGCYTAYATWWTRGC	2296-4007	1712	50	Sense	Yoke-Fun & Abu Bakar, 2006
		EV4R	GCTGTYTTBGMYTTRAYCCAVGC				Antisense	
		MJP-VP1F	CACCCTTGTAATACCATGGATCAG	2172-3391	1220	53	Sense	Chua *et al.*, 2008
		MJP-P1R	GTCGCGRGAGCTGTYYTCCCA				Antisense	
	3D	EV3DF	TWGCHTTTGAYTACWCNGGNTATGA	6590-7168	579	50	Sense	This study
		EV3DR	TAYTCBTSYTCNCCRTTRTGCCA				Antisense	
		EV8F	CATYAAGAARARRGACATYYTBG	6273-7191	919	50	Sense	Yoke-Fun & Abu Bakar, 2006
		EV8R	TTGCTATTCTGGTTATAACAAATTTACCC				Antisense	
CV-A16	VP4	EntabF	TCCTCCGGCCCCTGAATGCGGCTAAT	452-1171	720	55	Sense	This study
		CVVP2R	CCCAAGATTTAGTATCTAGCGTGAA				Antisense	
		CV2F	ACTTTGGGTGTCCGTGTTTC	552-1034	483	60	Sense	
		CV2R	TGTGTCGTGATGGTGGAGTT				Antisense	
	VP1	CVVP1F	CACAGAGGACATTGAGCAAAC	2412-3401	990	55	Sense	This study
		CVVP1R	AGGTGCCGATTCACTACCC				Antisense	
		CV6F	GCGTCGTGTAATGCTAGTGAC	2150-3401	1252	60	Sense	
		CV7R	AGGTGCCGATTCACTACCC				Antisense	
	3D	CV3DF	GGTCTGCAGTAGGGTGCAAT	6563-7389	827	55	Sense	This study
		CV3DR	CCCCCACCAGTCATTAACAC				Antisense	
		CV15F	GGATGACTTTTGGGCATCTC	6505-7353	849	60	Sense	
		CV16R	CCCATCATGTGTATCGGAAC				Antisense	

Hilden, Germany) and were sequenced with ABI PRISM 377 DNA sequencer (Applied Biosystems, California, USA).

Sequence identity analysis

Sequences were trimmed and assembled using Geneious 5.0.3 (Biomatters Ltd., Auckland, New Zealand). After trimming, EV-71 sequences of the complete VP4, partial VP1 and partial 3D were 207 bp, 824 bp (position in VP1, 42-867) and 537 bp (position in 3D, 715-1020), respectively. For CV-A16, the complete VP4 was 207 bp, complete VP1 was 891 bp, and partial 3D was 708 bp (position in 3D, 639-1386). The amino acid differences for all the genes were compared with other genotypes.

Phylogenetic analysis

Multiple alignment was performed using ClustalX 2.012 (Larkin *et al*., 2007). Phylogenetic trees were constructed using Kimura two parameter and bootstraps of 1000 replicates were used to test the robustness of the trees. The trees were displayed using MEGA 5 (Tamura *et al*., 2011), with CV-A16/G10 (CV-A16 prototype) as the outgroup for EV-71 datasets, and BrCr (EV-71 prototype) as the outgroup for the CV-A16 datasets. Previously published sequences from Malaysia and other reference sequences were also downloaded for the analyses.

Selective pressure analysis

Selective pressure analysis was performed using codon-based maximum likelihood methods implemented in the Datamonkey web server (Delport *et al*., 2010). Amino acids were only selected when positively identified by all three different codon-based maximum likelihood methods, which were fixed effects likelihood (FEL), internal branches FEL (iFEL) and single likelihood ancestor counting (SLAC). Random effects likelihood (REL) was also used to determine positive selection.

Nucleotide accession numbers

The sequences have been deposited into GenBank with accession numbers JN248387-JN248422 and JN316029-JN316208 (Table 2).

RESULTS

Isolation of EV-71 and CV-A16 in UMMC

From 1997 to 2008, 1248 samples from patients suspected with enterovirus infection were received by the virology laboratory at UMMC, and of these, 203 (16.3%) samples were culture-confirmed EV-71, and 48 (3.6%) samples were culture-confirmed CV-A16. Of these, 42 EV-71 and 36 CV-A16 isolates were randomly chosen for amplification and sequencing (Table 2). Two fatal EV-71 cases were from the 1997 outbreak. All the CV-A16 sequences in the present study were from mild HFMD cases.

Phylogenetic analysis of EV-71 and CV-A16 in VP4 and VP1

EV-71 sequences from this study (n=42) were aligned with other previously published EV-71 sequences from Malaysia. Based on the VP4 and VP1 trees, our isolates were grouped in subgenotypes B3, B4, B5, C1 and C2. Subgenotypes B3, B4, C1 and C2 were the cause of the 1997 severe outbreak, while subgenotypes B4 and C1 were co-circulating during the 2000 and 2001 outbreaks.

Subgenotypes B3 and C2 were only present in the 1997 outbreak and were not isolated after that. Subgenotypes B4 and C1 also disappeared after 2001 and 2003, respectively. Since then, subgenotype B5, which first appeared in 2000, became the subgenotype causing outbreaks in 2006. This is the sole subgenotype currently circulating in Malaysia (Figure 1, Figure 2). The presence of Malaysian isolates in other subgenotypes such as C4/D and C5 were only seen in VP4, however this was not bootstrap supported (Figure 1). The isolates clustered according to the years and not according to different geographical states in Malaysia.

Similarly, sequences of CV-A16 isolates from this study (n=36) were aligned with other previously published CV-A16 from Malaysia. Based on the VP4 and VP1 trees, our sequenced isolates were grouped into subgenotypes B2a and B2c (Figure 3). A few Sarawak isolates from 1998 and 2000 were grouped in genotype B1. Subgenotype B2a isolates were circulating throughout the

Table 2. Enterovirus 71 and coxsackievirus A16 isolates sequenced in the present study

Isolate name*	Accession number			Genotype	State	Gender	Age (years)	Specimen	Diagnosis
	VP4	VP1	3D						
EV-71									
PM-13091-97	JN316124	JN316108	JN316195	B4	Selangor	M	3	Biopsy	Fatal
PM-13473-97	JN316145	JN316110	JN316162	B3	Selangor	M	16 months	Stool	HFMD
PM-13899-97	JN316146	JN316109	JN316163	B3	Melaka	F	4	Cerebellum	Fatal
PM-14283-97	JN316131	JN316071	JN316168	C1	Selangor	F	2	Throat swab	HFMD
PM-14716-97	JN316152	JN311611	JN316164	B3	Selangor	F	2	Throat/rectal swab	HFMD
PM-17557-98	JN316147	JN316067	JN316175	C1	Selangor	M	NA	Rectal swab	HFMD
PM-17808-98	JN316142	JN316069	JN316172	C1	Selangor	M	6	Throat swab	Meningitis
PM-17838-98	JN316143	JN316068	JN316173	C1	Selangor	M	7	Throat/rectal swab	HFMD
PM-10749-99	JN316148	JN316070	JN316174	C1	Selangor	M	7	Vesicle swab	Meningitis
PM-12615-99	JN316112	JN316104	JN316201	B4	Sabah	M	11 months	Rectal swab	Enteroviral myocarditis
PM-12627-99	JN316113	JN316105	JN316202	B4	Selangor	F	NA	Stool	Stevens-Johnson syndrome
PM-12919-99	JN316119	JN316106	JN316203	B4	Sabah	M	8	Rectal swab	HFMD
PM-15774-00	JN316144	JN316079	JN316177	C1	Johor	M	1	Throat/vesicle swab	HFMD
PM-15948-00	JN316151	JN316073	JN316181	C1	Kedah	M	5	Rectal swab	HFMD
PM-16042-00	JN316115	JN316107	JN316197	B4	Selangor	M	1	Rectal swab	HFMD
PM-17113-00	JN316113	JN316076	JN316164	C1	Johor	M	1	Rectal/throat swab	HFMD
PM-17164-00	JN316116	JN316099	JN316206	B4	Johor	M	3	Throat swab	HFMD
PM-17177-00	JN316140	JN316095	JN316194	B5	Johor	M	4	Rectal swab	HFMD
PM-17181-00	JN316150	JN316080	JN316176	C1	Johor	F	2	Vesicle swab	HFMD
PM-17204-00	JN316153	JN316077	JN316166	C1	Johor	M	3	Vesicle swab	HFMD
PM-17431-00	JN316121	JN316100	JN316198	B4	Johor	M	2	Throat/vesicle swab	HFMD
PM-17467-00	JN316117	JN316097	JN316199	B4	Kedah	F	2	Throat swab	HFMD
PM-19229-01	JN316137	JN316081	JN316178	C1	Kedah	F	3	Vesicle swab	HFMD
PM-19552-01	JN316127	JN316072	JN316169	C1	Kedah	F	2	Throat/vesicle swab	HFMD
PM-20045-01	JN316120	JN316102	JN316204	B4	Kedah	F	9 months	Vesicle swab	HFMD

PM-20680-01	JN316114	JN316101	JN316205	B4	Kedah	M	16	Rectal swab	HFMD
PM-20756-01	JN316118	JN316103	JN316207	B4	Kedah	M	2	Throat swab	HFMD
PM-20822-01	JN316122	JN316098	JN316200	B4	Selangor	M	4	Stool	HFMD
PM-24886-03	JN316129	JN316075	JN316180	C1	Selangor	M	1	Rectal swab	NA
PM-25405-03	JN316141	JN316074	JN316179	C1	Selangor	F	NA	Rectal swab	NA
PM-26165-03	JN316136	JN316083	JN316187	B5	Selangor	M	2	Stool	NA
PM-32308-05	JN316139	JN316086	JN316190	B5	Selangor	F	1	Nasopharyngeal secretion	Bronchiolitis
PM-32286-05	JN316134	JN316085	JN316189	B5	Selangor	F	1	Nasopharyngeal secretion	Pneumonia
PM-33034-05	JN316133	JN316084	JN316188	B5	Selangor	F	10 months	swab	NA
PM-34242-06	JN316135	JN316088	JN316192	B5	Selangor	M	2	Rectal swab	NA
PM-34589-06	JN316132	JN316082	JN316191	B5	Selangor	M	6	Stool	NA
PM-35017-06	JN316155	JN316093	JN316186	B5	Selangor	M	5	Stool	HFMD
PM-1657636-06	JN316154	JN316091	JN316182	B5	Selangor	NA	NA	Rectal swab	NA
PM-1657640-06	JN316158	JN316092	JN316183	B5	Selangor	NA	NA	Rectal swab	NA
PM-1673313-06	JN316159	JN316094	JN316185	B5	Selangor	NA	NA	Rectal swab	NA
PM-1687413-06	JN316157	JN316090	JN316184	B5	Selangor	NA	NA	Rectal swab	NA
PM-2219140-08	JN316126	JN316096	JN316193	B5	Selangor	NA	NA	Vesicle swab	NA
CV-A16									
PM-13884-97	JN215396	JN248409	JN316039	B2a	Selangor	F	1	Stool	HFMD
PM-14194-97	JN215497	JN248410	JN316041	B2a	Selangor	F	5	Throat swab	HFMD
PM-14660-97	JN215395	JN248408	JN316044	B2a	Selangor	M	3	Throat/rectal swab	HFMD
PM-16809-98	JN215393	JN248411	JN316051	B2a	Kuala Lumpur	M	NA	Rectal swab	HFMD
PM-16985-98	JN215394	JN248412	JN316053	B2a	Kuala Lumpur	F	NA	Stool	HFMD
PM-12727-99	JN215371	JN248406	JN316037	B2a	Kuala Lumpur	F	NA	Throat swab	HFMD
PM-12284-99	JN215370	JN248405	JQ746661	B2a	Selangor	M	1	Stool	HFMD
PM-12593-99	JN215387	JN248404	JN316036	B2a	Selangor	M	2	Vesicle swab	HFMD
PM-12969-99	JN215369	JN248407	JN316038	B2a	Kuala Lumpur	F	1	Throat swab	HFMD
PM-13998-00	JN215384	JN248396	JN316040	B2a	NA	NA	6	Ulcer swab	HFMD
PM-14200-00	JN215385	JN248413	JN316042	B2a	NA	NA	7	Vesicle swab	HFMD
PM-15765-00	JN215372	JQ746666	JN316045	B2a	Johor	M	5	Throat/rectal swab	HFMD

PM-15771-00	JN215378	JN248392	JN316046	B2a	Johor	F	NA	Vesicle swab	HFMD
PM-15773-00	JN215373	JN248399	JN316047	B2a	Johor	M	1.5	Throat/vesicle swab	HFMD
PM-15912-00	JN215374	JN248403	JN316048	B2a	Johor	M	2	Throat swab	HFMD
PM-15922-00	JN215379	JN248398	JN316049	B2a	Johor	M	6 months	Vesicle swab	HFMD
PM-17106-00	JN215375	JN248401	JN316054	B2a	Johor	M	3	Throat swab	HFMD
PM-17264-00	JN215376	JN248402	JN316055	B2a	Johor	M	4 months	Vesicle swab	HFMD
PM-17368-00	JN215377	JN248400	JN316056	B2a	Johor	F	4	Throat swab	HFMD
PM-22159-02	JN215388	JN248387	JN316029	B2a	Selangor	M	5	Throat swab	HFMD
PM-22217-02	JN215389	JN248388	JN316030	B2a	Selangor	F	3	Vesicle swab	HFMD
PM-22339-02	JN215392	JN248391	JN316033	B2a	Selangor	NA	NA	Throat swab	HFMD
PM-22241-02	JN215390	JN248390	JN316031	B2a	Selangor	NA	NA	Throat swab	HFMD
PM-22264-02	JN215391	JN248390	JN316032	B2a	Selangor	M	3	Throat swab	HFMD
PM-23208-02	JN215382	JN248397	JN316060	B2a	Selangor	M	2	Ulcer swab	HFMD
PM-31033-05	JN215368	JN248415	JN316061	B2a	Selangor	M	1	Stool	HFMD
PM-31131-05	JN215401	JN248418	JN316062	B2a	Selangor	M	NA	Throat swab	HFMD
PM-31174-05	JN215381	JN248394	JN316063	B2a	Selangor	M	5	Stool	HFMD
PM-31376-05	JN215383	JN248416	JN316064	B2a	Selangor	NA	NA	Rectal swab	HFMD
PM-35210-06	JN215402	JN248420	JN316065	B2c	Selangor	M	NA	Rectal swab	HFMD
PM-1651402-06	JN215398	JN248421	JN316050	B2C	Selangor	M	2	Rectal swab	HFMD
PM-1694925-06	JN215403	JN248419	JN316052	B2C	Selangor	M	9 months	Rectal swab	HFMD
PM-00033-07	JN215367	JN248414	JQ746660	B2a	NA	NA	NA	NA	HFMD
PM-1791021-07	JN215380	JN248393	JN316057	B2a	Selangor	M	NA	Rectal swab	HFMD
PM-1795457-07	JN215399	JN248417	JN316058	B2C	Selangor	M	NA	Rectal swab	HFMD
PM-1824818-07	JN215400	JN248422	JN316059	B2C	Selangor	F	NA	Stool	HFMD

*The last two digits of the isolate name refer to the year of isolation
NA: data not available

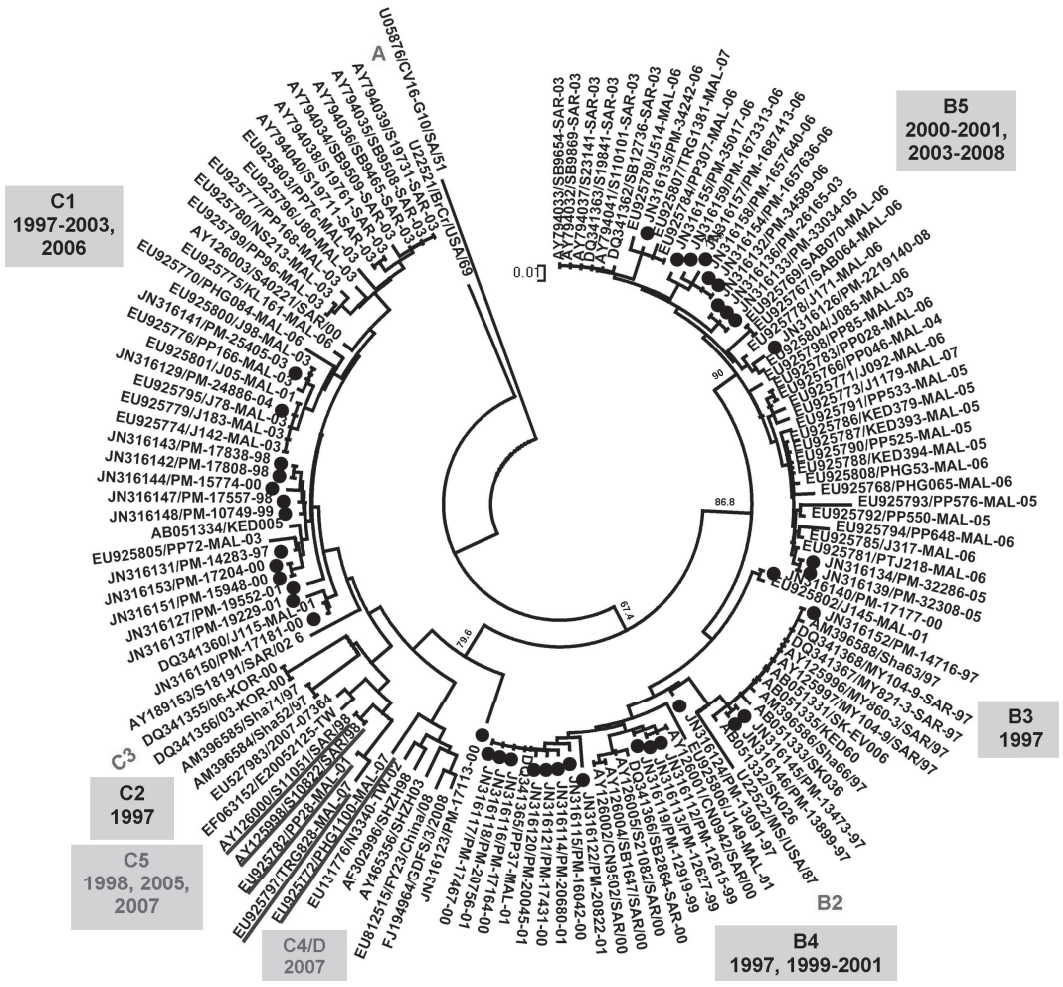

Figure 1. Phylogenetic tree of EV-71 VP4. The tree was drawn using VP4 gene sequences from this study (●) and also other Malaysian sequences available in GenBank. The tree was outgrouped with CV-A16/G10. All the subgenotypes and years of isolation of Malaysian isolates are labeled. Isolates from subgenotype C4/D and C5 are underlined in black

study period and were dominant in 1997-2003, 2005 and 2007. Subgenotype B2c only emerged after 2005 and was isolated again in 2007 (Figure 3).

Non-consistent phylogeny observed at 3D of EV-71 and CV-A16

The 3D regions for EV-71 and CV-A16 were trimmed to 306 nucleotides for comparison with other previously published Malaysian isolates and other HEV-A isolates (Figure 4). As previously reported, three EV-71 isolates of subgenotype B3 were grouped with CV-A16/G10 (Yoke-Fun & Abu Bakar, 2006). Apart from this, other isolates were grouped according to the subgenotypes previously shown with VP1.

Comparison of genetic changes in EV-71 and CV-A16

Comparison of two viruses was performed to identify possible genetic signatures that contribute to virus virulence. Two fatal EV-71 isolates were from subgenotypes B3 and B4. Other non-fatal isolates from B3 and B4 also have very similar sequences to these two fatal isolates. We found that EV-71 and CV-A16 were genetically distinct and the similarities at nucleotide and amino acid levels were only 67-74% and 72-79% in VP4,

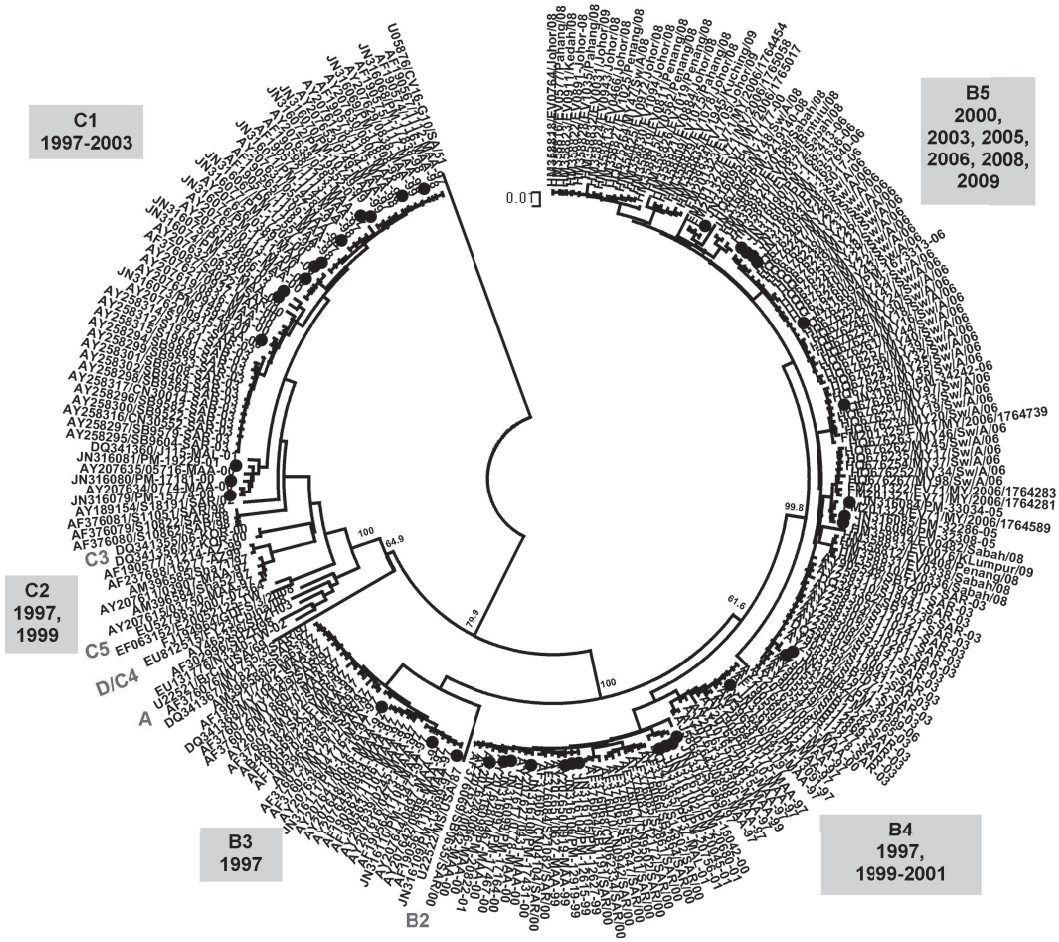

Figure 2. Phylogenetic tree of EV-71 VP1. The tree was drawn using partial VP1 gene sequences from this study (●) and also other Malaysian sequences available in GenBank. The tree was outgrouped with CV-A16/G10. All the subgenotypes and years of isolation of Malaysian isolates are labeled

64-68% and 71-74% in VP1, and 74-83% and 93-98% in 3D, respectively.

The VP4, VP1 and 3D nucleotide and amino acid similarities of EV-71 and CV-A16 isolates from this study are summarised in Table 3. The EV-71 isolates showed greater genetic diversity, while the CV-A16 isolates were generally better conserved. For example, for VP4, the genetic similarities within EV-71 isolates were 78-100% and 89-100% at nucleotide and amino acid levels, respectively, while in CV-A16 isolates, the genetic similarities were higher at 87-100% and 98-100%, respectively.

The amino acids of different genotypes for the three genomic regions, VP4, VP1 and 3D were compared (Figure 5). There were many specific mutations attributed to each subgenotype of EV-71 and CV-A16. No mutation was found in EV-71 VP4, while one mutation, I61M was seen in VP4 of isolates from subgenotype B2c (Figure 5g). Many mutations were observed in VP1 of Malaysian EV-71 (16 mutations) and CV-A16 (29 mutations) (Figure 5a-5c). For EV-71, six mutations were due to differences with the prototype BrCr virus (genotype A), while in CV-A16, there were 26 mutations contributed by differences with prototype CV-A16/G10 (genotype A). Both EV-71 and CV-A16 have similar mutations at positions 22, 43 and 240. For 3D, EV-71 and CV-A16 have 12 and 16 mutations, respectively, and five similarities at positions 368, 370, 383, 396 and 410 were

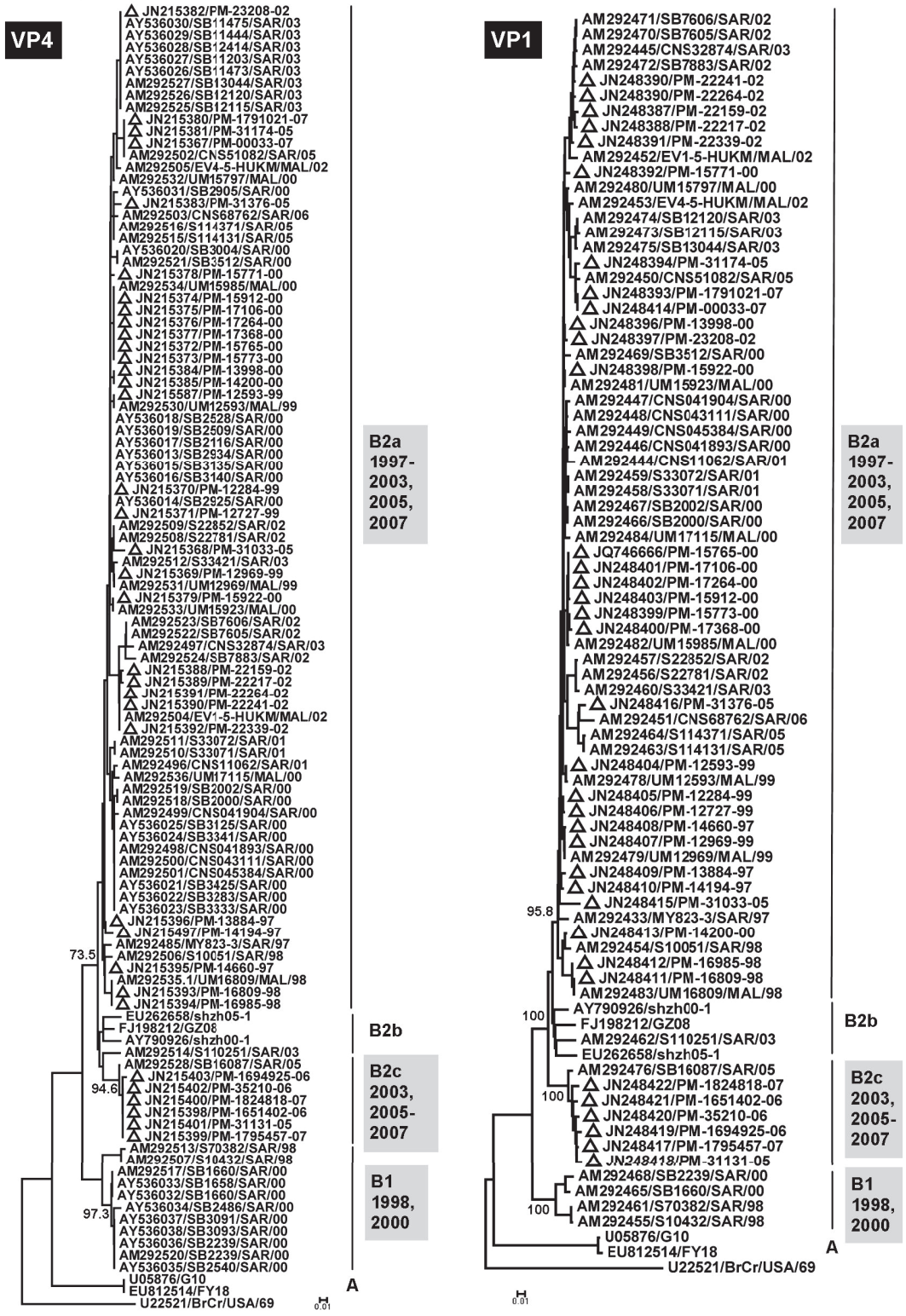

Figure 3. Phylogenetic trees of CV-A16 VP4 and VP1. The trees were drawn using VP4 and VP1 gene sequences from this study (Δ) and also other Malaysian sequences available in GenBank. The tree was outgrouped with EV-71 BrCr. All the subgenotypes and years of isolation of Malaysian isolates are labeled

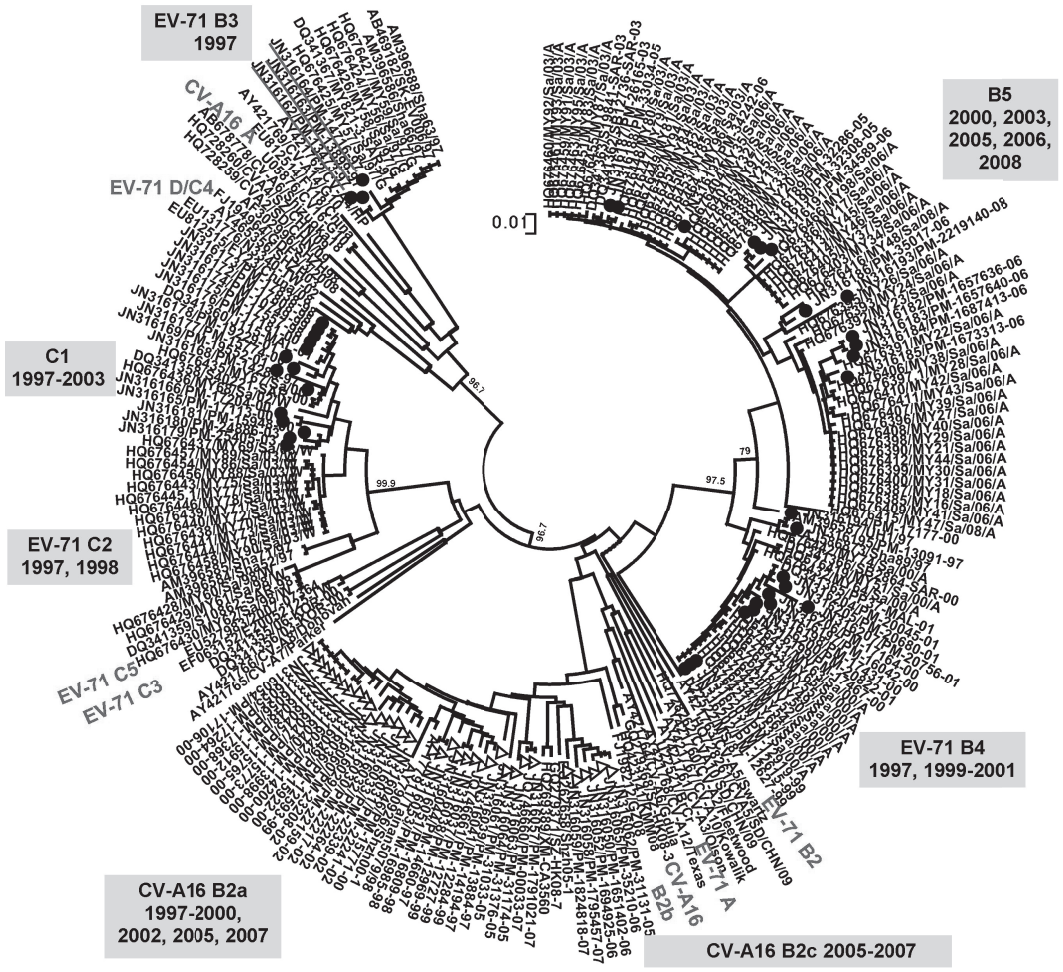

Figure 4. Phylogenetic tree of EV-71 and CV-A16 3D. The tree was drawn using partial 3D gene sequences of 306 bp. 3D sequences of EV-71 (●) and CV-A16 (Δ) from this study were compared with other HEV-A sequences available in GenBank. The tree was mid-point rooted. All the subgenotypes and years of isolation of Malaysian isolates are labeled. The three recombinant B3 isolates are underlined in black

Table 3. Comparison of nucleotide and amino acid similarities of EV-71 and CV-A16 sequenced in the present study

Similarity	VP4 (%)	VP1 (%)	3D (%)
EV71			
Nucleotide	78-100	80-100	73-100
Amino acid	89-100	89-100	93-100
CV-A16			
Nucleotide	87-100	88-100	91-100
Amino acid	98-100	97-100	96-100

shared (Figure 5d-f). These common sites for mutations showed that these amino acids were hot spots for mutations to occur.

Recently, the crystal structure of EV-71 capsid was elucidated (Wang *et al.*, 2012; Plevka *et al.*, 2012). Based on that, two observed amino acids changes in VP1 of EV-71, positions 98 and 184, were located at the immunogenic BC loop and FG loop, respectively (Figure 5b). More VP1 amino acid changes of CV-A16 were located at the immunogenic regions, position 101 in the BC loop, 183 in the FG loop, and positions 213, 215, 217 and 220 in the GH loop

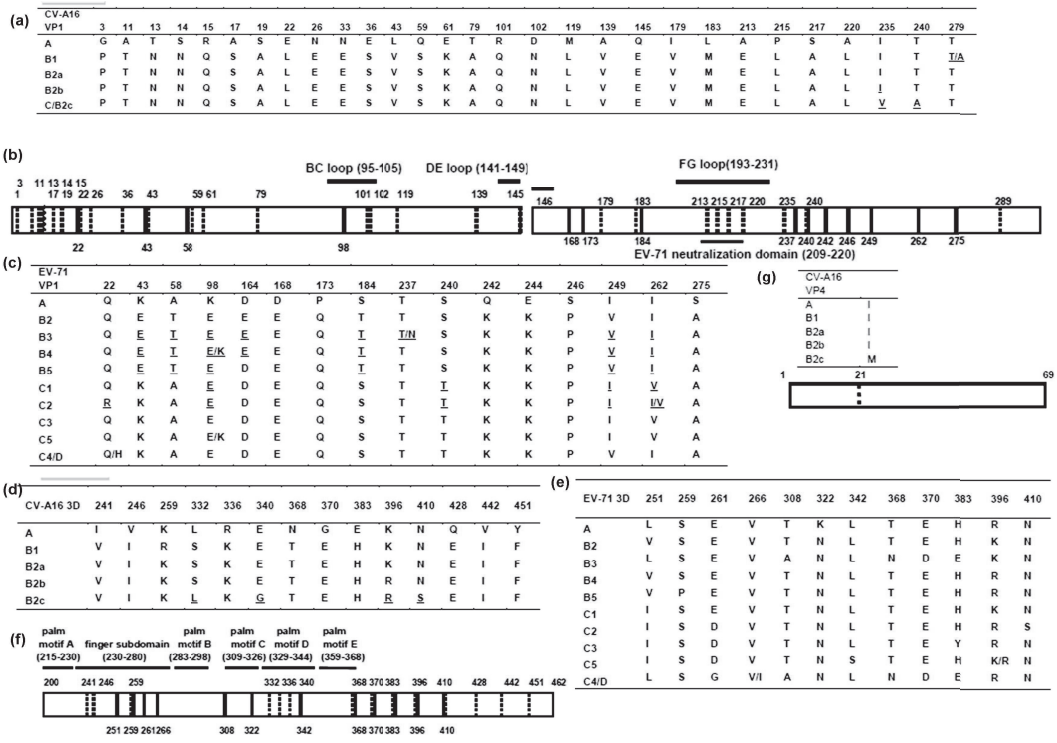

Figure 5. Genetic changes in EV-71 and CV-A16. Comparison of VP1 amino acid changes of CV-A16 (a) and EV-71 (c), and the graphical representation of VP1 showing the antigenic domains, neutralization epitopes and location of mutations (b). Comparison of 3D amino acid changes of CV-A16 (d) and EV-71 (e), and the graphical representation of 3D showing the functional domains and location of mutations (f). A single CV-A16 VP4 amino acid change was in position 21 (g). No amino acid changes were found in EV-71 at VP4 gene region. The solid lines represent mutations observed in CV-A16 and the dotted lines are mutations observed in EV-71. Amino acid changes observed in the present study are underlined

(Figure 5b). There were five amino acid differences between EV-71 and CV-A16 in the neutralization epitopes (209-220), which suggested that both EV-71 and CV-A16 have specific neutralization epitopes (Figure 5b).

Selective pressures on EV-71 and CV-A16 genes

It was of interest to understand the type of selective pressures acting on the Malaysian EV-71 and CV-A16 genes. No positive selection was detected. In EV-71 VP4, partial VP1 and partial 3D, 39% (29/69), 32.5% (89/274) and 53.7% (101/188) of the amino acids were under purifying selection, respectively. In CV-A16, VP4, VP1 and partial 3D, only 2.9% (2/69), 4.7% (14/297) and 7.6% (18/236) of the amino acids were under purifying selection, respectively. EV-71 showed strong purifying selection in all the three genes as compared to CV-A16.

DISCUSSION

The present study was aimed at integrating the molecular epidemiological information known about EV-71 and CV-A16 in Malaysia. Malaysia has experienced at least five HFMD outbreaks in the last ten years. Major fatal HFMD outbreaks occurred in 1997 and 2000, and were caused by EV-71. Other outbreaks occurred in 2003, 2006 and 2008/2009 (reviewed in Chan *et al.*, 2011; Podin *et al.*, 2006). Like many other studies, we found no associations between subgenotype and clinical severity (reviewed in Chan *et al.*, 2011). However, this could also be due to the

small number of fatal and severe EV-71 cases in this study.

Three genomic regions, VP4, VP1 and 3D were chosen for sequencing to provide further data on evolution of the viruses. VP4 is usually buried within and not likely to be under immune pressure. Interestingly, no amino acid mutations were observed in EV-71 and there was only one mutation in CV-A16. This highlights the importance of the functionally conserved VP4 for uncoating. VP1 is the most immunodominant protein and always under high immune selection. This study showed that many of the mutations were located at the loops that are highly immunogenic. 3D is the viral RNA polymerase, which is functionally conserved and provides evidence of recombination. In the 3D, the mutations were mainly located at the finger subdomain, palm motif C and D. It is likely that mutations alone may not be sufficient to confer different virulence, which may also be dependent on host factors and immune response.

VP1 and VP4 are the most commonly used gene regions for genotyping. We have previously showed that both VP1 and 3D phylogenies provided more accurate genotyping which better reflects genotyping with complete genomes, as compared to VP4 (Chan *et al.*, 2010). We have also observed that some isolates in VP4 were not clustered according to their genotypes as identified by VP1 in the present study.

Many EV-71 subgenotypes circulating worldwide are not present in Malaysia. After 2003, the circulating subgenotypes were C1 and C2 in Europe, C1, C2, C4/D and C5 in Thailand and C4/D in China (Chan *et al.*, 2011). China has recently reported the presence of subgenotype C2 at low levels (Tao *et al.*, 2012). Japan has reported frequent importations and exportations of EV-71 to and from neighbouring countries (Mizuta *et al.*, 2005). We only observed the presence of subgenotypes C5 and C4/D from a few previously sequenced isolates. This suggests that importation or exportation of our Malaysian isolates to/from other countries were not common or present at very low levels. A similar phenomenon was observed with CV-A16. Subgenotype B2c seen in Malaysia was not observed in China (Li *et al.*, 2005; Zhang *et al.*, 2010; Zong *et al.*, 2011).

It is unclear why there were many subgenotypes of EV-71 circulating in 1997, but later only subgenotype B5 emerged to become the sole currently circulating virus. The persistence of subgenotype B5 may be due to the presence of high levels of population immunity towards the previous genotypes. In contrast, Taiwan demonstrated many episodes of genotype displacements, from B1 in 1986, C2 in 1998, B4 in 2000 and 2001, C4 in 2004 and 2005, and finally B5 in 2008. Huang and colleagues (2009) used antigenic cartography mapping to demonstrate that subgenotype B5 was antigenically distinct from subgenotype B4 and C. The mechanism of genotype displacement may be due to low neutralization antibody levels against the new antigenic variant in that population.

Recombination is common in enteroviruses (Santti *et al.*, 1999) and has been reported in EV-71 and CV-A16 (Yoke-Fun & Abu Bakar, 2006; Zhao *et al.*, 2011). As in our previously published study, we have observed recombination in EV-71 isolates from subgenotype B3 (Yoke-Fun & Abu Bakar, 2006). These isolates clustered together with prototype CV-A16/G10 in the 3D region. This seems to correlate with our previous findings that all the current genotypes have emerged sometime ago through recombination with other prototype HEV-A. Apart from EV-71 subgenotype B3, no other EV-71 or CV-A16 isolates showed significant incongruence in the phylogeny. This may imply that recombination only plays a minor role in generating the genetic diversity in the circulating EV-71 and CV-A16. However, recombination that drives the diversification of EV-71 may actually render the virus less fit. Subgenotype B3 was only isolated in 1997 and not seen after that. We have also previously showed that recombinant B3 was less virulent in mice (Chan & Abu Bakar, 2005).

Genetic diversity serves as a way for viruses to adapt to changing environments. High genetic diversity was observed in the Malaysian EV-71, but diversity was lower in

CV-A16. High genetic diversity in EV-71 may allow the virus to increase fitness and evade host immune response. Low genetic diversity in CV-A16 may correlate with the low mutation rates. The average evolutionary rate of CV-A16 was only 0.91 X 10^{-2} nucleotide substitutions per year, compared to a rate of 1.35 X 10^{-2} in EV-71 (Brown *et al.*, 1998; Zhang *et al.*, 2010). In this study, the major driver of diversification for EV-71 and CV-A16 is likely to be purifying selection. Strong purifying selective pressures were observed in EV-71 but less so in CV-A16. CV-A16 has undergone very few amino acid changes, and very few sites were under purifying selection. As a consequence of the strong purifying selection, EV-71 subgenotypes B3 and C2 disappeared after the 1997 outbreak, and were followed by B4 and C1 later. Hence, purifying selection has resulted in extinction of these less-adapted variants. Strong purifying selection has also been found in human rhinovirus (Kristler *et al.*, 2007) and foot, mouth and disease virus (Carillo *et al.*, 2005). In both viruses, the purifying selection was strongest in 3D as this gene needs to be functionally conserved for virus replication.

There are some limitations in our study. Occurrence of recombination was not common in EV-71 and not obvious in CV-A16. This could be due to the short gene regions used. It was previously reported that recombination breakpoints occurred at the 2B gene region (Huang *et al.*, 2008; Zhao *et al.*, 2011), which was not sequenced in the study. Hence, the elucidation of recombination is best performed with the complete genome. We did not sequence all the isolates from the study period due to lack of funding, and may have missed isolates from some genotypes. We would also have missed very mild cases who did not attend the hospital. Nonetheless, our present study has helped us to understand the genetic diversity and evolutionary forces acting on VP4, VP1 and 3D of EV-71 and CV-A16 in Malaysia. With all the ongoing vaccine trials in Singapore, China and Taiwan, it is important to monitor the genetic mutations that may lead to antigenic changes and emergence of new subgenotypes in these two viruses.

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