Molecular characterization of enterovirus 71 and coxsackievirus A16 using the 5' untranslated region and VP1 region

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Enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) are members of the species *Human enterovirus A*, and are both major and independent aetiological agents of hand-foot-and-mouth disease. The human enterovirus (HEV) 5' untranslated region (UTR) is fundamentally important for efficient virus replication and for virulence, whilst the VP1 region correlates well with antigenic typing by neutralization, and can be used for virus identification and evolutionary studies. A comparison was undertaken of the 5'UTR and VP1 nucleotide sequences of five EV71 clinical isolates and 10 CVA16 clinical isolates from one laboratory with the 5'UTR and VP1 sequences of 104 EV71 strains and 45 CVA16 strains available in GenBank. The genetic relationships were analysed using standard phylogenetic methods. The EV71 phylogenetic analysis showed that the VP1 sequences were clustered into three genogroups, A, B and C, with genogroups B and C further divided into five subgenogroups, B1–B5 and C1–C5, respectively. All EV71 strains were clustered similarly in the 5'UTR and VP1 trees, except for one Taiwanese strain, which demonstrated different clustering in the two trees, suggesting a recombination event in the phylogeny. The CVA16 phylogenetic analysis showed that the VP1 sequences were clustered into two genogroups, A and B, with genogroup B further divided into B1 (B1a and B1b), B2 and a possible B3; and that a similar pattern and grouping of all strains were displayed in the 5'UTR tree. This study demonstrated that comparing the two regions provides evidence of epidemiological linkage of HEV-A strains, and that mutation in the two regions plays a vital role in the evolution of these viruses. The combination of molecular typing and phylogenetic sequence analysis will be beneficial in both individual patient diagnosis and public health measures.

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INTRODUCTION

Enterovirus 71 (EV71) and coxsackievirus A16 (CVA16), members of the species *Human enterovirus A* in the genus *Enterovirus*, family *Picornaviridae*, are both major and independent aetiological agents of large outbreaks of hand-foot-and-mouth disease (HFMD). In contrast to CVA16, EV71-associated HFMD is more frequently associated with

serious neurological complications and fatalities (McMinn *et al.*, 2001b). Initial EV71 isolates were identified in the USA and Australia in the early 1970s, and in HFMD outbreaks in Sweden and Japan (Wong *et al.*, 2010). Since 1997, the Asia-Pacific region has had several large EV71 epidemics, including in the Chinese mainland, Taiwan, Singapore, Malaysia and Japan. Several outbreaks have also been recorded in Australia (Gilbert *et al.*, 1988; Sanders *et al.*, 2006; McMinn *et al.*, 2001b). Co-circulation of EV71 and CVA16 has contributed to serious HFMD outbreaks in China in 2007 (Zhang *et al.*, 2009), Taiwan in 1998 (Lin *et al.*, 2003), and Malaysia in 1997, 2000 and 2003 (Podin *et al.*, 2006).

The enterovirus genome is a positive ssRNA molecule of approximately 7500 nt, comprising a single ORF flanked 5'

Abbreviations: CVA16, coxsackievirus A16; EV71, enterovirus 71; HEV, human enterovirus; HFMD, hand-foot-and-mouth disease; PV, poliovirus; UTR, untranslated region.

The GenBank/EMBL/DDBJ accession numbers for the 5'UTR sequences of EV71 and CVA16 are GU236109–GU236113 and GU236100–GU236108, respectively; and for the VP1 sequences are FJ868281 and GU142870–GU142873, and FJ868280, GU142867–GU142869 and GU232771–GU232775, respectively.

and 3' by untranslated regions (UTRs). In the 5'UTR, the cloverleaf structure of the domain (stem-loop) I is important for virus replication (Bailey & Tappich, 2007), whilst domains II–VI encompass the internal ribosome entry site, which directs translation of the mRNA by internal ribosome binding (Fernández-Miragall *et al.*, 2009). The 5'UTR is relatively conserved at the nucleotide level and is often used in nucleic acid testing to diagnose enterovirus infections (Romero, 1999; Thoelen *et al.*, 2003). However, numerous examples have shown that mutations in the 5'UTR markedly decrease multiplication efficiency (Willian *et al.*, 2000), alter cell tropism (Shiroki *et al.*, 1997) and attenuate virulence (Tu *et al.*, 1995; DeJesus *et al.*, 2005). Moreover, recombination can occur in the 5'UTR (Adu *et al.*, 2007; Yang *et al.*, 2003).

The enterovirus coding region, divided into three sub-regions (P1–P3), encompasses a single ORF encoding a single polyprotein. The P1 region encodes four structural proteins (VP4, VP2, VP3 and VP1), whilst the non-structural proteins are encoded in the P2 (2A, 2B and 2C) and P3 (3A, 3B, 3C and 3D polymerase) regions. VP1, VP2 and VP3 are located at the viral capsid surface and are exposed to immune pressure, whereas VP4 is internal (Muir *et al.*, 1998). The VP1 capsid protein is the most external and immunodominant of the picornavirus capsid proteins (Rossmann *et al.*, 1985) and contains the most neutralization epitopes.

Human enterovirus (HEV) serotypes are classified into four species, *Human enterovirus A*, *B*, *C* and *D*, based on genome organization, sequence similarity and biological properties (Pallansch & Roos, 2007). EV71 and CVA16 belong to *Human enterovirus A*. VP1 sequences correlate well with antigenic typing by neutralization, and can be used for virus identification and evolutionary studies (Oberste *et al.*, 1999, 2004). Molecular typing methods depend largely on RT-PCR amplification and nucleotide sequencing of the entire or 3' half of the VP1 gene (Pallansch & Roos, 2007). Comparison of individual VP1 sequences with databases of VP1 sequences of HEV prototype and variant strains allows genotype assignment and identification of new enteroviruses (Oberste *et al.*, 2004).

The 20 most frequently identified HEV serotypes in our laboratory between 1979 and 2007 included EV71 and CVA16. A total of 91 EV71 and 177 CVA16 clinical isolates were identified during this period (Zhou *et al.*, 2009). In addition, 3.6% (227/6383) of HEVs were non-serotypable (not identifiable by standard neutralization procedures), and 9/92 non-serotypable clinical isolates were genotyped as EV71 or CVA16 (Zhou *et al.*, 2010). To investigate the molecular features of EV71 and CVA16, we compared the 5'UTR and VP1 regions of five EV71 and 10 CVA16 clinical isolates in our laboratory ('local isolates') with the 5'UTR and VP1 regions of 104 EV71 strains and 45 CVA16 strains available in GenBank.

METHODS

Viruses. Our laboratory database contains 6383 HEV isolates collected between 1979 and 2007, including 227 non-serotypable isolates (Zhou *et al.*, 2010). Viruses were stored as unpurified cell culture supernatants at -70°C . There were five EV71 local isolates (including four non-serotypable isolates; Zhou *et al.*, 2010) and 10 CVA16 local isolates (including three non-serotypable isolates; Zhou *et al.*, 2010) available for analysis (Table 1).

PCR and sequencing primers for 5'UTR and VP1. To design HEV-specific 5'UTR PCR and sequencing primers, the 5'UTR sequences of 83 HEV prototype strains (Pallansch & Roos, 2007) (not available for EV78 and EV96) plus EV92 (GenBank accession no. EF667344), EV94 (DQ916376), EV98 (AB426608), EV107 (AB426609), poliovirus (PV) Sabin 1 strain (V01150), PV Sabin 2 strain (X00595) and PV Sabin 3 strain (X00925) were aligned using CLUSTAL W (accurate) provided on Biomanager (<http://biomanager.info>). This allowed the design, in highly conserved regions of the 5'UTR, of two PCR amplification primers (5UTR-S and 5UTR-A1) and two sequencing primers (5UTR-S and 5UTR-A2) to cover all known HEV serotypes (Table 2). Primer sequences were evaluated using the Sigma DNA Calculator (<http://www.sigma-genosys.com/calc/dnalc.asp>) and synthesized by Sigma-Aldrich.

Published primers 423–426, 480–483 and 486–489 for PCR amplification and/or sequencing of the complete VP1 were obtained (Oberste *et al.*, 2006), as well as PCR amplification and sequencing primers 292 and 222 for analysis of partial VP1 sequences (Oberste *et al.*, 2006).

RT-PCR for 5'UTR and VP1. Reverse transcription was performed as described previously (Zhou *et al.*, 2009). HEV-specific 5'UTR PCR (using primers 5UTR-S and 5UTR-A1, Table 2) for all 15 local HEV isolates and partial VP1 PCR (using primers 292 and 222) for five CVA16 isolates was performed using a HotStarTaq DNA polymerase kit (Qiagen) according to the manufacturer's instructions with the following conditions: 95°C for 15 min; 35 cycles of 94°C for 30 s, 42°C for 30 s and 72°C for 1 min; 72°C for 10 min; and a hold at 22°C . For the HEV-specific 5'UTR PCR, the PCR mixture contained: 2 μl template cDNA, 0.3 μl each primer 5UTR-S (100 pmol μl^{-1}) and primer 5UTR-A1 (100 pmol μl^{-1}), 1 μl dNTPs (2.5 mM each dNTP), 2.5 μl $10\times$ PCR buffer, 0.2 μl Qiagen HotStarTaq DNA polymerase (5 U μl^{-1}) and water to 25 μl . For the partial VP1 PCR, the PCR mixture contained: 2 μl template cDNA, 0.3 μl each primer 292 (100 pmol μl^{-1}) and primer 222 (100 pmol μl^{-1}), 1 μl dNTPs (2.5 mM each dNTP), 2.5 μl $10\times$ PCR buffer, 0.2 μl Qiagen HotStarTaq DNA polymerase (5 U μl^{-1}) and water to 25 μl . The methods for complete VP1 PCR for the five EV71 isolates and five CVA16 isolates were as described previously (Zhou *et al.*, 2009).

Sequencing and analysis for 5'UTR and VP1. The sequencing methods have been described previously (Zhou *et al.*, 2009). For 5'UTR sequencing of all 15 local isolates, primers 5UTR-S and 5UTR-A2 (Table 2) were used; for partial sequencing of VP1 of five CVA16 isolates, primers 292 and 222 were employed. The complete VP1 gene for each of the five EV71 isolates and five CVA16 isolates was sequenced in two fragments using primers 486–489, followed by assembly of the fragment sequences. The previously tested serotype for each of eight serotyped isolates (one EV71 isolate and seven CVA16 isolates; Table 1) or the genotype for each of seven non-serotypable isolates (four EV71 and three CVA16 isolates; Table 1) was confirmed by pairwise comparison of the complete or partial VP1 sequence with a database containing VP1 sequences for the prototype and variant strains of all known HEV serotypes (Oberste *et al.*, 2004).

Table 1. Details of the 15 HEV local isolates used for analysis of 5'UTR and VP1 regions

Serotype or genotype	Short name in Fig. 1 or Fig. 2	Laboratory identifier	Date of isolation (year)	Specimen type	Clinical association	Genogroup/subgenogroup	5'UTR GenBank accession no.	VP1 GenBank accession no.
EV71	#1-EV71	98.163.0453*	1998	Stool	Meningitis	C2	GU236109	GU142871
	#2-EV71	98.338.1853*	1998	Throat	Meningitis	C2	GU236110	GU142872
	#3-EV71	99.040.0971*	1999	Throat	Meningitis	C2	GU236111	GU142870
	#4-EV71	95.318.0855*	1995	Throat	HFMD	C2	GU236112	GU142873
	#5-EV71	20.2.04	2004	Stool	HFMD	C1	GU236113	FJ868281
CVA16	#1-CVA16	00.108.3206*	2000	Stool	HFMD	B1a	GU236100	GU142867
	#2-CVA16	00.143.2668*	2000	Vesicle	HFMD	B1a	GU236101	GU142868
	#3-CVA16	05.194.4135	2005	Vesicle	HFMD	B1b	GU236102	FJ868280
	#4-CVA16	05.210.2819	2005	Vesicle	HFMD	B1b	GU236103	GU232772
	#5-CVA16	05.354.3788	2005	Mouth	HFMD	B1b	GU236104	GU232773
	#6-CVA16	05.158.4065	2005	Vesicle	HFMD	B1b	GU236105	GU232771
	#7-CVA16	06.103.3121	2006	Vesicle	HFMD	B1b	GU236106	GU232774
	#8-CVA16	99.132.2264*	1999	Vesicle	HFMD	B1a	GU236107	GU142869
	#9-CVA16	62918.89	1989	Mouth	HFMD	B3	GU236108	GU232775
	#10-CVA16	05.194.4166†	2005	Vesicle	HFMD	B1b	–	–

*These seven HEV local isolates were non-serotypable (Zhou *et al.*, 2010).

†The 5'UTR and VP1 nucleotide sequences of this CVA16 isolate (05.194.4166) were 100% identical to those of another CVA16 isolate (05.194.4135).

Comparison with GenBank database sequences. The 5'UTR and VP1 regions of the five local EV71 isolates and 10 CVA16 isolates were compared with the 5'UTR and VP1 regions of 104 EV71 strains (Fig. 1) and 45 CVA16 strains (Fig. 2) available in GenBank. The length for each of these 149 GenBank sequences was at least 3300 nt, including both 5'UTR and VP1 regions. Sequences were trimmed and downloaded separately into two databases for the 5'UTR and VP1 using Biomanager. Most of the GenBank sequences represented strains isolated from disease outbreaks from different locations over time, and a small number were laboratory-modified strains (Chua *et al.*, 2008). Where available, further information (including isolation dates and countries of origin) that could not be found directly from GenBank was obtained from the associated references.

Phylogenetic analysis of 5'UTR and VP1. Alignment of all 5'UTR and VP1 nucleotide sequences was undertaken intraserotypically for EV71 or CVA16 using the CLUSTAL W (accurate) program on Biomanager. Phylogenetic trees of the 5'UTR and VP1 regions for EV71 or CVA16 were constructed by neighbour joining using the nucleotide/Kimura two-parameter method with MEGA, version 4.1

(Kumar *et al.*, 2008). Bootstrap analysis with 1000 pseudoreplicates provided an estimate of reliability for phylogenetic reconstructions. The bootstrap values in 1000 pseudoreplicates within trees are shown as percentages.

RESULTS

Sequencing results for the 5'UTR and VP1

The length of the 5'UTR sequences for the 15 local isolates ranged between 495 and 498 nt, covering the relatively conserved major part of the internal ribosome entry site. The relatively long 5'UTR sequences amplified by one consensus primer pair (5UTR-S and 5UTR-A1) provided a more accurate and systematic interpretation for the 5'UTR.

The deduced amino acid sequences from the partial or complete VP1 sequences included the B–C loop, a region

Table 2. Oligonucleotide primers for PCR amplification and sequencing of 5'UTR

Primer*	Target	T _m (°C)	GenBank accession no.	Sequence (5'→3')†
5UTR-S‡§	5'UTR	58.62	U22521 (EV71)	⁶⁵ GRNAYYYTTGKRNGCCTGTTT ⁸⁶
5UTR-A1‡	5'UTR	61.39	U22521 (EV71)	⁶⁰¹ TYGTCACCATWWGCAGYCA ⁵⁸³
5UTR-A2§	5'UTR	64.03	U22521 (EV71)	⁵⁵⁵ CCCAAAGTASTCGGTTCCG ⁵³⁷

*S, Sense; A, antisense.

†R, A or G; W, A or T; S, C or G; Y, C or T; K, G or T; N, A, C, G or T. Numbers represent the base positions at which primer sequences start and finish, starting at position 1 of the corresponding GenBank sequence.

‡Primers 5UTR-S and 5UTR-A1 were used for PCR amplification of the 5'UTR.

§Primers 5UTR-S and 5UTR-A2 were used for sequencing of the 5'UTR.

Fig. 1. Phylogenetic trees showing the genetic relationships among 107 EV71 strains based on alignment of the partial 5'UTR (496 nt) (a) and complete VP1 (891 nt) (b) sequences. This included five local isolates (short names #1-EV71 to #5-EV71, labelled 'a' and highlighted with arrows, with details provided in Table 1) and four other Australian strains (labelled 'b') whose sequences have already been deposited in GenBank. Trees were constructed by neighbour joining using the nucleotide/Kimura two-parameter method with MEGA, version 4.1 (Kumar *et al.*, 2008). Bootstrap values (percentage of 1000 pseudoreplicate datasets) of $\geq 75\%$ supporting each cluster are shown at the nodes. The scale bars represent the genetic distance. The CVA16 prototype strain (G-10) was included as an outgroup for the 5'UTR and VP1 trees. The corresponding VP1 genogroups

or subgenogroups are indicated. Subgenogroups B4, B5 and C4 are compressed into triangles to save space. Eight strains isolated in Malaysia, Singapore or Taiwan from 1997 to 2002 belonged to B4. B5 consisted of five strains from Malaysia, Singapore or Taiwan isolated from 2000 to 2008. C4 included 48 strains isolated between 1998 and 2009 from the Chinese mainland, in addition to four Taiwanese strains isolated between 2002 and 2004. Strain names indicate the GenBank accession number, country or area, and year of isolation. AUS, Australia; JAP, Japan; MAL, Malaysia; SK, South Korea; SA, South Africa; SIN, Singapore; NOR, Norway; TW, Taiwan. A '?' indicates that the year of isolation is unknown.

needed for type-specific antibody reactivity (Norder *et al.*, 2003). The VP1 genotypes of all eight serotyped isolates corresponded with their respective serotypes; for the seven non-serotypable isolates, genotypes were determined by VP1 sequencing (Table 1).

Clinical features and genogroups/subgenogroups

Clinical features and the genogroups/subgenogroups of the 15 local EV71 or CVA16 isolates are presented in Table 1. Of the five EV71 local isolates from stool or throat specimens, three were from cases with severe neurological disease (meningitis) and all were subgenogroup C2. Ten CVA16 local isolates, cultured from stool, mouth or vesicles, were all from patients with HFMD (three subgenogroup B1a, six B1b and one B3). All 15 local isolates were from children between 1 and 8 years old (data not shown).

Phylogenetic analysis for EV71

In the EV71 VP1 tree (Fig. 1b), VP1 sequences were clustered into three genogroups, A, B and C. Genogroup A contained the EV71 prototype strain (BrCr) (GenBank accession no. U22521) and differed from all others by 16.0–19.1%. Genogroup B strains differed from those of genogroup C by 11.9–19.2%. Genogroups B and C were each classified further into five subgenogroups. Of the five local isolates (labelled 'a'), one (#5-EV71) from 2004 was identified as subgenogroup C1, whilst the other four (#1-EV71 to #4-EV71) isolated between 1995 and 1999 were identified as C2. Of another four Australian strains already in GenBank (labelled 'b'), one (GenBank accession no. EU364841) (Chua *et al.*, 2008) was identified as B3, one (GenBank accession no. DQ341361) as C1 and the other two (GenBank accession nos DQ341357 and DQ381846) as C2.

Genogroup C dominated the VP1 tree. Subgenogroup C1 included three Malaysian strains, one local isolate, another Australian strain and one Norwegian strain obtained from an asymptomatic child (Witsø *et al.*, 2007), differing by 2.4–7% within the cluster. Three main clusters within C2, supported by high bootstrap values, were evident on the VP1 dendrogram. The first C2 cluster (bootstrap value 100%) was made up of Taiwanese strains from 1998. Another branch separated the other sequences into two more C2 clusters. One cluster showed a linkage between six Australian isolates (including four local isolates) from the years 1995–1999 and two Malaysian strains from 1997, with nucleotide identities within this cluster of between

97.1 and 99.7%. The third C2 cluster contained two recent Singaporean strains. The largest subgenogroup was C4, which included 19 strains isolated between 1998 and 2004 (Li *et al.*, 2005) and 29 strains isolated in 2008 or 2009 (Ding *et al.*, 2009) from the Chinese mainland, in addition to four Taiwanese strains isolated between 2002 and 2004 (data not shown). This suggested that C4 has predominantly been responsible for EV71 infections on the Chinese mainland for over 10 years (Yang *et al.*, 2009).

The EV71 5'UTR phylogenetic tree (Fig. 1a) illustrated the same classification as the VP1 tree (i.e. genogroup A, subgenogroups B1–B5 and C1–C5), although the relative positions of some subgenogroups were exchanged. All strains were clustered into their respective genogroups or subgenogroups except for one strain (AF119795-TW-1998, labelled 'c') when compared with the VP1 tree. This strain, which was assigned to genogroup B in the VP1 analysis, was clustered with subgenogroup C2 in the 5'UTR analysis. A few strains exhibited shifts in branch positions within subgenogroups.

Phylogenetic analysis for CVA16

The two dendrograms in Fig. 2 were composed primarily of Chinese strains and our local Australian isolates. The CVA16 VP1 tree (Fig. 2b) demonstrated two distinct genogroups, A and B. Surprisingly, genogroup A included not only the CVA16 prototype strain (G-10) (GenBank accession no. U05876) but also another recent Chinese strain (EU812514-CHN-2008, labelled 'b'). Comparison of these two complete VP1 nucleotide and deduced amino acid sequences showed 99.4% (886/891 nt) and 98.7% (293/297 aa) identity, respectively. These two genogroup A strains had 20.4–24.7% nucleotide difference relative to other CVA16 strains, but the genetic variation among all other CVA16 strains was less than 13.7%.

Genogroup B was classified into subgenogroups B1, B2 and possibly B3. The largest subgenogroup was B1, which could be further divided into B1a and B1b. B1a comprised Chinese strains from 1999 to 2008, as well as three local isolates (labelled 'a') from 1999 to 2000. In contrast, B1b was made up of six local isolates (labelled 'a') collected from 2005 to 2006, although a Chinese strain isolated in 2004 was also present. The subgenogroup B2 included four Chinese strains isolated in 1999 or 2000. Only one local isolate (#9-CVA16, labelled 'a') belonged to the possible B3 subgenogroup, supported by a high bootstrap value (99%). The nucleotide divergence between B3 and the other two subgenogroups (B1 and B2) was 9.0–13.4%.

Fig. 2. Phylogenetic trees showing the genetic relationships among 55 CVA16 strains [including 10 local isolates (short names #1-CVA16 to #10-CVA16, labelled 'a' and highlighted with arrows, with details provided in Table 1)] based on alignment of partial 5'UTR (498 nt) (a) and partial VP1 (301 nt) (b) sequences. Trees were constructed by neighbour joining using the nucleotide/Kimura two-parameter method with MEGA, version 4.1 (Kumar *et al.*, 2008). Bootstrap values (percentage of 1000 pseudoreplicate datasets) of $\geq 75\%$ supporting each cluster are shown at the nodes. The scale bars represent the genetic distance. The EV71 prototype strain (BrCr) was included as an outgroup for the 5'UTR and VP1 trees. The corresponding VP1 genogroups or subgenogroups are indicated. Strain names indicate the GenBank accession number, country or area, and year of isolation. CHN, Chinese mainland; SA, South Africa.

In general, a similar pattern and grouping of 55 CVA16 strains were displayed in the 5'UTR tree (Fig. 2a) as seen in the VP1 tree. All strains in the 5'UTR tree were clustered into their respective genogroup or subgenogroup, although some strains exhibited exchanges in branch positions within subgenogroups (especially evident for B1a strains). Likewise, the local isolate (#9-CVA16, labelled 'a') also formed the possible B3 subgenogroup, comparable with the finding for VP1. The recent Chinese strain (EU812514-CHN-2008, labelled 'b') still belonged to genogroup A, together with the CVA16 prototype strain (G-10). Comparison of the two complete 5'UTR nucleotide sequences showed 99.7% (749/751 nt) identity. Furthermore, comparison of complete genomic nucleotide sequences for these two strains demonstrated 99.8% (7401/7414 nt) identity.

DISCUSSION

To understand the molecular epidemiology and evolution of EV71, several distinct regions of the EV71 genome have been employed for analysis, such as the 5'UTR (AbuBakar *et al.*, 1999), 3D polymerase region (Bible *et al.*, 2008), VP4 region (Chu *et al.*, 2001) and VP1 region (Brown *et al.*, 1999; McMinn *et al.*, 2001a; Tee *et al.*, 2010). However, the VP1 gene is considered to be the most informative and robust region for evolutionary study due to a high degree of diversity and lack of involvement in recombination (Bible *et al.*, 2007). To date, three EV71 genogroups (A, B and C) have been described (Brown *et al.*, 1999), with genogroups B and C further divided into five subgenogroups, B1–B5 and C1–C5, respectively. An epidemiological study of EV71 infections in The Netherlands from 1963 to 2008 demonstrated a new subgenogroup B0 (van der Sanden *et al.*, 2009). More recently, the analysis of complete genome sequences of EV71 has suggested that subgenogroup C4 should be classified as a new genotype, D (Chan *et al.*, 2010).

Analysis of the EV71 VP1 tree showed three genogroups, A, B and C (Fig. 1). The 5'UTR tree demonstrated the same classification as the VP1 tree. This generally corresponded with an earlier study, which showed that the analysis of all available EV71 sequences of the 5'UTR V, VI and VII domains correlated with the VP1 subgenogroup clustering (Witsø *et al.*, 2007). Australian EV71 C2 strains have been detected since 1995 (Brown *et al.*, 1999), as reflected by the 1995 local strain (#4-EV71, labelled 'a') in subgenogroup C2. Additionally, two local strains (#1-EV71 and #2-EV71, labelled 'a') were isolated in 1998 when C2 strains were causing a large outbreak in Taiwan (Shih *et al.*, 2000). One year later (1999), an outbreak involving C2 and B3 strains occurred in Perth, Australia (McMinn, 2002). The three Australian C2 strains (#3-EV71, labelled 'a', and two GenBank strains, labelled 'b') and one B3 strain (labelled 'b') isolated in 1999 were probably the representatives of the 1999 outbreak.

EV71 intraserotypic and interserotypic recombination have been reported (Chan & AbuBakar, 2004; Bible *et al.*, 2008; Chen *et al.*, 2010). Recombination would be facilitated by the presence of several subgenogroups circulating during the same period in one country or region, such as the co-circulation of the four subgenogroups B3, B4, C1 and C2 in Malaysia between 1997 and 2000 (Herrero *et al.*, 2003) (Fig. 1). All EV71 strains were clustered similarly in the 5'UTR tree and the VP1 tree; thus, no hints of recombination were found. However, we observed a significant incongruence between the 5'UTR and VP1 trees regarding the Taiwanese strain (AF119795-TW-1998, labelled 'c' in Fig. 1), which was assigned to genogroup B in the VP1, but clustered with subgenogroup C2 in the 5'UTR. This observation agrees with another study, where this strain was grouped as genogroup B based on VP1 analysis, but was repositioned to subgenogroup C2 in the 3D polymerase region (Bible *et al.*, 2008). Analysis of the full-length genome of this strain using the similarity plot method, as well as other C2 and B sequences, indicated that it resulted from intraserotypic recombination (Bible *et al.*, 2008). The analysis of EV71 based on the VP1 region, in combination with the 5'UTR in the current study or with the 3D polymerase region (Bible *et al.*, 2008), could provide more objective information on the molecular epidemiology and evolution of EV71, including the demonstration of possible recombination. Sequencing multiple regions of circulating strains would be required to characterize EV71 in more detail.

The overall rapid evolutionary rate of the EV71 VP1 gene has been estimated to be 4.2×10^{-3} and 3.4×10^{-3} (Brown *et al.*, 1999) or $4.5\text{--}4.6 \times 10^{-3}$ and 4.2×10^{-3} (Tee *et al.*, 2010), all substitutions per nucleotide per year, for genogroups B and C, respectively. Of nine Australian strains (five local strains, labelled 'a', and four GenBank strains, labelled 'b', in Fig. 1), only one C2 GenBank strain (DQ381846) possessed the Ala→Val substitution at VP1 position 170, which appears to be associated with increased neurovirulence (McMinn *et al.*, 2001a). In addition, the nucleotide mutation (A→G) at position 485 in the 5'UTR may be one of the molecular determinants of EV71 attenuation of neurovirulence in cynomolgus monkeys (Arita *et al.*, 2005). However, we found that the nucleotide at this position was a highly conserved A in all five EV71 local isolates (identical to the virulent EV71 prototype BrCr strain), although the five patients had different clinical features (three cases were meningitis and two had HFMD; Table 1). This finding corresponded with the results of another study (Ortner *et al.*, 2009).

In contrast to the extensive analysis of EV71 worldwide, little attention has been paid to the molecular evolution of CVA16. This is reflected in the shortage of complete CVA16 nucleotide sequences in GenBank from countries other than China. This may be due partly to most CVA16-associated disease being mild (Li *et al.*, 2005). The VP1 and VP4 genes are used mostly for classifying CVA16 (Perera *et al.*, 2007; Li *et al.*, 2005; Iwai *et al.*, 2009). It has been

classified on VP1 analysis into genogroups A and B, with genogroup B further divided into subgenogroups B1 (including B1a, B1b and possibly B1c) and B2 (Zhang *et al.*, 2010).

This study noted that all CVA16 strains were clustered into genogroups A and B in VP1; however, genogroup B could be further divided into B1 (B1a and B1b), B2 and a possible B3 (Fig. 2). The pattern and grouping of the 55 CVA16 strains in the 5'UTR and VP1 trees were generally concordant. Molecular analysis of VP1, combined with the 5'UTR, may enhance the reliability of molecular surveillance and our understanding of the genetic evolution of CVA16. For instance, both VP1 and 5'UTR analysis suggested the possible new subgenogroup B3, although more examples in addition to this single local strain (#9-CVA16, labelled 'a') need to be identified.

In addition, the recent strain EU812514-CHN-2008 (labelled 'b' in Fig. 2) belonged to genogroup A on both the VP1 and 5'UTR dendrograms, together with CVA16 prototype strain G-10. Comparison of the two complete VP1 and 5'UTR sequences showed 99.4 and 99.7% nucleotide identity, respectively. Further comparison of complete genomic nucleotide sequences for these two strains demonstrated 99.8% identity. The mean evolutionary rate calculated for CVA16 B1a and B2 was 0.91×10^{-2} synonymous substitutions per nucleotide per year based on the VP1 gene (Zhang *et al.*, 2010), which is slightly lower than the value of 1.35×10^{-2} calculated for EV71 (Brown *et al.*, 1999). The evolutionary rate of CVA16 is relatively slow given that the prototype CVA16 strain was first identified in South Africa in 1951, and that all other CVA16 strains have formed the single genogroup B after approximately 60 years of evolution (Zhang *et al.*, 2010). Whilst this recent virus (EU812514-CHN-2008) and the CVA16 prototype strain were isolated 57 years apart, they display an anomalous similarity. This is surprising because enteroviruses display a high evolutionary rate; a laboratory contamination may have occurred. Additional investigations (for example, analysis of more strains taken from the surrounding area during the same period) would be needed to elucidate this observation.

Molecular typing approaches allow the rapid and accurate identification of HEVs. Nucleotide sequencing of the VP1 gene is especially useful for the identification of non-serotypable isolates. Phylogenetic analysis of sequences is the best method to discriminate between variants within a serotype, to confirm the linkage of isolates during an outbreak and to study the evolution of a given serotype or among different serotypes. A combination of molecular typing and phylogenetic sequence analysis will assist with both individual patient diagnosis and public health measures. The genetic comparison of different HEV genes (such as the 5'UTR and VP1) enhances the epidemiological linkage of HEV strains, as shown in Figs 1 and 2.

The evolution of HEVs occurs through genetic drift and, over much longer periods, antigenic diversification in the

structural gene region encoding the virus capsid (including VP1) (Brown *et al.*, 1999; Martin *et al.*, 2000). Mutation during chain elongation and lack of 3'→5' exonuclease proofreading ability in RNA polymerases can cause very high error rates; there is a spontaneous mutation rate of approximately one mutation per genome per replication (Drake & Holland, 1999). The evolutionary diversity of HEVs is also due to intraserotypic or interserotypic recombination between the structural and non-structural coding regions and the 5'UTR (Chevaliez *et al.*, 2004; Lukashev *et al.*, 2003; Simmonds & Welch, 2006).

In conclusion, the sequence analyses of the 5'UTR and VP1 regions of 109 EV71 strains and 55 CVA16 strains belonging to the species *Human enterovirus A* demonstrated that comparing the two regions provides evidence of epidemiological linkage of HEV-A strains, and mutation in the two regions plays a vital role in their evolution. The combination of molecular typing and phylogenetic sequence analysis will assist with both individual patient diagnosis and public health measures.

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