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Molecular identification of coxsackievirus A24 variant, isolated from an outbreak of acute hemorrhagic conjunctivitis in Singapore in 2005

D. S.-Y. Yeo, S. G.-K. Seah, J. S.-W. Chew, E. A.-S. Lim, J. C.-W. Liaw, J.-P. Loh, B.-H. Tan

Detection and Diagnostics Laboratory, DSO National Laboratories, Defence Medical and Environmental Research Institute, Singapore

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Summary

An outbreak of acute hemorrhagic conjunctivitis (AHC) was reported in Singapore military camps in the year 2005. A total of 103 conjunctival swab specimens were collected from military personnel diagnosed clinically with AHC. PCR testing on these conjunctival specimens revealed the presence of an enterovirus, and this was confirmed by virus isolation. Molecular typing using a partial VP1 gene confirmed a variant of coxsackievirus A24 (CA24v) as the most likely etiological agent for the outbreak. Full-length genome sequencing was carried out on 2 selected virus strains, DSO-26SIN05 and DSO-52SIN05. Sequence comparison and phylogenetic analyses of the VP4, VP1 and 3Cpro gene regions were performed, clustering the Singapore CA24v strains with viruses originating from Asia in the post-2000 era. In addition, we report evolution rates of 4.2×10^{-3} and 1.0×10^{-3} nucleotide/year, respectively, for the VP4 capsid and 3Cpro gene regions. Our result shows a focal evolutionary point around 1965–1966, suggesting that the CA24v virus has been evolving constantly since its emergence in Singapore, nearly 40 years ago.

Correspondence: Dr. Boon-Huan Tan, PhD, Head, Detection and Diagnostics Laboratory, DSO National Laboratories, Defence Medical and Environmental Research Institute, #13-00, 27 Medical Drive, Singapore 117510
e-mails: tboonhua@dso.org.sg, tanboonhuan@yahoo.com.sg

Introduction

Enterovirus (EV) 70 and a variant of coxsackievirus A24 (CA24v), both members of the family *Picornaviridae*, are the common primary causes of acute hemorrhagic conjunctivitis (AHC), and have been implicated in epidemics since the 1970s [21, 5, 14]. EV70 belongs to the species *Human enterovirus D*, while CA24v, formerly known as echovirus 34, falls under the human EV group C [17]. However, CA24v has been reported to cause more explosive and extensive outbreaks of AHC in Asia. The first isolate of a CA24v was obtained during an outbreak in Singapore in 1970 [21]. Since then, Singapore has had periodical outbreaks of AHC [43, 42, 13] that have spread to Malaysia [11], Brunei [2] and Thailand [19], and to other countries around Asia such as Taiwan [22, 23], Korea [35], Japan [41] and India [4]. Starting from 1986, worldwide reports of AHC outbreaks due to CA24v have been reported from American Samoa in the Pacific Ocean to the Caribbean islands [37], Central America [8] and other parts of America [40].

The EV genome is a single, positive-stranded RNA molecule of approximately 7500 nucleotides (nt) in length. It comprises a single open reading frame, flanked at its 5′ and 3′ ends by the untranslated regions (UTRs), and a short poly (A) tail at the 3′-UTR [26, 28]. The encoded polyprotein is subsequently cleaved to produce 4 structural capsid proteins, VP4, VP2, VP3 and VP1 proteins, and 7 nonstructural proteins: a protease, 2Apro; the proteins 2B and 2C; the 3B virus-encoded protein (VPg) precursor (3AB); the major viral protease (3Cpro); and the RNA-dependent RNA polymerase (3Dpol). The EVs were traditionally serotyped by virus neutralization using antisera raised against prototype EVs, a time-consuming and costly process. However, recent reports have indicated that partial or complete sequence analysis of the VP1 gene could provide an alternative method of serotyping within the picornavirus classification [29–31]. For example, VP1 gene region analysis was repeatedly used in the epidemiological study of CA24v involved in recent epidemics of AHC [35, 8, 40, 34]. The results of molecular typing correlated well with serotyping using virus neutralization, and this is not surprising since the VP1 protein remains the most external and immunodominant picornavirus capsid proteins [25].

In the year 2005, there was a national outbreak of AHC in Singapore, affecting more than 10,000 people [12]. The AHC outbreak was also reported in military camps, located in different parts of Singapore. Previous epidemic conjunctivitis in military camps has been reported with a significant loss of manpower-days [33]. It was essential to identify the etiological agent responsible for the Singapore outbreak so that disease control strategies could be rapidly implemented. Polymerase chain reaction (PCR) tests for EV were successfully performed on the conjunctival swab specimens collected from military personnel diagnosed clinically with AHC. Sequence analysis at the partial VP1 regions of the PCR-positive specimens further identified CA24v as the likely etiological agent for the AHC outbreak. Full-length genome sequencing was carried out on 2 virus strains that were successfully isolated in cell culture. Phylogenetic analyses were performed, confirming the Singapore strains as CA24v originating from the prototype strain EH24/70 (accession number D90457), first isolated in Singapore in 1970 [38]. This is the first comprehensive report of detailed sequence comparison and analysis of CA24v strains from different geographical and temporal lineages, as well as from isolates with different clinical manifestations for AHC.

Material and methods

Clinical specimens

A total of 103 conjunctival swab specimens were collected from military personnel diagnosed clinically with AHC from 5 military camps. Dacron swabs (Remel, Lenexa, Kans, USA) were used to soak up the eye discharges and placed in viral transportation medium (VTM). One portion of the VTM specimen was used for viral culture and another portion for the extraction of total DNA/RNA for PCR testing.

Viral culture and immunofluorescence assay

Two hundred microliter of specimen was inoculated onto human lung carcinoma A549 cells [European Collection of Cell Cultures (ECACC) number 86012804] and human laryngeal carcinoma Hep-2 cells (ECACC number 85020207), in Dulbecco's Modified Eagle Medium (Invitrogen Corporation, CA, USA) supplemented with 2% fetal bovine serum (Invitrogen Corporation, CA, USA) and 100 U/ml penicillin/streptomycin (Invitrogen Corporation, CA, USA) at 37 °C. Five days after inoculation, the infected cells were harvested, resuspended in 1X phosphate-buffered saline and spotted onto glass slides for IFA. The slide preparations were fixed with acetone for 30 min and processed for IFA testing with antibodies for pan-EV screening and EV subtyping, and adenovirus (Chemicon International, Inc.).

RNA extraction, reverse transcription and PCR tests

Each clinical specimen was subjected to 2 PCR assays, with primers EV2F/EV1R, targeting the highly conserved 5′-UTR of EVs [31], and primers HEX3F/HEX4R, specific for the hexon gene of adenovirus [9] (Table 1). For PCR testing of EVs, RNA was extracted from clinical specimens using the RNeasy mini kit (Qiagen Inc., Valencia, CA, USA), eluted in 50 µl nuclease-free water and stored at −80 °C until needed. First-strand cDNA synthesis was performed using 8 µl of RNA and 50 ng of random hexamers in the presence of Superscript II RT (Invitrogen Corporation, CA, USA) according to the manufacturer's protocol. PCR testing for EVs was performed in a 50-µl reaction mixture containing 0.2 µM of each primer (set EV2F/EV1R), 2 U

Table 1. Details of primers used for PCR identification and serotyping of clinical specimens, as well as primers used in full-length sequencing of CA24v Singapore 2005 strains, DSO-26SIN05 and DSO-56SIN05

Primer names	Virus	Gene target	Nt position*	Sequence 5′ to 3′	Applications
EV2F	Enterovirus	5′-	453–471	TCCGGCCCCTGAATGCGGC	PCR testing of
EV1R		UTR+	566–540	ACACGGACACCCAAAGTAGTCGGTCC	clinical specimens
HEX3F	Adenovirus	Hexon	NA	GACATGACTTTCGAGGTCGATCCCATGGA	PCR testing of
HEX4R				CCGGCTGAGAAGGGTGTGCGCAGGTA	clinical specimens
EV188modF	CA24	VP1	2623–2642	ACTGCAGTAGAGACTGGTGC	molecular typing
EV222modR			2987–2968	GCGCCAGGTGGTATGTACAT	
Ent-1040R	DSO-SIN05	VP2	1048–1026	CATTCGCTGCCTCTTGAGTAGTG	PCR-amplification with GeneRacer5′ for the 5′-UTR
Ent-940F	DSO-SIN05	VP4	939–969	GACTGCTCCAGCCCTAAACTC	PCR-amplification
Ent-6760R		3D	6763–6742	CCATCTTGAGAGCTTCGAACCA	from 939 to 6763 nt position
Ent-6650F	DSO-SIN05	3D	6634–6654	GCTGTAGGATGTGACCCAGAC	PCR-amplification with GeneRacer3′ for the 3′-UTR
Ent-1620F	DSO-SIN05	VP2	1613–1636	GTATGGCCAAACACAACAACTGGG	sequencing
Ent-2030F	DSO-SIN05	VP3	2032–2049	CCCGGCCTCCGATAAGAG	sequencing
Ent-2900F	DSO-SIN05	VP1	2873–2895	CCTACTCCAGATTTGACCTAGAG	sequencing
Ent-3050R	DSO-SIN05	VP1	3032–3014	GTGGTGCACTGCCGTACGT	sequencing
Ent-3405F	DSO-SIN05	2A	3409–3431	GGTTTGGACACCAGAACATGGC	sequencing
Ent-4700R	DSO-SIN05	2C	4679–4702	GACAGAACAGCTTCATGTCGTTGC	sequencing
Ent-5395R	DSO-SIN05	3A	5394–5377	CTGGTGCCCAGCAAACAGTTTG	sequencing
Ent-6145R	DSO-SIN05	3D	6127–6144	GGTGAGAGCTGCTGGTTC	sequencing

* Primer location relative to the CA24v genome for strain EH24/70, accession number D90457, is indicated in the primer name. *F* Denotes primer in the forward or positive strand, and *R* denotes primer in the reverse or negative strand of the viral genome. GeneRacer5′ and 3′ primers were obtained from the GeneRacer kit, Invitrogen. *DSO-SIN05* refers to the CA24v strains isolated during the course of this investigation. +*UTR* Represents untranslated region. *NA* not applicable.

Platinum Taq DNA polymerase (Invitrogen Corporation, CA, USA), 0.2 mM dNTPs, and 1.5 mM $MgCl_2$. The reaction was amplified by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, and extension at 72 °C for 30 sec. The final extension step was extended to 7 min. PCR testing for adenovirus was performed similarly with primers HEX3F/HEX4R. The PCR products from both tests were analysed in a 2% agarose gel in the presence of ethidium bromide, with visualization under UV illumination.

Molecular typing using the partial VP1 gene region

The partial VP1 gene regions were PCR-amplified using cDNAs synthesized from viral RNAs with primers EV188modF/EV222modR, modified from [31] (Table 1), as described above. PCR products were purified using the QIA quick PCR purification kit (Qiagen Inc., Valencia, CA, USA) and sequenced (Applied Biosystems, USA) using the same primers.

Full-length sequencing of the viral genome

The ~7.5-kb genome was subjected to 3 different strategies of PCR amplification, using RNA extracted from virus-infected Hep-2 cells. To obtain the cDNA fragment at the 5′ end of the genome, total RNA was first treated with calf intestinal phosphatase to remove the 5′ phosphates. Tobacco acid pyrophosphatase was next added to remove the 5′ cap structure from the intact, full-length RNA. The GeneRacer RNA Oligo (GeneRacer kit, Invitrogen Corporation, CA, USA) was then ligated to the 5′ end of the RNA using T4 RNA ligase according to the manufacturer's recommendations. First-strand cDNAs were synthesized next using random hexamers. Double-stranded DNAs were amplified using primers Ent-1040R/GeneRacer5′ (GeneRacer kit, Invitrogen Corporation, CA, USA). The cDNA fragment comprising the middle portion was PCR-amplified from first-strand cDNAs using GeneRacer Oligo dT primer, with primers Ent-940F/Ent-6760R. Lastly, the cDNA fragment for the 3′ end of the genome were also PCR-amplified from first-strand cDNAs

using GeneRacer Oligo dT primer, but with primers Ent-6650F/GeneRacer3′ (GeneRacer kit, Invitrogen Corporation, CA, USA). PCR reactions in 50-µl volumes were carried out with 0.3 µM of each primer, 0.5 mM dNTPs, the Expand Long Template buffer 3 and enzyme mix (Roche Diagnostics). The details of the primers used for PCR and sequencing reactions are described in Table 1. Primers for sequencing the internal region of DNA fragments were designed by 'primer-walking'. The PCR products were gel-purified using the QIAquick gel extraction purification kit, sequenced directly, or cloned into TOPO-pCR2.1 vectors (Invitrogen Corporation, CA, USA) and the recombinant vectors subjected to sequencing. All sequencing reactions were performed with ABI Prism BigDye (Applied Biosystems, USA) in the presence of 1 µM of each respective primer. Each portion of the viral genome was sequenced at least 5 times.

Sequence, phylogenetic and evolutionary rate analyses

The sequences of partial VP1 genes from Singapore 2005 CA24v strains and the full-length genome sequences were assembled and aligned using SeqMan (DNASTAR, Lasergene version 7). The individual gene and protein sequences [at the nt and amino acid (aa) levels, respectively] were predicted by the alignment and comparison with the CA24v strain EH24/70. For phylogenetic analyses, nt sequences of partial VP1, VP4 and 3C genes from other CA24v strains were obtained from GenBank and aligned against the Singapore strains using the algorithm CLUSTALW method in the program MEGALIGN (DNASTAR, Lasergene version 7). Percent (%) sequence homology and phylogenetic trees were calculated and constructed using the neighbour-joining method at the nt level, with bootstrap analysis performed on 1000 replicates (DNASTAR, Lasergene version 7). Phylogenetic trees were viewed using TreeExplorer (version 2.12, http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html), assigning CA24v strain EH24/70 as the root in all trees. The evolutionary rates for the VP4 and 3Cpro genes were calculated as regression coefficient of nt substitution. For each gene, the number of nt differences of each Singapore strains with respect to EH24/70 was plotted along the Y-axis, and against their year of isolation along the X-axis. A regression analysis was performed by fitting a linear trend line to the points, and the rate of nt change per year and the evolutionary focal point were calculated.

Nucleotide accession numbers

The partial VP1 gene sequences from Singapore 2005 CA24v strains, DSO-27SIN05, DSO-28SIN05, and DSO-60SIN05 have been deposited in GenBank under the accession numbers DQ901736, DQ901737, and DQ901738, respectively; and that for DSO-26SIN05 and DSO-52SIN05 have been deposited together with the full-length genome sequences under the accession numbers, DQ443002 and DQ443001, respectively.

Results

Virus isolation and PCR tests

Thirty-one specimens were randomly selected and subjected to virus isolation on Hep-2 and A549

Table 2. Results of CPE and IFA in Hep-2 cells and PCR tests on 6 selected specimens. For enterovirus IFA, a pan-enterovirus screen (EV panel) was first done, followed by EV subtyping: i) echo (echovirus 4, 6, 9, 11, 30, 34, and CA24), ii) CB (CB1, 2, 3, 4, 5, 6), EV (EV70 and EV71) and iii) polio (poliovirus 1, 2, 3)

Strain names	Viral cultures using Hep-2 cells						PCR results	
	IFA results						Enterovirus (EV2F/EV1R)	Adenovirus (HEX3F/HEX4R)
	EV panel	Echo	CB	EV	Polio	Adenovirus		
DSO-24SIN05	+++	++	−	−	−	−	+	−
DSO-26*SIN05	+++	+++	−	−	−	−	+	−
DSO-52*SIN05	+++	+++	−	−	−	−	+	−
DSO-60SIN05	+++	++	−	−	−	−	+	−
DSO-64SIN05	+++	++	−	−	−	−	+	−
DSO-66SIN05	+++	+++	−	−	−	−	+	−

* Full-length sequencing of isolates DSO-26SIN05 and DSO-56SIN05 was carried out (GenBank accession numbers are DQ443002 and DQ443001, respectively).
For IFA, "−" represents no fluorescent cells, "+" represents few fluorescent cells, "++" represents a moderate number of fluorescent cells, and "+++" represents a large number of fluorescent cells.
For PCR, "−" indicates a negative result while "+" indicates a positive result.

cells. The cells were monitored daily, and on the fifth day of infection, CPE was observed in at least 4 Hep-2 cultures. The virus-infected cells were harvested for IFA with the pan-EV screen, and 6 out of 31 specimens tested positive (Table 2). The 6 specimens were further subtyped with each of the EV subtyping antibodies. The echovirus blend, specific for echoviruses 4, 6, 9, 11, 30 and CA24, showed strong positive IFA for all 6 specimens. Twenty-four out of 103 conjunctival specimens tested PCR positive for the presence of EV, using EV2F/EV1R primers. As past AHC outbreaks had implicated adenovirus as an alternate etiological agent, we subjected the specimens for adenovirus testing by viral isolation using A549 cells and PCR assays. Both viral cultures and PCR tests were negative for the presence of adenovirus for all of the specimens. All test results concurred for the presence of EV, indicating the strong possibility of CA24v as the causative agent for the conjunctivitis outbreak.

Molecular typing of clinical specimens

All 24 EV PCR-positive clinical specimens were successfully amplified with modified primers EV188modF/EV222modR, specific for the VP1 region of Singapore 2005 CA24v, to yield a 363-bp product. The PCR products were directly sequenced and compared to each other, as well as to published EV sequences from GenBank. We found 5 unique VP1 gene sequences (from the 24 sequenced), out of which 3 sequences were representatively reported to GenBank, and the other 2 sequences were reported as part of the full genome data after full genome sequencing, described in the following section. Analysis of these 5 unique sequences revealed nt differences at 5 positions, whereas aa alignment showed 100% identity. Further analysis with other EV sequences confirmed the identity of the recent Singapore strains to be CA24v. Comparing the Singapore sequences in the VP1 region to CA24v prototype strain EH24/70, a 78.1–79.2% pairwise nt identity and 91.7% aa identity were observed. This is higher than the reported molecular typing criteria of 75% nt and 88% aa identities between a clinical isolate and its prototype strain [29–31]. These results, taken together, confirmed that CA24v could be the likely etiological agent of the AHC outbreak and that the same strain was circulating in the 5 Singapore military camps.

Genome comparison of DSO-26SIN05 and DSO-52SIN05

Two virus strains (DSO-26SIN05 and DSO-52SIN05) were selected for full-length viral genome sequencing. There were only 3 complete genomes of CA24v reported – the first strain, EH24/70, isolated in Singapore in 1970 [38], and 2 strains, Hangzhou (accession number AY876913) and Ningbo (accession number AY876912) from China. The deposition of the 2 new Singapore strains, DSO-26SIN05 and DSO-52SIN05, in GenBank, contributed the fourth and fifth complete CA24v genomes. The CA24v genomes from both DSO-26SIN05 and DSO-52SIN05 strains were 99.6% similar at the nt level and 100% identical at the aa level. Each genome is 7461 nt long with 750 nt at the 5′-UTR, and a short, 69-nt polyadenylated sequence at the 3′ end. The polyprotein precursor is encoded by a long open reading frame of 6642 nt, corresponding to 2214 aa. The individual protein positions and sequences were deduced from strain EH24/70 and other closely related HEV-C group viruses. As shown in Table 3, sequence comparison of individual genes/proteins against those of the CA24v strain EH24/70, revealed nt identities between 81.8 and 87.8%, while aa identities stood at 90.7–99.2%. When the sequence comparison was made with CA24v strains Hangzhou13-02 and Ningbo3-02 against strain EH24/70, the nt and aa identities were 82.1–87.8% and 90.7–99.2%, respectively, almost the same as those obtained with the Singapore strains.

Sequence comparison and phylogenetic analysis

To further investigate the genetic relationship of the Singapore 2005 CA24v (DSO-26SIN05 and DSO-52SIN05) with existing CA24v sequences, we performed sequence comparison and phylogenetic analysis for the gene regions encoding VP4, VP1 and 3Cpro. Sequence analysis in the VP4 gene region has been reported to correlate well with typing

Table 3. Predicted gene positions and protein sequences in CA24v Singapore isolate DSO-26SIN05 and CA24v China isolates Hangzhou and Ningbo. The prediction with isolate DSO-52SIN05 was similar, and the results are not presented here. Nt and aa percent identities of the Singapore strain DSO-26SIN05 and China strains against the CA24v strain EH24/70 were computed

Gene/ Protein	Nt position	Number of aa	DSO-26SIN05 % identity		Hangzhou % identity		Ningbo % identity	
			Nt	Aa	Nt	Aa	Nt	Aa
5′-UTR	1–750	NA	84.0	NA	83.8	NA	83.8	NA
VP4	751–957	69	84.5	97.1	84.1	97.1	84.1	97.1
VP2	958–1770	271	87.5	97.0	87.1	97.4	86.7	97.4
VP3	1771–2490	240	87.8	99.2	87.8	99.2	87.8	99.2
VP1	2491–3405	305	85.8	95.4	85.9	95.1	86.2	95.4
2A	3406–3855	150	84.0	92.7	85.6	92.7	85.6	92.7
2B	3856–4146	97	82.8	90.7	82.1	90.7	82.1	90.7
2C	4147–5133	329	85.6	97.3	85.8	97.6	85.7	97.6
3A	5134–5394	87	85.4	92.0	86.2	93.1	86.2	93.1
3B	5395–5460	22	81.8	95.5	83.3	95.5	83.3	95.5
3C	5461–6009	183	86.5	96.2	87.2	96.7	87.2	96.7
3D	6010–7392	461	86.5	98.0	86.7	98.7	86.8	98.7
3′-UTR	7393–7461	NA	98.6	NA	98.6	NA	98.6	NA

* *NA* not applicable.

of EVs into CA24v [16]. In addition, recent studies on AHC have identified CA24v as the causative agent by molecular typing at the VP1 [35, 8, 40] and 3Cpro [23, 8] gene regions.

The VP4 genes were aligned with Singapore CA24v strains isolated from year 1970 to 2005. Percent nt identities in the VP4 capsid gene ranged from 80.7 to 99.5%, while aa identities ranged from

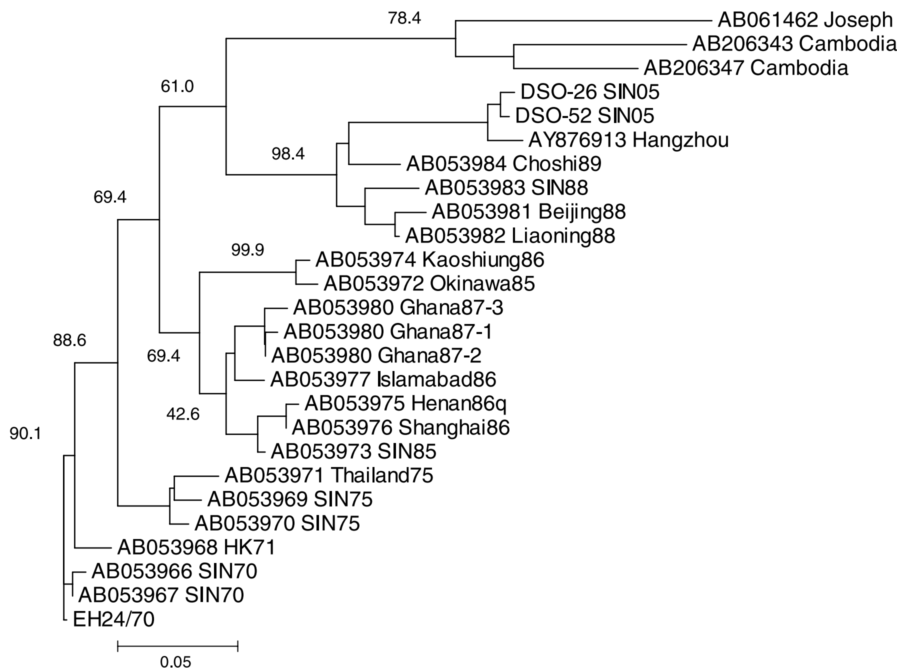

Fig. 1. Phylogenetic analysis of the VP4 capsid gene region for DSO-26SIN05 and DSO-52SIN05 strains with different CA24v strains, including the prototype CA24v strain EH24/70 (accession number D90457) as the root. The bootstrap values in 1000 replicates for major lineages are shown as percentages. The location and year of isolation of each strain (where known) are reported next to its GenBank accession number. Abbreviations used are: *HK* HongKong, *SIN* Singapore

95.7 to 100%. Our results correlate well with the reported nt identity between CA24v strains and prototype strain EH24/70 [16]. When the VP4 genes from other CA24v strains of different geographical origins were compared, the phylogenetic analysis clustered DSO-26SIN05 and DSO-52SIN05 together (Fig. 1). As expected, the Singapore 2005 viruses were found to group most closely to the Hangzhou strain, isolated in China during the post-2000 period.

We analysed the 5′ half of the VP1 capsid gene region (2677 to 2964 nt) by aligning the sequences of 5 Singapore virus strains (DS0-26SIN05 to DSO-28SIN05, DSO-52SIN05 and DS0-60SIN05) with CA24v isolated from different geographical locations. Percent nt identities ranged from 74.3 to 86.1% in the 5′-VP1 region, corresponding to a 77.9-to-94.8% aa identity in the N-terminus half of the VP1 protein. Phylogenetic analysis of the CA24v strains showed 2 distinct genetic groups: group I, consisting of post-2000 "Asian" strains from documented AHC cases; and group II, consisting of more diverse strains and including some AHC isolates (pre-2000 and "non-Asian") as well as some "untypable" isolates with unknown or unrelated clinical manifestations. (Fig. 2). From the phylogenetic analysis, the Singapore 2005 isolates clustered into group I, with CA24v strains associated with AHC epidemics isolated from Korea [35], Tunisia, Guadeloupe, and French Guiana [8] that were isolated after the year 2000.

The 3Cpro gene regions of all the Singapore strains isolated from the year 1970 onwards as well as other strains isolated worldwide [23, 41, 8] were studied. Percent nt identity of all strains with respect to strain EH24/70, ranged from 86.3 to 100%. Further analysis from the aa alignment of Singapore CA24v strains (isolated from 1970 to 2005) highlighted 13 aa points of substitution over

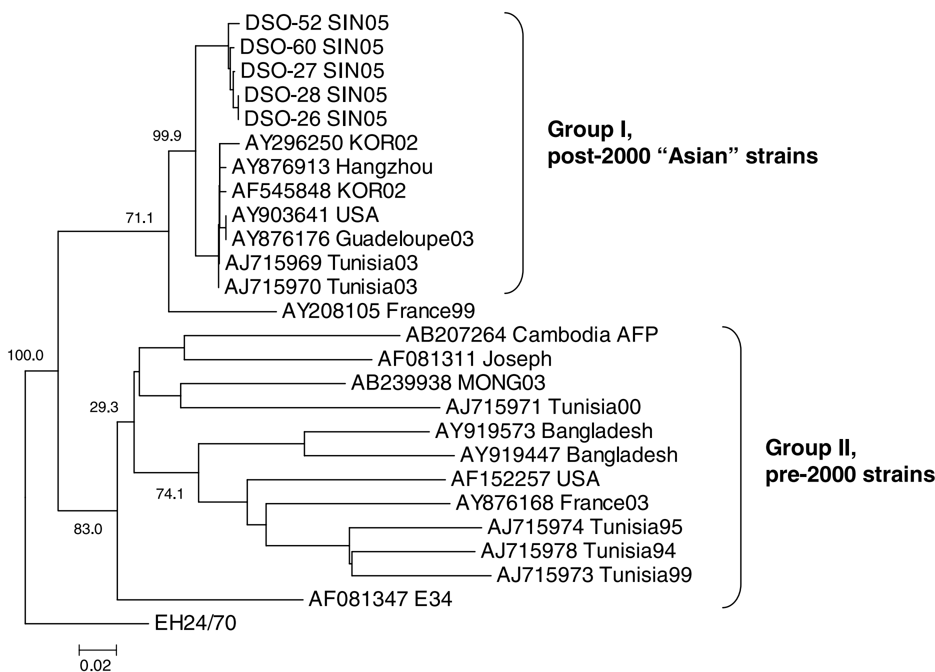

Fig. 2. Phylogenetic analysis of the 5′ half of the VP1 capsid gene region (spanning nt 2677–2964) for DSO-26SIN05 to DSO-28SIN05, DSO-52SIN05 and DS0-60SIN05 with different CA24v strains, including the prototype CA24v strain EH24/70 (accession number D90457) as the root. CA4v strains associated with AHC as a clinical symptom are shaded. The bootstrap values in 1000 replicates for major lineages are shown as percentages. The location and year of isolation for each strain (where known) are reported next to its GenBank accession number. Exceptions include "E34", which represents the former echovirus 34, now reclassified as a CA24v [30]. "AFP" stands for "acute flaccid paralysis", which was the reported clinical diagnosis for the isolate, and "Joseph" refers to the strain name. Abbreviations used are: *MONG* Mongolia, *KOR* Korea, *USA* United States of America, *SIN* Singapore

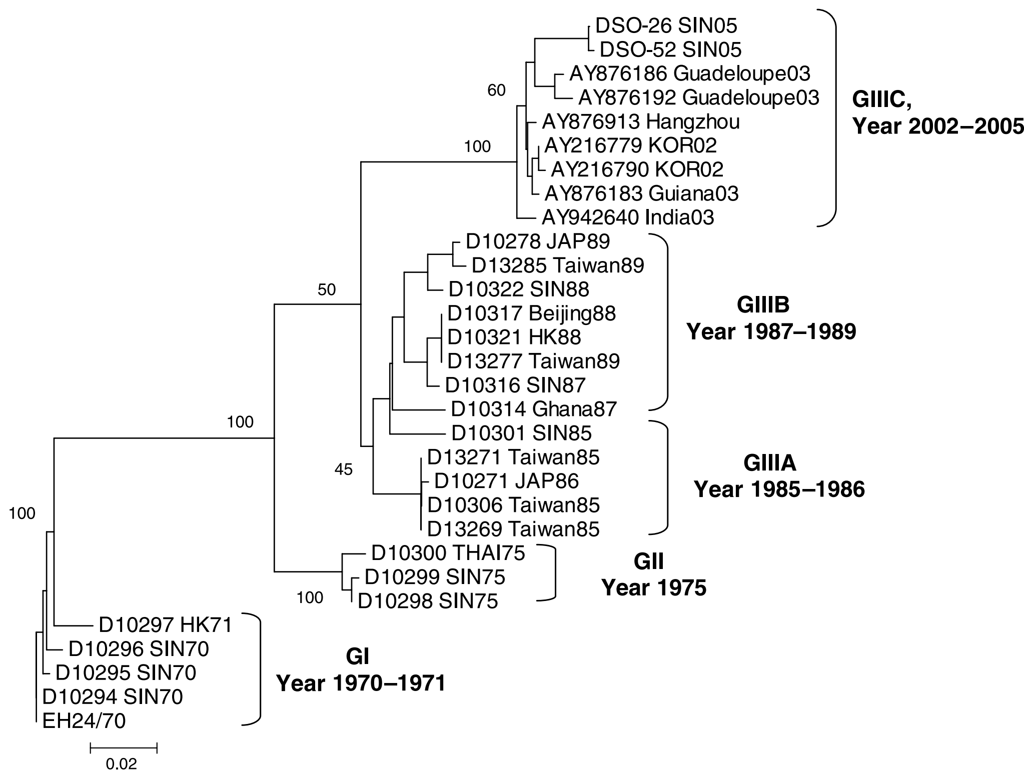

Fig. 3. Phylogenetic analysis of the 3Cpro gene region (511 nt) for DSO-26SIN05 and DSO-52SIN05 strains with different CA24v strains, including the prototype CA24v strain EH24/70 (accession number D90457) as the root. Comparison of aa sequence of Singapore CA24v strains, isolated from the years 1970 to 2005, revealed temporal evolutionary trends over 3½ decades. The bootstrap values in 1000 replicates for major lineages are shown as percentages. The location and year of isolation of each strain are reported next to its GenBank accession number. Abbreviations used are: *HK* Hong Kong, *JAP* Japan, *KOR* Korea, *SIN* Singapore, *THAI* Thailand

a period of 3½ decades. Phylogenetic analysis identified 3 main genetic genotypes clustered according to their year of isolation from varying geographical locations (Fig. 3). They are group I, containing CA24v isolated from 1970 to 1971; group II, from 1975; and group III, from 1985 to 2005. Group III is further divided into 3 sub-clusters: group IIIA, containing strains isolated from 1985 to 1986; group IIIB, from year 1987 to 1989; and group IIIC, from 2002 to 2005. The recent Singapore 2005 strains fall into group IIIC, clustering with CA24v strains associated with AHC isolated after the year 2000. The 3 main genotypes, groups I to III, were also shown in similar phylogenetic studies performed in [23, 8]. Furthermore, as reported in Ref. [8], a sub-cluster containing CA24v strains isolated from different geographical locations after the year 2000 was split from genotype group III. This sub-cluster [8] corresponds to group IIIC in our study (Fig. 3), further indicating the possibility of re-classifying this sub-cluster as the fourth genotype for CA24v.

Molecular evolution of the VP4 and 3C gene regions

The evolution rate of CA24v was analysed at the nt level based on VP4 capsid and 3Cpro genes for Singapore strains, isolated during the past 35 years (Fig. 4A and B). The nt substitution rate of the VP4 capsid gene was estimated at 4.2×10^{-3} nt/year, and that of the 3Cpro gene at 1.0×10^{-3} nt/year. The same figure also established a common focal point around the years 1965–1966, when CA24v first emerged.

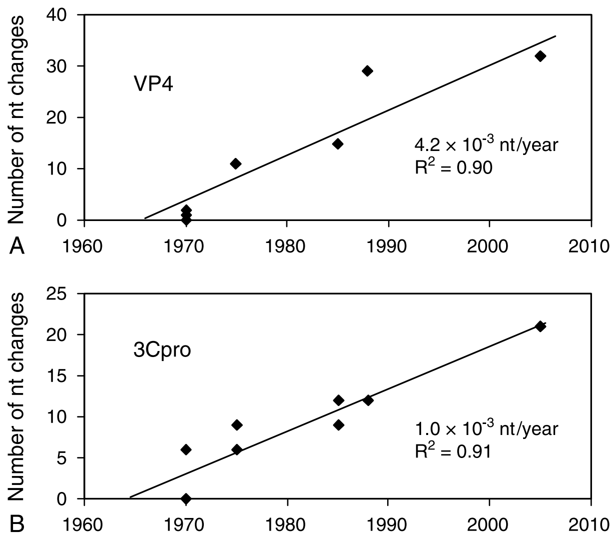

Fig. 4. Estimation of the evolutionary rate of the (*A*) VP4 capsid and (*B*) 3Cpro gene regions. Analysis of the rate of nt changes for Singapore CA24v strains in the VP4 (207 nt) and 3C (511 nt) regions revealed a focal point around the mid-1960s. Evolutionary rates for VP4 and 3C genes were calculated to be 4.2×10^{-3} nt/year and 1.0×10^{-3} nt/year, respectively

Discussion

During the national outbreak of AHC in Singapore in 2005, similar outbreaks were also reported in military camps, located in different parts of Singapore. A total of 103 conjunctival swab specimens were collected from military personnel diagnosed clinically with AHC from 5 military camps, during the months of September, October and November 2005. We used primers EV2F/EV1R, targeting the 5′-UTR of EV, to screen genetic material extracted from the 103 specimens, and we used another set of primers for typing the positive EV specimens at the VP1 gene region. Thirty-one specimens were also innoculated into cell culture for virus isolation. Comparing the rates of virus isolation to PCR-positive results, we had 6/31 (19%) positive with IFA results, and 24/103 (23%) PCR-positive with primers EV2F/EV1R. Our findings demonstrate that direct EV detection from clinical specimens by molecular diagnostics and sequence determination at the VP1 gene region prove to be an efficient and rapid alternative method compared to the traditional method of virus neutralisation. Molecular typing using partial VP1 gene region with primers containing deoxyinosine and mixed bases for codon degeneracy has been reported to be almost 100% specific [29–31]. In our initial reactions, we applied the same degenerate primers directly to the clinical specimens as described in Ref. [34]. However, we failed to recover products from any PCR amplifications. To investigate the sequence divergence, if any, from the newly isolated viruses, we subjected DSO-26SIN05 and DSO-52SIN05 to full-genome analysis (Table 3). Sequence comparison of DSO-26SIN05 and DSO-52SIN05 did not contain any insertions or deletions but showed similar divergence values with the Singapore strain, EH24/70, isolated in 1970, in both the structural and nonstructural proteins, e.g. the 3Dpol gene. Most of the variation seen in the protein-coding region were silent. A smilar observation was made when Ningbo and Hangzhou strains, isolated from China, were compared to EH24/70, indicating that the sequence divergence in the Singapore 2005 strains were not significant.

Analyses of the CA24v strains at the VP4, 5′-VP1 and 3Cpro gene regions revealed genetic clustering of the post-2000 "Asian" strains, from the pre-2000 and "non-Asian" strains. Although it was not possible to use the same set of viruses for all of the analysis due to the restricted availability of sequences, the Singapore 2005 strains remained clustered with the CA24v that were the post-2000 "Asian" strains (Figs. 1–3). These post-2000 "Asian" strains included isolates from Tunisia and Guadeloupe that had a high nt sequence identity with concurrently circulating strains in Asia and were suspected to have been imported from Asia [8]. On the other hand, the pre-2000 and "non-Asian" strains were initially "untypable" using traditional virus neutralization tests [1, 20, 36, 32]. These strains were eventually typed by sequence determination of the VP1 gene and included CA24v strains from Bangladesh, Cambodia, and Mongolia, isolated as non-poliomyelitis-EV strains during surveillance for poliomyelitis. However, molecular typing of some EVs as CA24v remains unrelated to the clinical disease. For example, a CA24v strain denoted as AB207264 Cambodia, was reported to mimic polioviruses in cases of

acute flaccid paralysis (AFP) [3]. The observation that some newly classified CA24vs have unrelated clinical manifestations, is not new. The same phenomenon has been reported for the cardioviruses, in particular the encephalomyocarditis viruses [7], also members of the family *Picornaviridae*.

In CA24v, the reported rates of genome evolution were estimated based on the non-structural 3Cpro gene. The evolution rate for the Singapore CA24v 3Cpro gene was estimated to be 1.0×10^{-3} nt/year, slightly lower than the rates for other non-Singapore CA24v, at 3×10^{-3} nt/year [23], 3.7×10^{-3} nt/year [15], and 4.1×10^{-3} nt/year [27] (Fig. 4B). Our results indicate that the Singapore CA24v has been evolving gradually at a slower rate since its first isolation in Singapore more than 40 years ago. However, once the virus emerged outside Singapore, it could be subjected to different selection pressures in a new environment, leading to a slightly higher evolutionary rate. To date, no evolutionary rates have been estimated for the structural genes of CA24v. In our study, we were able to provide an estimation based on the Singapore CA24v VP4 capsid gene. The VP4 capsid gene was estimated to evolve at a rate of 4.2×10^{-3} nt/year, higher than that calculated for the 3Cpro gene (Fig. 4A). This is in concordance with the fact that structural proteins are subjected to stronger evolutionary pressures than the nonstructural proteins. Variation in the capsid proteins may be advantageous, enabling the virus to escape from host immune responses, while variation in the nonstructural proteins, many of which are enzymes, might be deleterious. In spite of the different evolutionary rates for both the VP4 and 3Cpro genes, a common focal point around the years 1965–1966 was estimated for the emergence of the Singapore 2005 CA24v, corresponding approximately to when the first world epidemic of AHC was reported in the late 1960s [18].

Evolution rates for other HEVs, including EV70 (human EV group D) and EV71 (human EV group A), and polioviruses have also been reported. The evolutionary rate reported for the VP1 capsid gene of EV70 strains collected worldwide was 3.7×10^{-3} nt/year [39], that for the VP4 capsid gene of EV71 genotype B was 3.9×10^{-3} (strains isolated from United States, Japan, and Taiwan, prior to 1990), and that for genotype C was 1.4×10^{-3} (strains isolated after 1990) [6]. We note with interest that the evolution rates of the VP1 gene in EV70 and VP4 gene for EV71 genoptye B closely ressemble the estimated evolution rate of the capsid VP4 gene in the Singapore CA24v and could be attributed to similar evolutionary pressures. In the case of wild-type polioviruses, the evolution rates estimated for the VP1 gene region was at 0.86×10^{-2} nt/year, and for the 2AB gene region it was 1.63×10^{-2} nt/year [10]. These estimated evolutionary rates for polioviruses were higher than that reported for the EVs, including the rates estimated for the Singapore CA24v, probably due to the presence of a higher recombination rate, a well-known characteristic associated with poliovirus genetics (see Ref. [24] for review).

In conclusion, we have identified the likely etiological agent from an outbreak of AHC in the year 2005 from Singapore to be CA24v and have characterized the full genome of 2 CA24v strains. Our sequence comparison, phylogenetic analyses, and evolutionary studies reveal that the CA24v has been evolving constantly since its first emergence in Singapore in the 1960s.

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