Arch Virol (2007) 152: 1201–1208
DOI 10.1007/s00705-006-0934-5
Printed in the Netherlands

Archives of Virology

Brief Report

Molecular phylogeny of modern coxsackievirus A16

D. Perera[1], **M. A. Yusof**[1,2], **Y. Podin**[1], **M. H. Ooi**[1,3], **N. T. T. Thao**[4], **K. K. Wong**[5], **A. Zaki**[6], **K. B. Chua**[7], **Y. A. Malik**[5,8], **P. V. Tu**[4], **N. T. K. Tien**[4], **P. Puthavathana**[9], **P. C. McMinn**[10], and **M. J. Cardosa**[1]

[1] Institute of Health and Community Medicine, Universiti Malaysia Sarawak, Sarawak, Malaysia
[2] Institute for Medical Research, Kuala Lumpur, Malaysia
[3] Sibu Hospital, Sarawak, Malaysia
[4] Pasteur Institute, Ho Chi Minh City, Vietnam
[5] Faculty of Medicine, Universiti Kebangsaan Malaysia, Malaysia
[6] Dr. Fakeeh Hospital, Jeddah, Saudi Arabia
[7] National Public Health Laboratory, Sungai Buloh, Selangor, Malaysia
[8] International Medical University, Kuala Lumpur, Malaysia
[9] Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[10] Telethon Institute for Child Health Research, Perth, Western Australia

Received November 14, 2006; accepted December 21, 2006; published online February 19, 2007

Summary

A phylogenetic analysis of VP1 and VP4 nucleotide sequences of 52 recent CVA16 strains demonstrated two distinct CVA16 genogroups, A and B, with the prototype strain being the only member of genogroup A. CVA16 G-10, the prototype strain, showed a nucleotide difference of 27.7–30.2% and 19.9–25.2% in VP1 and VP4, respectively, in relation to other CVA16 strains, which formed two separate lineages in genogroup B with nucleotide variation of less than 13.4% and less than 16.3% in VP1 and VP4, respectively. Lineage 1 strains circulating before 2000 were later displaced by lineage 2 strains.

Author's address: Mary Jane Cardosa, Institute of Health and Community Medicine, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia. e-mail: jane.cardosa@gmail.com

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Hand, foot, and mouth disease (HFMD) is a common febrile illness of children associated with infections of species A enteroviruses from the genus *Enterovirus* within the family *Picornaviridae*. Lesions on the skin and oral mucosa typically characterize the illness, with herpangina also presented in some patients. Several enterovirus serotypes have been associated with this disease, the majority of these being members of human enterovirus A, such as coxsackieviruses (CV) A2, A4, A5, A8, A10, A16 and human enterovirus (HEV) 71 [17, 20, 15]. Of these, CVA16 and HEV71 are the major causative agents associated with HFMD, and co-circulation of both of these serotypes during outbreaks of HFMD

has been described in Malaysia, Taiwan, and China [17, 8, 9]. Although both viruses appear to co-circulate temporally and geographically, recent HFMD outbreaks in the Asia–Pacific region associated with neurological complications and a large number of fatalities have been attributed largely to HEV71 [8, 9, 4]. In contrast, CVA16-associated HFMD has a milder outcome, with much lower incidence of complications [5].

Phylogenetic clustering based on the VP1 (891 bp) genome region of HEV71 has been used to describe different genotypes of the virus [18, 3, 11, 2]. Previous research has shown a strong correlation between serotype identity and VP1 sequences of human enteroviruses [12], and as such, VP1 nucleotide sequences have proven useful in describing different genogroups of HEV71 strains. Such classifications have proven useful in tracking HEV71-associated HFMD genotypes over different temporal and geographical outbreaks [3]. A study by Cardosa et al. also showed that the much smaller VP4 gene (207 bp) could be used to quickly identify HEV71 genogroups during an outbreak, and these genogroups were verified by phylogenetic analysis of the VP1 gene as well [3]. Recently, an attempt to classify CVA16 strains was reported [8] using VP4-based phylogenetic clusterings of several Chinese CVA16 sequences and GenBank deposits. The authors showed three different major clusters that they called lineages A, B, and C. A corresponding analysis based on VP1 was hampered due to the lack of VP1 sequences available in GenBank.

The aim of this study was to present comparative phylogenetic analyses of both VP1 and VP4 nucleotide sequences of recent CVA16 strains and to determine their relationships to nucleotide sequences of earlier strains published in GenBank for which only VP4 sequences were available. To do this, we determined the VP1 and VP4 nucleotide sequences of 52 CVA16 strains isolated between 1997 and 2006 from 5 different countries. The phylogenetic relationships of both VP1 and VP4 nucleotide sequences generated in this study and others obtained from GenBank were determined. Our study showed that CVA16 strains fall into two genetic clusters that we have called genogroups A and B.

All CVA16 strains characterised in this study were isolated from stool, throat, vesicle or oral swabs of HFMD patients. These viruses were from different geographical locations and include isolates from Malaysia (Peninsular Malaysia as well as the state of Sarawak on the island of Borneo), South Vietnam, Western Australia, Saudi Arabia and Thailand (Table 1). Viruses were propagated in rhabdomyosarcoma (RD) or Vero cells using conventional cell culture methods. Extraction of total RNA from supernatant of infected cell cultures were performed using either the TRI REAGENTS-LS (Molecular Research Centre Inc.) or High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's instructions. RNA was extracted from 200-µl supernatants of infected cell cultures. RT-PCR of the VP4 gene was performed as previously described [7]. The complete VP1 gene was amplified by RT-PCR using the sense primer 051 (5′-TSAARYTGT GCAARGACAC-3′) and antisense primer 011 (5′-GCICCIGAYTGITGICCRAA-3′) [12]. All PCR products were examined by gel electrophoresis and gel-purified using the GENECLEAN III kit (Q-BIOgene). VP4 and VP1 amplicons were sequenced in both directions using the respective PCR primer sets that generated these products. Additionally, two internal sequencing primers, CA16_intR (5′-GCCCCTGGCGGGACATACAT-3′) and CA16_intF (5′-TGTGTGTTGAACCATCACT CCAC-3′) were used to generate complete nucleotide sequences of VP1. Both of these primers were designed based on the VP1 nucleotide sequence of the prototype G-10 strain (GenBank accession, U05876) and are located at nucleotide positions 2924–2905 and 2647–2669, respectively, of the prototype genome. Sequencing was carried out using the Big Dye Cycle Sequencing kit version 3.0 (Applied Biosystems) and performed using the ABI377 automated DNA sequencer (Applied Biosystems).

Nucleotide sequences of VP4 and VP1 generated in this study and others obtained from GenBank (Table 1) were aligned separately using the ClustalX software [19]. Phylogenetic analysis was performed using the neighbor-joining method in the PHYLIP package (version 3.6), and the reliability of the trees was tested using bootstrap analysis of 1000

Table 1. List of CVA16 strains examined in this study

Isolate	Year of isolation	Location (abbreviation)	Source	GenBank accession no. (Gene/s)
MY823-3	1997	Sarawak, Malaysia (SAR)	This study	AM292485(VP4), AM292433(VP1)
S10432	1998	Sarawak, Malaysia (SAR)	This study	AM292507(VP4), AM292455(VP1)
S70382	1998	Sarawak, Malaysia (SAR)	This study	AM292513(VP4), AM292461(VP1)
S10051	1998	Sarawak, Malaysia (SAR)	This study	AM292506(VP4), AM292454(VP1)
UM16809	1998	Peninsular Malaysia (MAL)	This study	AM292535(VP4), AM292483(VP1)
UM12593	1999	Peninsular Malaysia (MAL)	This study	AM292530(VP4), AM292478(VP1)
UM12969	1999	Peninsular Malaysia (MAL)	This study	AM292531(VP4), AM292479(VP1)
0001	1999	Perth, Western Australia (AUS)	This study	AM292486(VP4), AM292434(VP1)
UM15985	2000	Peninsular Malaysia (MAL)	This study	AM292534(VP4), AM292482(VP1)
UM15797	2000	Peninsular Malaysia (MAL)	This study	AM292532(VP4), AM292480(VP1)
UM15923	2000	Peninsular Malaysia (MAL)	This study	AM292533(VP4), AM292481(VP1)
CNS041893	2000	Sarawak, Malaysia (SAR)	This study	AM292498(VP4), AM292446(VP1)
CNS043111	2000	Sarawak, Malaysia (SAR)	This study	AM292500(VP4), AM292448(VP1)
CNS045384	2000	Sarawak, Malaysia (SAR)	This study	AM292501(VP4), AM292449(VP1)
CNS041904	2000	Sarawak, Malaysia (SAR)	This study	AM292499(VP4), AM292447(VP1)
SB2000	2000	Sarawak, Malaysia (SAR)	This study	AM292518(VP4), AM292466(VP1)
SB2002	2000	Sarawak, Malaysia (SAR)	This study	AM292519(VP4), AM292467(VP1)
SB1660	2000	Sarawak, Malaysia (SAR)	This study	AM292517(VP4), AM292465(VP1)
SB2239	2000	Sarawak, Malaysia (SAR)	This study	AM292520(VP4), AM292468(VP1)
UM17115	2000	Peninsular Malaysia (MAL)	This study	AM292536(VP4), AM292484(VP1)
TS1-2000	2000	Thailand (THAI)	This study	AM292529(VP4), AM292477(VP1)
2055	2001	Saudi Arabia (SA)	This study	AM292494(VP4), AM292442(VP1)
CNS11062	2001	Sarawak, Malaysia (SAR)	This study	AM292496(VP4), AM292444(VP1)
S33071	2001	Sarawak, Malaysia (SAR)	This study	AM292510(VP4), AM292458(VP1)
S33072	2001	Sarawak, Malaysia (SAR)	This study	AM292511(VP4), AM292459(VP1)
S22781	2002	Sarawak, Malaysia (SAR)	This study	AM292508(VP4), AM292456(VP1)
S22852	2002	Sarawak, Malaysia (SAR)	This study	AM292509(VP4), AM292457(VP1)
EV4-5-HUKM	2002	Peninsular Malaysia (MAL)	This study	AM292505(VP4), AM292453(VP1)
SB7605	2002	Sarawak, Malaysia (SAR)	This study	AM292522(VP4), AM292470(VP1)
SB7606	2002	Sarawak, Malaysia (SAR)	This study	AM292523(VP4), AM292471(VP1)
SB7883	2002	Sarawak, Malaysia (SAR)	This study	AM292524(VP4), AM292472(VP1)
EV1-5-HUKM	2002	Peninsular Malaysia (MAL)	This study	AM292504(VP4), AM292452(VP1)
S33421	2003	Sarawak, Malaysia (SAR)	This study	AM292512(VP4), AM292460(VP1)
SB12115	2003	Sarawak, Malaysia (SAR)	This study	AM292525(VP4), AM292473(VP1)
SB12120	2003	Sarawak, Malaysia (SAR)	This study	AM292526(VP4), AM292474(VP1)
SB3512	2003	Sarawak, Malaysia (SAR)	This study	AM292521(VP4), AM292469(VP1)
CNS32874	2003	Sarawak, Malaysia (SAR)	This study	AM292497(VP4), AM292445(VP1)
S110251	2003	Sarawak, Malaysia (SAR)	This study	AM292514(VP4), AM292462(VP1)
5338	2003	Saudi Arabia (SA)	This study	AM292495(VP4), AM292443(VP1)
1018T	2005	South Vietnam (VNM)	This study	AM292493(VP4), AM292441(VP1)
521 V	2005	South Vietnam (VNM)	This study	AM292488(VP4), AM292436(VP1)
535 V	2005	South Vietnam (VNM)	This study	AM292489(VP4), AM292437(VP1)
576T	2005	South Vietnam (VNM)	This study	AM292491(VP4), AM292439(VP1)
577T	2005	South Vietnam (VNM)	This study	AM292492(VP4), AM292440(VP1)
546T	2005	South Vietnam (VNM)	This study	AM292490(VP4), AM292438(VP1)
SB13044	2005	Sarawak, Malaysia (SAR)	This study	AM292527(VP4), AM292475(VP1)
CNS51082	2005	Sarawak, Malaysia (SAR)	This study	AM292502(VP4), AM292450(VP1)
S114131	2005	Sarawak, Malaysia (SAR)	This study	AM292515(VP4), AM292463(VP1)
S114371	2005	Sarawak, Malaysia (SAR)	This study	AM292516(VP4), AM292464(VP1)
SB16087	2005	Sarawak, Malaysia (SAR)	This study	AM292528(VP4), AM292476(VP1)

(*continued*)

Table 1 (*continued*)

Isolate	Year of isolation	Location (abbreviation)	Source	GenBank accession no. (Gene/s)
0033	2005	Perth, Western Australia (AUS)	This study	AM292487(VP4), AM292435(VP1)
CNS68762	2006	Sarawak, Malaysia (SAR)	This study	AM292503(VP4), AM292451(VP1)
G-10	1951	South Africa (SAF)	GenBank	U05876(VP4, VP1)
1547	1979	Japan (JPN)	GenBank	E11656(VP4)
4057	1981	Japan (JPN)	GenBank	E11657(VP4)
Aichi	1984	Japan (JPN)	GenBank	AB053286(VP4)
Aichi-4	1990	Japan (JPN)	GenBank	AB053285(VP4)
0241	1991	Japan (JPN)	GenBank	E11659(VP4)
51	1995	Japan (JPN)	GenBank	AB061505(VP4)
21	1995	Japan (JPN)	GenBank	AB061504(VP4)
V-15599	1998	Japan (JPN)	GenBank	AB094773(VP4)
5079	1998	Taiwan (TWN)	GenBank	AF177911(VP4, VP1)
V-15487	1998	Japan (JPN)	GenBank	AB094772(VP4)
Shzh99-48	1999	China (CHN)	GenBank	AY895116(VP4)
V-16192	1999	Japan (JPN)	GenBank	AB094776(VP4)
V-16472	1999	Japan (JPN)	GenBank	AB094778(VP4)
Shzh99-83	1999	China (CHN)	GenBank	AY821797(VP4, VP1)
Epsom-15290	1999	United Kingdom (UK)	GenBank	AJ297109(VP4)
V-16473	1999	Japan (JPN)	GenBank	AB094779(VP4)
IS_99_240	1999	Japan (JPN)	GenBank	AB094781(VP4)
Shzh00-2	2000	China (CHN)	GenBank	AY895127(VP4, VP1)
Pool-46	2001	Scotland (SCOT)	GenBank	DQ251294(VP4)
Pool-67	2001	Scotland (SCOT)	GenBank	DQ251298(VP4)
Pool-53	2001	Scotland (SCOT)	GenBank	DQ251295(VP4)
Pool-37	2001	Scotland (SCOT)	GenBank	DQ251292(VP4)
Shzh01-77	2001	China (CHN)	GenBank	AY895097(VP4, VP1)
Shzh01-66	2001	China (CHN)	GenBank	AY895105(VP4, VP1)
P-1757A	2002	Japan (JPN)	GenBank	AB094784(VP4)
Shzh02-124	2002	China (CHN)	GenBank	AY895094(VP4, VP1)
Shzh02-16	2002	China (CHN)	GenBank	AY895100(VP4, VP1)
Shzh02-12	2002	China (CHN)	GenBank	AY895098(VP4, VP1)
Shzh02-14	2002	China (CHN)	GenBank	AY895110(VP4, VP1)
P-1752A	2002	Japan (JPN)	GenBank	AB094783(VP4)
Shzh03-13	2003	China (CHN)	GenBank	AY895114(VP4)
Shzh03-10	2003	China (CHN)	GenBank	AY895095(VP4, VP1)
Shzh04-J31	2004	China (CHN)	GenBank	AY821796(VP4)
Shzh04-4	2004	China (CHN)	GenBank	AY895121(VP4)
Shzh04-40	2004	China (CHN)	GenBank	AY895120(VP4)

pseudo replicate data sets [6]. Trees were drawn using the TREEVIEW program [14]. Genogroups were described in a similar way to that originally used to describe HEV71 [2]. A difference of at least 15% in the VP1 gene was used to distinguish genogroups.

All VP4 and VP1 sequences determined in this study have been given accession numbers AM292485 to AM292536 and AM292433 to AM292484, respectively. Detailed information for accession numbers of each CVA16 strain is provided in Table 1.

Nucleotide sequences of both VP4 and VP1 genes obtained in this study and others obtained from GenBank were aligned separately, and phylogenetic trees were constructed (Fig. 1). VP1 sequences were used to describe the phylogenetic relation-

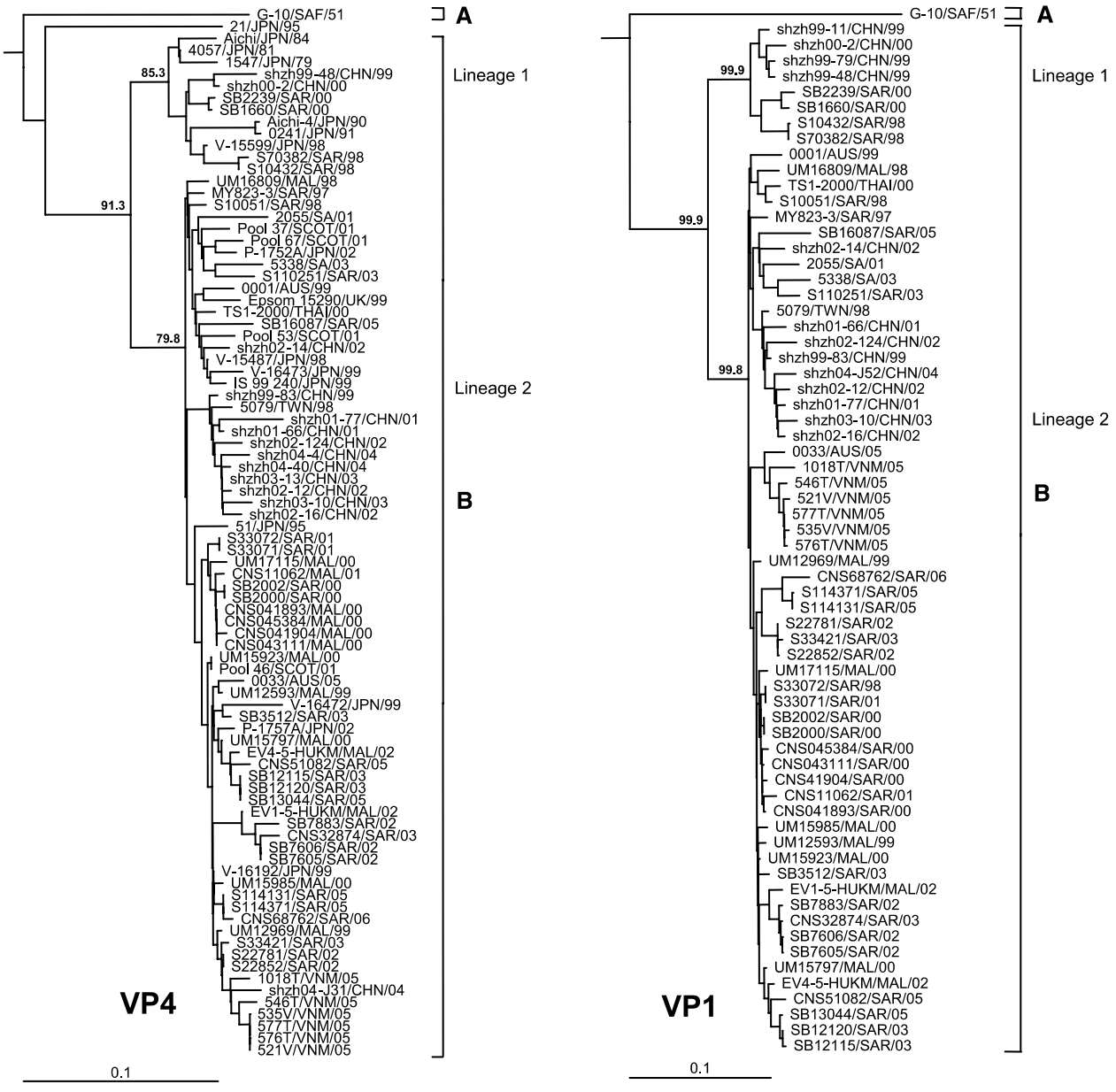

Fig. 1. Phylogenetic trees based on the VP1 and VP4 nucleotide sequences of CVA16. Both the VP4 (left) and VP1 (right) distance trees were rooted with the prototype HEV71 strain, BrCr (GenBank accession: U22521). To save space, the root was edited from both tree figures. CVA16 strains are labeled using the following convention: ''isolate name''/''country of origin''/''year of isolation''. Details of each strain can be found in Table 1. Genogroups (A and B) are indicated to the right of each tree and bootstrap values (% of 1000 pseudoreplicates) shown at the nodes of major clades. The scale at the bottom indicates a measurement of relative phylogenetic distance

ship between CVA16 strains by defining different genogroups of these viruses. Based on the nucleotide alignment and phylogenetic analysis of VP1 sequences, the VP1 tree showed that the G-10 prototype strain clustered separately from all other CVA16 strains analysed in this study. The G-10 strain differed from other strains by 27.7–30.2%. As such, the prototype G-10 strain was designated

as the sole member of genogroup A. The genetic variation between all other CVA16 strains was fewer than 13.4% nucleotide differences. Based on this, all other CVA16 strains were assigned as members of genogroup B. Viruses in genogroup B form two separate clusters in the phylogenetic tree with bootstrap support of >99% (Fig. 1). We have named these clusters lineages 1 and 2 (Fig. 1).

Sequences generated in this study were used to anchor both the VP1 and VP4 trees to allow the description of phylogenetic relationships of recent CVA16 strains to older strains for which only VP4 nucleotide sequences were available. Based on the alignment of VP4 nucleotide sequences and the VP4 tree (Fig. 1), the prototype G-10 strain was also the only member of genogroup A with between 19.9 and 25.2% nucleotide differences to other CVA16 strains. A single strain, 21/JPN/95, differed from the prototype strain by 20.6% and from all other CVA16 strains by 16.5–22.9%. Close relatives of this strain have not been found among sequenced strains and may represent an emerging genogroup. All other CVA16 strains formed genogroup B, with genetic variation of VP4 nucleotide sequences between 0 and 16.3%. Similar to the VP1 tree, genogroup B viruses formed two separate lineages in the VP4 tree with bootstrap support of >79% (Fig. 1).

Based on the analysis of VP1 and VP4 nucleotide sequences generated in this study, CVA16 strains appear to be monophyletic in both of these genome regions. This is apparent in that strains that cluster together in the VP1 phylogenetic tree also cluster together in the VP4 tree. For example, genogroup B strains S10432/SAR/98, shzh99-48/CHN/00 and SB1660/SAR/00 in lineage 1 and strains UM16809/MAL/00, 576T/VNM/05, and 001/AUS/99 in lineage 2 cluster together in both the VP1 and VP4 trees. In view of this observation, a more comprehensive epidemiological history of CVA16 strains can be inferred from both the VP1 and VP4 phylogenetic trees. The prototype South African strain, G-10, isolated in 1951, is the only member of genogroup A. This virus has not appeared in the sequence databases since it was first described. Viruses in genogroup B separate into two separate lineages (1 and 2) with Japanese strains isolated in the late 70s and early 80s (Aichi/JPN/84, 4057/JPN/81, 1547/JPN/91) and strains from China, Malaysia, Taiwan and Japan isolated in the 90s and in 2000 forming one lineage and the majority of CVA16 strains isolated recently forming the second lineage. Lineage 1 strains were last isolated in 2000 in China and Malaysia. These strains appear to have given way to lineage 2 strains that appear to be the dominant circulating strain isolated from 2000 onwards in the region sampled in this study. Viruses in this group (lineage 2) are also widely distributed, with strains isolated in Europe (e.g. Epsom-15290/UK/99, Pool-46/SCOT/01), the Middle East (e.g. 2055/SA/01, 5338/SA/03), Asia (e.g. S33072/SAR/01, TS1-2000/THAI/00, EV4-5-HUKM/MAL/02) and Western Australia (e.g. 001/AUS/99, 0033/AUS/05).

In recent years, large outbreaks of HEV71-associated HFMD in the Asia–Pacific region [10, 3, 17], often coupled with severe clinical manifestations, have drawn a lot of attention to this virus. This increased interest in HEV71 strains associated with these outbreaks has generated a lot of viral sequence data that has been used to describe different circulating genotypes of the virus [10, 3, 11, 2]. In contrast, CVA16 appears to have drawn very little interest, probably due to its association with often mild and benign clinical symptoms. As such, very little sequence data has been made available for CVA16 strains, although it has been observed that both HEV71 and CVA16 often co-circulate during HFMD outbreaks [17, 8, 9]. In this study, we have attempted to provide a phylogenetic description of CVA16 strains in line with that available for HEV71. A total of 52 CVA16 strains isolated from five different geographical locations and spread over a ten-year period from 1997 to 2006 were examined. We have improved on a similar study done by Li et al. [8] by including an analysis of the VP1 gene together with VP4. Based on analysis of VP1 sequences, we have shown that CVA16 strains cluster into two distinct genogroups, A and B (Fig. 1). Our study adds to this earlier work and suggests that CVA16 strains belonging to lineages B and C described by Li et al. (based on VP4 nucleotide sequences) actually constitute a single genogroup, which we have described here as genogroup B.

Lineage B and C viruses in the Li study represent lineage 1 and 2 viruses, respectively, in genogroup B as determined in this study using complete VP1 sequences. These results suggest that while it may be faster to analyse the VP4 gene due to its smaller size, a more accurate description of different genotypes should be determined from the VP1 gene.

Our analysis of recent CVA16 strains suggests that although these viruses are geographically broadly dispersed, genetically, the virus has undergone far fewer changes when compared to HEV71. Unlike HEV71 strains that have evolved (since the 1970s) into two co-circulating genogroups (B and C) with several genetically distinct sub-genogroups [10, 18, 3, 11, 2], the evolution of CVA16 strains over the same timeframe has been less remarkable. Similar to HEV71, the prototype CVA16 strain appears to have given way to more modern strains that are genetically distinct. All CVA16 strains isolated since then appear to be from a single genogroup. Of these, CVA16 strains that began to circulate from 1970 to 2000 were later displaced by strains that started to emerge in the mid-90s to become the dominant circulating genotype from 2000 onwards.

In addition to being used to describe CVA16 strains, VP1 nucleotide sequences generated in this study may be useful for other purposes, such as in detection methods for CVA16. Several different RT-PCR methods that target the VP1 gene for CVA16 detection have been published [13, 1, 21]. Some of these methods have been designed as tools to differentiate between HEV71 and CVA16 strains [1, 21] during HFMD outbreaks. Primer design for these methods has mostly been dependent on limited CVA16 nucleotide sequence data available in GenBank. The lack of available CVA16 nucleotide sequences, particularly for RT-PCR primer design, has led to a least one report of HEV71-specific primers losing specificity when tested on Asian CVA16 strains [16]. As such, we hope that by contributing CVA16 VP1 sequences of strains from diverse geographical locations generated in this study, these methods can be further improved or newer methods developed to better detect different genogroup strains of CVA16.

Acknowledgements

This study was supported by grants from the Ministry of Science, Technology and Innovation, Government of Malaysia, 06-02-09-002BTK/ER/003 and The Wellcome Trust, 071588/Z/03/Z. We thank Professor Lam Sai Kit for helpful input.

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