

Complete genome sequence analysis of two human coxsackievirus A9 strains isolated in Yunnan, China, in 2009

Jiansheng Liu · Yanju Zhu · Yue Pan ·
Zhengling Liu · Chen Guo · Shaohui Ma

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Abstract Human coxsackievirus A9 (CVA9) is a member of *Enterovirus B* species and may cause aseptic meningitis. The complete genome analyses of two strains CVA9 A242/YN/CHN/2009 and A108/YN/CHN/2009 isolated from aseptic meningitis cases in Yunnan Province, China, in 2009 were performed. These two strains shared 81.3 and 80.7, 81.0 and 81.1 % nucleotide similarity with prototype strain Griggs in the VP1-encoding sequence and the complete genome sequence, respectively. Through phylogenetic analysis and homogeneity analysis for twenty-eight VP1-encoding sequences, CVA9 strains could be divided into four genotypes and the Chinese strains might belong to genotype D. Similarity plot and bootscanning analyses showed evidence of recombination with other EVB viruses. In conclusion, persistent surveillance of circulating enterovirus might help understand the enterovirus evolution.

Jiansheng Liu and Yanju Zhu have contributed equally to this study and are the first authors.

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J. Liu · Y. Zhu · Y. Pan · Z. Liu · C. Guo · S. Ma (✉)
Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College (CAMS & PUMC), 935 Jiao Ling Road, Kunming 650118, Yunnan, People's Republic of China
e-mail: shaohuima70@hotmail.com

J. Liu · Y. Zhu · Y. Pan · Z. Liu · C. Guo · S. Ma
Yunnan Key Laboratory of Vaccine Research Development on Severe Infectious Disease, Kunming 650118, People's Republic of China

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Introduction

Human enteroviruses (HEVs) are single-stranded, positive-sense, non-enveloped small RNA viruses, which are members of the genus *Enterovirus*, family *Picornaviridae*. HEVs have been classified into four species: Enterovirus A, B, C, and D [1]. These species are comprised more than 100 serotypes. Species B consists of all echoviruses (E) and coxsackieviruses B group (CVB), coxsackievirus A9 (CVA9), enterovirus B69 (EVB69), EVB73 -B75, EVB77-88, EVB93, EVB97-98, EVB100-101, EVB106-107, EVB110 [2]. Most enterovirus B infections are asymptomatic, but also associated with the common cold, hand-foot-mouth disease, acute flaccid paralysis, exanthematous disease, aseptic meningitis, and encephalitis [3–5]. E30, E6, CVB3, CVB5, and CVA9 of the enterovirus B species could frequently cause sporadic cases and outbreaks of aseptic meningitis [6–16].

The viral RNA of CVA9 is approximately 7.5 kb long, which contains a long open reading frame (ORF) flanked by a 5' un-translated region (5'-UTR) and a 3'-UTR. The ORF encodes a single polyprotein of 2201 amino acids which is first cleaved into three polyprotein precursors: P1, P2, and P3. P1, P2, and P3 are processed to yield the four structural proteins (VP1–VP4), the non-structural proteins 2A–2C and 3A–3D, respectively [9]. At present, there have already been reports of recombination-related disease outbreaks of HEV [17, 18]. Recombination of HEVs is frequently shown in the genome region encoding the non-structural proteins [19, 20].

In this study, we determined the complete genomes of the CVA9 strains isolated from two patients suffering from

aseptic meningitis in Yunnan, China, and compared the two strains to previously reported strains.

Materials and methods

Virus isolation

CVA9 A242/YN/CHN/2009 (abbreviated to A242) and A108/YN/CHN/2009 (abbreviated to A108) strains were isolated from a 4-year-1-month-old and another 4-year-10-month-old boys in 2009 during meningitis surveillance in Yunnan Province, respectively. The patients were diagnosed with critically severe type of aseptic encephalitis. The stool specimens were collected from patients and processed according to standard protocols. RD (human rhabdomyosarcoma cells), KMB17 (human embryonic lung diploid fibroblast cells), and A549 (human lung cancer cells) cell lines were used to isolate the viruses [21]. Within three passages, samples that induced the cytopathic effect were considered positive and the supernatants were harvested and stored at -80°C .

VP1-encoding sequence RT-PCR, sequencing, and typing

Viral RNA was extracted from cell culture supernatants with a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. RT-PCR was carried out using a PrimeScript One Step RT-PCR Kit Ver. 2 (TAKARA, Dalian, China).

Primer pairs 222 and 224 were used to amplify the partial VP1 gene [22], and primers for amplifying the complete genome were newly designed based on the published sequence of the Griggs strain and part primers were designed by “primer-walking” strategy (Supplementary materials Table 1), the positive PCR products were sequenced at BGI Sequencing Company (Beijing, China). VP1-encoding sequence and complete genome were compared with sequences available in GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>), and the partial VP1-encoding sequences were analyzed using the Enterovirus Genotyping Tool for serotyping [23]. Virus strain showing $>75\%$ nucleotide sequence identity with known enterovirus serotypes was designated the same serotype of HEVs. The complete genomic sequences of the A242 and A108 strains described in this study were deposited in the GenBank database under the accession numbers KM890277 and KM890278.

Phylogenetic analysis

Phylogenetic analyses were conducted using Molecular Evolutionary Genetic Analysis (MEGA) version 5.1

software as described previously [24]. The plot of nucleotide similarity between strains A108 (A242) and 6 (5) strains of HEV-B mentioned above were created using Simplot software version 3.5.1, respectively, with a sliding window of 200 nucleotides moving in steps of 20 nucleotides [25]. Pairwise alignment of the sequences was performed by Geneious Basic 5.6.5 software [26].

Results

Nucleotide sequencing and comparison with CVA9 strains

The complete nucleotide sequences of strains A242 and A108 consisted of 7,447 and 7,425 nucleotides, respectively. The genomes consisted of a 5'UTR of 743 and 742 nucleotides followed by an ORF that encoded a polyprotein of 2201 amino acids and a 3'UTR of 98 and 77 nucleotides, respectively. The variations between these two strains at the nucleotide and amino acid level were 10.4 and 3.8 % in the complete genome and complete coding nucleotide sequence, respectively.

A comprehensive comparison of the nucleotide sequences and deduced amino acid sequence identities between the two Yunnan CVA9 strains and prototype Griggs strain is shown in Table 1. The results indicated that in the structural regions, the two Yunnan CVA9 isolates were closer to each other than to the prototype strain.

Phylogenetic analysis

Due to only 26 VP1-encoding sequences (including 16 Chinese strains) of CVA9 strains were available in GenBank, the phylogenetic tree (Fig. 1) was conducted with 18 Chinese CVA9 strains (Fig. 2, including two novel strains), 9 strains from other countries and the prototype strain based on the VP1-encoding sequences.

The phylogenetic tree indicated that all CVA9 strains could be divided into four genotypes (A–D), and D genotype could be further divided into D1 and D2 subgenotypes (Fig. 1). The prototype strain Griggs and Cuba 163 of 91 belonged to genotype A, while the CO62 and CO79 strains belonged to genotypes B and C, respectively. 18 Chinese strains, two Canada strains (CVA9 Alberta 2010 and 2003), two American strains (Cuba 270 of 90 and Cuba 450 of 90), one France strain (CVA9 CF027040 FRA07), and one Australian strain (06.109.3344) belonged to genotype D and the two strains determined in this study belonged to genotype D2.

Comparisons of the VP1-encoding nucleotide and amino acid homology between the CVA9 genes clusters were performed (Table 2). The VP1-encoding sequences

Table 1 Nucleotide and amino acid identities between the novel CVA9 isolates and the prototype strain Griggs in all sequenced genomic region

Genomic region	A108 vs Griggs		A242 vs Griggs		A108 Vs A242	
	%Nucleotide identity	%Amino acid identity	%Nucleotide identity	%Amino acid identity	%Nucleotide identity	%Amino acid identity
5'UTR	82.2		83.2		91.7	
VP4	77.8	95.7	77.8	94.2	94.7	98.6
VP2	81.7	95.8	81.4	95.4	95.7	98.1
VP3	81.7	95.8	81.4	95.4	95.7	98.1
VP1	81.0	92.4	81.3	92.4	95.9	97.4
2A	78.2	94.6	78.0	95.2	96.8	99.3
2B	77.8	98.0	79.8	98.0	89.2	98.0
2C	81.7	97.6	77.7	84.2	80.0	84.8
3A	76.4	92.1	78.3	91.0	86.5	97.8
3B	80.3	95.5	83.3	95.5	86.4	100
3C	81.1	97.8	82.7	98.9	85.8	98.9
3D	82.5	97.2	80.7	96.3	83.7	97.6
3'UTR	82.5		85.0		83.8	
Complete genome	81.1	96.2	80.7	94.1	89.6	96.2

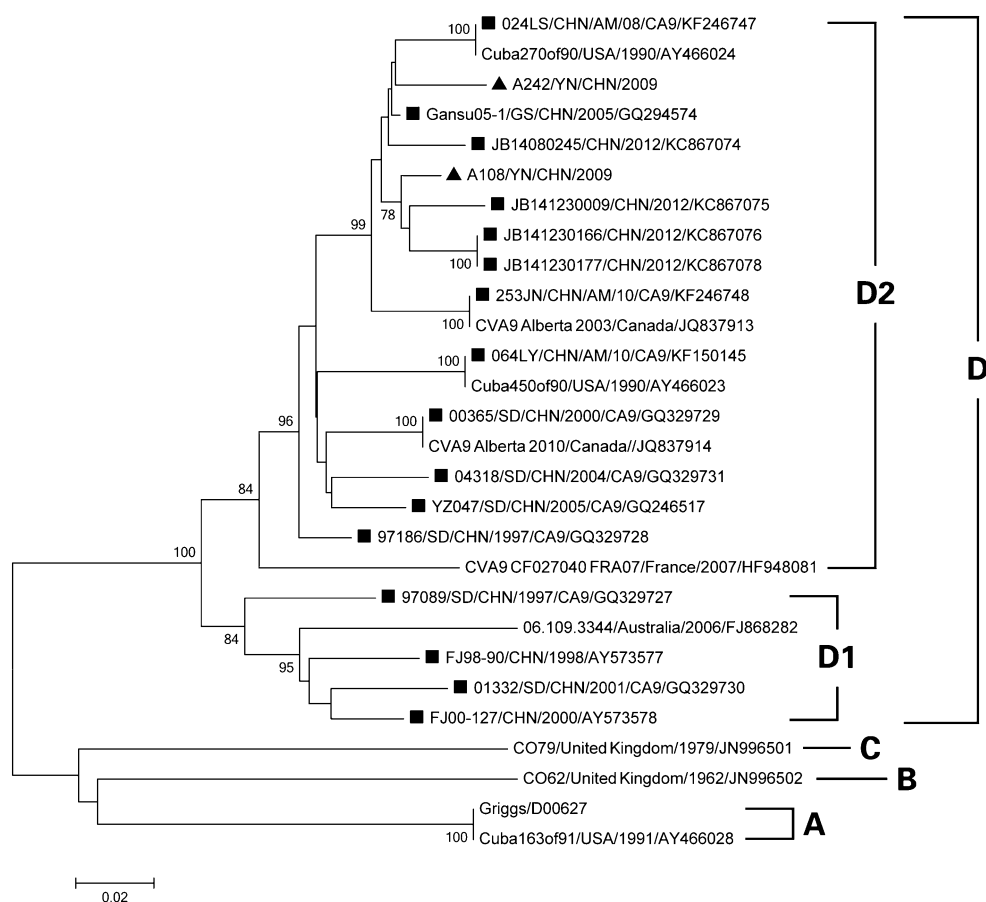
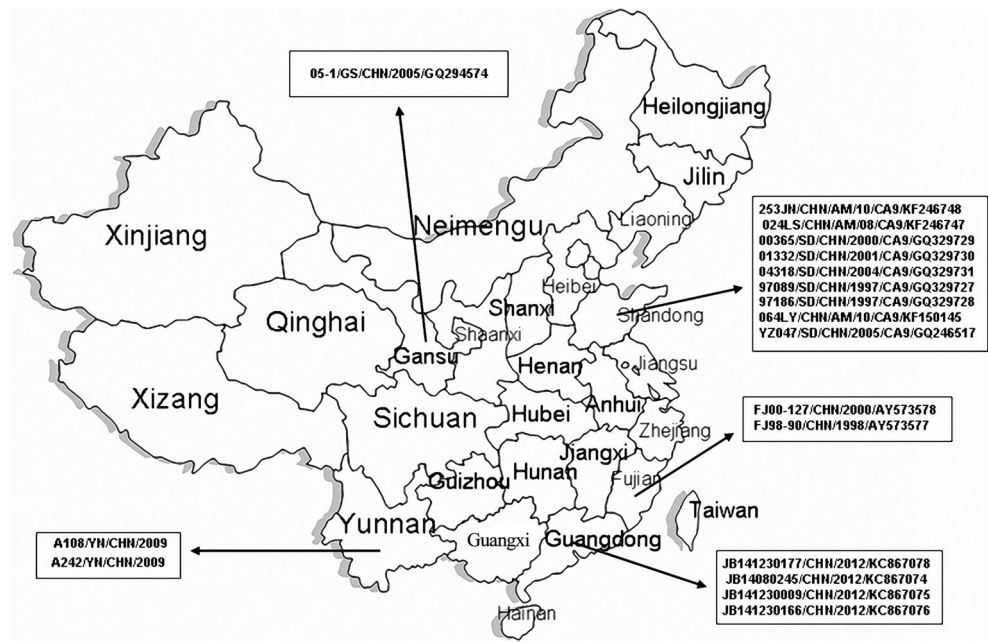
**Fig. 1** Phylogenetic analysis of the two Yunnan CVA9 strains and the reference strains from GenBank using the VP1-encoding sequences. The strains indicated by *triangles* are the CVA9 strains isolated in this study and other CVA9 strains indicated by *squares*

Fig. 2 Geographical distribution of 18 CVA9 strains isolated from different provinces in China, 1997–2012



divergences corresponded to the cut-off values of 25 % at the nucleotide level and 12 % at the amino acid level for human enterovirus identification [27]. The mean VP1-encoding nucleotide sequences divergence within the CVA9 genotypes was 18.7 % (17.5–19.9 %). However, genotype D1, which has nucleotide divergence of 9.4 % (6.3–12.5 %), when compared with genotype D2, was below the average 14.95 % cut-off divergence value assigned for EV71 subgenotyping [28], which was not consistent with the previous classification. The nucleotide and amino acid sequence similarities between strains A108 (A242) and other Chinese CVA9 strains within the VP1-encoding sequences were 86.6–97.1 % (87.8–97.5 %) and 95.0–99.0 % (94.4–97.4 %), respectively.

In addition, phylogenetic trees were constructed in the complete genome, P1, P2, and P3 genomic regions (Fig. 3). In the phylogenetic trees for the complete genome and P1, the isolates A242 and A108 clustered together with CVA9

isolates and the prototype strain Griggs, and in the phylogenetic trees for P2 and P3, A242 clustered with E30SD2010CHN, while A108 clustered with E30/FDJS0384 and Echo30/Zhejiang/17/03/CSF.

Recombination analysis

To further prove potential recombination events, the similarity plot and bootscanning analyses were performed by Simplot software version 3.5.1. Similarity plot and bootscanning analyses for strains A108 and A242 in comparison to 6 and 5 EVB strains are shown in Fig. 4, respectively. In the regions of P1 and P2, A242 and A108 strains were found to be most closely related to CVA9 strains, with similarities ranging from 84 to 98 %, while in the non-structural region P3, A242 had the highest nucleotide identity 94 % with the echovirus 30.

The bootscanning analyses were performed to investigate possible recombination sites within the genomes of strain A242 and A108, respectively. The analysis for A108 demonstrated that two recombination sites were probably located at 2C–3C junction region (between nucleotide positions 4500 and 5625) which was most closely related to E25 HN-2 and 3D region (between positions 6500 and 7300) which was most closely related to CVB1 MSH/KM9/2009 strain. For A242, a recombination site was probably located at 3C–3D region (between nucleotide positions 5000 and 7300) which was most closely related to E30SD2010CHN strain. The findings described above suggested that these two CVA9 strains from Yunnan, China, could be recombinant strains.

Table 2 The VP1-encoding nucleotide and amino acid sequences comparisons between the CVA9 gene clusters

Cluster	A	B	C	D1	D2
A		82.7	82.7	81.5–82.5	79.9–83.1
B	95.4		81.6	81.1–81.2	79.8–81.5
C	94.4	95.4		81.4–81.9	80.3–82.0
D1	92.7	95.4	95.7		87.5–93.3
D2	92.4–93.4	94.4–96.0	95.0–96.4	95.3–98.7	

The data in the upper right corner were for nucleotide homology analysis, and the data in the lower left corner were for amino acid homology analysis

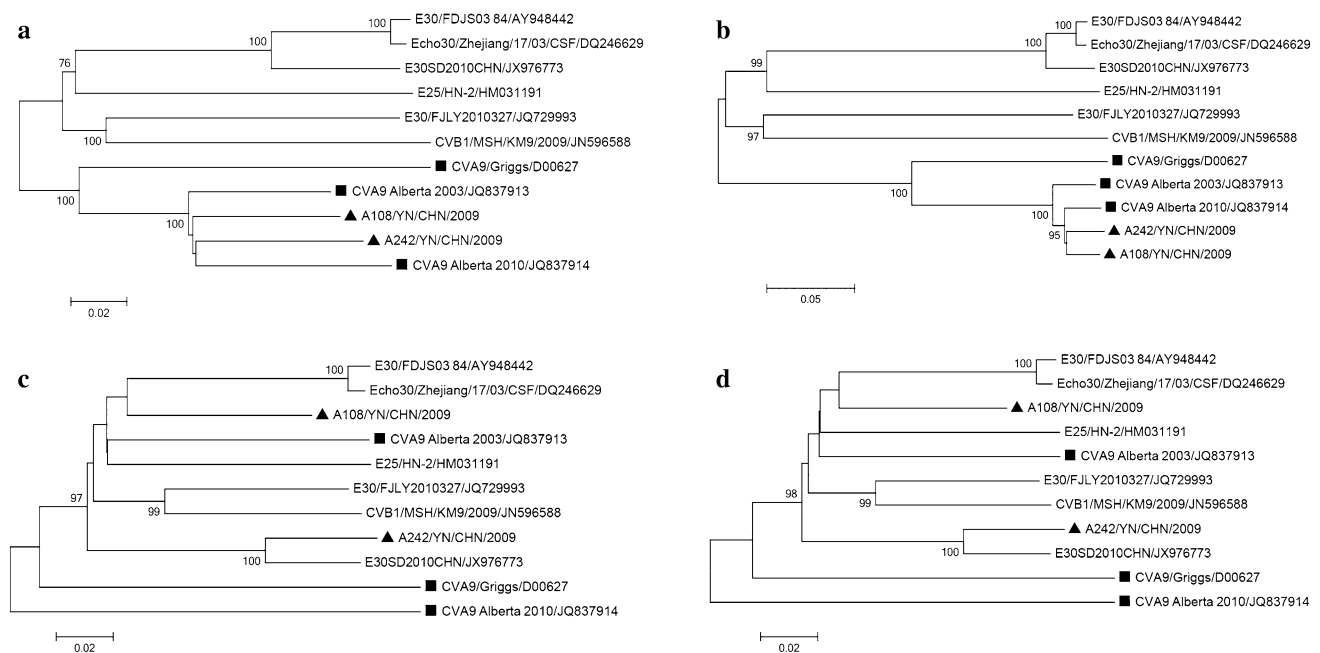


Fig. 3 Phylogenetic relationship of CVA9 strains and other *Enterovirus* generated from a nucleotide sequence alignment using the neighbor-joining algorithm of MEGA5.1 software. Numbers at the nodes indicate bootstrap support for that node (percent of 1000 bootstrap pseudoreplicates). The scale bars represent the genetic

distance. **a** complete genome sequences, **b** P1 coding sequences, **c** P2 coding sequences, and **d** P3 coding sequences. The strains indicated by triangles are the CVA9 strains isolated in this study and other CVA9 strains indicated by squares

Discussion

Due to the lack of proofreading function during RNA replication, like other RNA viruses, CVA9 have a high mutation rate. At present, 26 CVA9 strains were available in GenBank database, including 16 strains isolated in China. Although Chinese strains belonged to the same genotype, the genotype D was further divided into D1 and D2 subgenotypes.

It is reported that CVA9 strains could be assigned to 12 genotypes, which based on VP1/2A [14, 29]. We divided CVA9 strains into four genotypes according to the criteria designated for the enterovirus that strains sharing >85 % identity in the VP1-encoding sequence belonged to the same genotype, drawing a conclusion that Chinese strains belonged to the same genotype. By comparing the VP1-encoding sequence of the CVA9, it was found that Gansu05-1/GS/CHN/2005 strain could not be a new genotype, while it belonged to the same genotype with other Chinese strains, which was not consistent with previous report [14]. At that moment, few VP1-encoding sequences were available at GenBank. Moreover, there was no sequence of VP1/2A region for other Chinese CVA9 strains available in GenBank database. In that paper, phylogenetic analysis was conducted based on the VP1/2A sequences (VP1/2A, nt 3258-nt 3407, relative to strain Griggs) with 37 CVA9 strains. Strain Gansu05-1/GS/CHN/2005 formed an

independent branch in the phylogenetic tree compared with other genotypes of CVA9, but other Chinese CVA9 strains were unable to analyze for genotyping in that paper. So it cannot determine the molecular epidemiology of CVA9 in China. The new genotyping in this study was conducted on the basis of the VP1-encoding sequences of CVA9, and a phylogenetic tree built using the VP1-encoding nucleotide sequences is robust as it takes into consideration changes in the VP1-encoding sequences. Moreover, 18 CVA9 strains isolated from other regions of Chinese mainland and 10 CVA9 strains from other countries were selected. Through the new genotyping, it is easy to analyze the genetical linkage of the sequences between China and other countries and to determine the molecular epidemiology of Chinese CVA9 in China. This indicated that the partial VP1-encoding sequences could not fully reflect the genotyping information. However, due to few complete CVA9 VP1 sequences published in GenBank, it is difficult to know whether co-circulation of other CVA9 genotypes exist in China.

Recombination of HEVs is frequently shown in the non-structural region [19, 20]. By similarity plot and bootscanning analyses of the genomic sequences of Yunnan CVA9 strain A242 and A108 and other EVB strains, the results of our study indicated that A242 and A108 had recombined with other EVB serotypes, respectively. In the non-structural regions, recombination events were

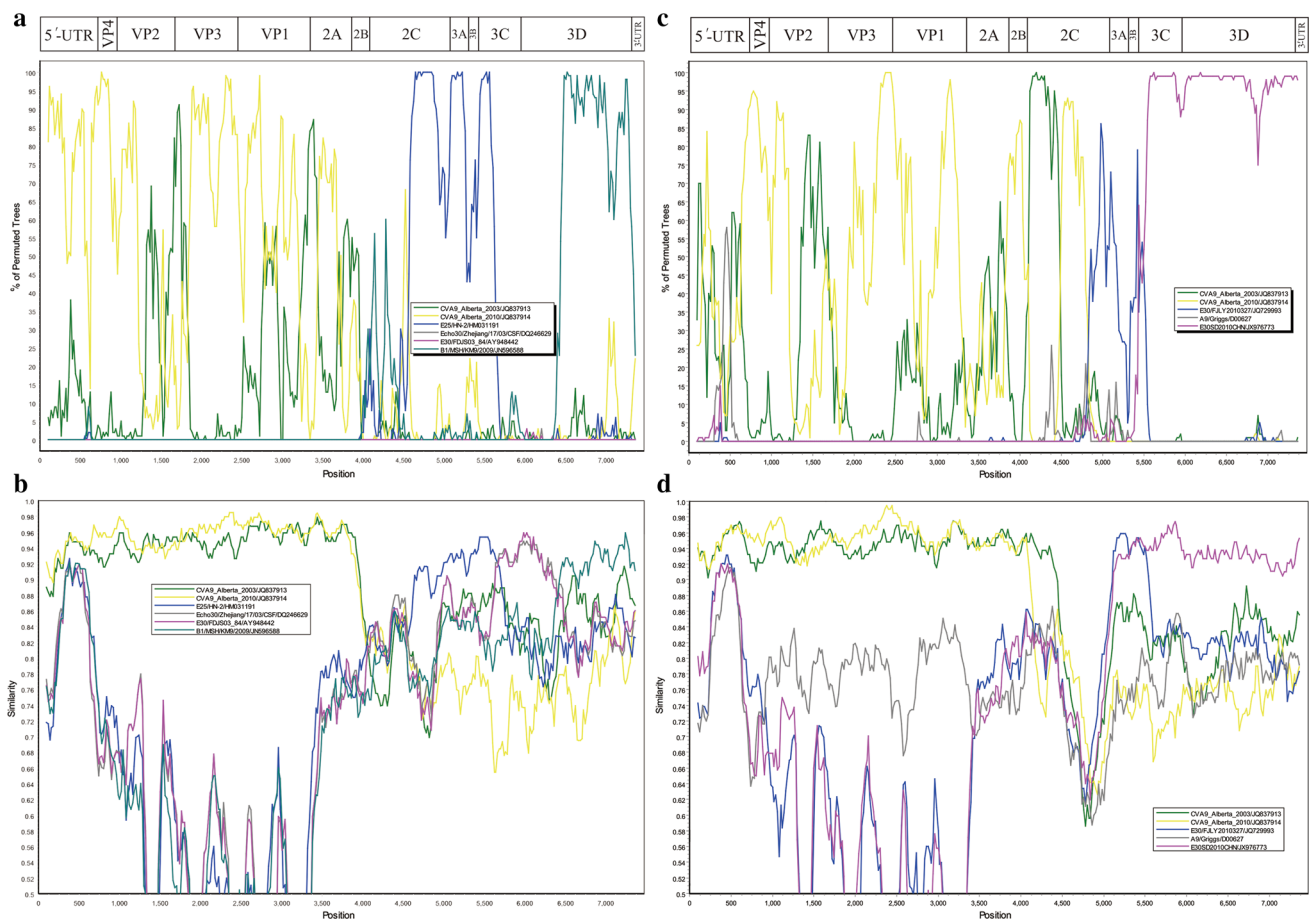


Fig. 4 Similarity plot and bootscanning analysis of the complete genome sequences of the two Yunnan CVA9 strains and other EVB strains. Gene structure organization and bootscanning analysis (*upper panel, a/c*), similarity plot (*lower panel, b/d*) of complete EVB genomes, using a sliding window of 200 nt moving in 20-nt steps.

The A108/YN/CHN/2009 isolate was used as a query sequence of the *left diagram (a, b)* and the A242/YN/CHN/2009 isolate was used as that of the *right one (c, d)*. For each bootscanning analysis, the names of the viruses used as the reference strains were indicated in the *square*

observed between A242 or A108 and some EVB strains, such as Henan (China) E25 strain isolated in 2008, Yunnan (China) CVB1 strain isolated in 2009, and Shandong (China) E30 strain isolated in 2010, with support value more than 90 %. In addition, in all phylogenetic trees for non-structural regions (P2 and P3), A108 and A242 were more closely related to the E30 strains and CVB1 strains. So, the two CVA9 strains from Yunnan, China, could be recombinant strains. Therefore, more genomic sequences of Chinese EVB strains are needed to identify the putative recombination partners and to understand the molecular evolution of the non-structural region. Two CVA9 strains in this study were isolated from patients with aseptic meningitis. However, we could not conclude that CVA9 was the causative agent of the diseases, for samples not taken from CSF, serum, and other sites of lesions in the two patients. Further surveillance might provide more valuable information.

So, analysis of the complete genome of CVA9 may help comprehend the evolution or diversity of the CVA9.

Although some studies on the CVA9 have been performed, further study of the relationships between biological function, virulence, and genetic information is required.

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References

1. C.W. Lo, K.G. Wu, M.C. Lin, C.J. Chen, D.M. Ho, R.B. Tang, Y.J. Chan, *J. Microbiol. Immunol. Infect.* **43**, 354–359 (2010)
2. A.M.Q. King, International Union of Microbiological Societies V.D. (eds.), *Virus taxonomy: classification and nomenclature of viruses : ninth report of the International Committee on Taxonomy of Viruses* (Academic Press, London, 2012)
3. F.B. Yen, L.Y. Chang, C.L. Kao, P.I. Lee, C.M. Chen, C.Y. Lee, P.L. Shao, S.C. Wang, C.Y. Lu, L.M. Huang, *J. Microbiol. Immunol. Infect.* **42**, 38–46 (2009)
4. Y. Aoki, A. Abe, T. Ikeda, C. Abiko, K. Mizuta, I. Yamaguchi, T. Ahiko, *Jpn. J. Infect. Dis.* **65**, 367–369 (2012)

5. C.A. Figueiredo, A. Luchs, D.H. Russo, C.C.R. de Cassia, A.M. Afonso, M.I. de Oliveira, S.P. Curti, J.C. de Moraes, C.M. Toscano, F.H. Ciccone, M.C. Timenetsky, *Arch. Virol.* **159**, 1445–1451 (2014)
6. Z. Mladenova, G. Buttinelli, A. Dikova, A. Stoyanova, M. Troyancheva, R. Komitova, M. Stoycheva, L. Pekova, K. Parmakova, L. Fiore, *Epidemiol. Infect.* **142**, 2159–2165 (2014)
7. H. Xiao, D. Guan, R. Chen, P. Chen, C. Monagin, W. Li, J. Su, C. Ma, W. Zhang, C. Ke, *Virol. J.* **10**, 263 (2013)
8. H.J. Kim, B. Kang, S. Hwang, J. Hong, K. Kim, D.S. Cheon, *Virol. J.* **9**, 38 (2012)
9. K. Pabbaraju, S. Wong, E.N. Chan, R. Tellier, *Virol. J.* **10**, 93 (2013)
10. P. Chen, Z. Tao, Y. Song, G. Liu, H. Wang, Y. Liu, L. Song, Y. Li, X. Lin, N. Cui, A. Xu, *J. Med. Virol.* **85**, 483–489 (2013)
11. M. Miyoshi, R. Komagome, S. Ishida, H. Nagano, K. Takahashi, M. Okano, *Arch. Virol.* **158**, 775–784 (2013)
12. A. Kumar, D. Shukla, R. Kumar, M.Z. Idris, P. Jauhari, S. Srivastava, T.N. Dhole, *Arch. Virol.* **158**, 211–215 (2013)
13. A.H. Wong, C.S. Lau, P.K. Cheng, A.Y. Ng, W.W. Lim, *J. Med. Virol.* **83**, 483–489 (2011)
14. A. Cui, D. Yu, Z. Zhu, L. Meng, H. Li, J. Liu, G. Liu, N. Mao, W. Xu, *Virol. J.* **7**, 72 (2010)
15. N. Mao, L. Zhao, Z. Zhu, X. Chen, S. Zhou, Y. Zhang, A. Cui, Y. Ji, S. Xu, W. Xu, *J. Med. Virol.* **82**, 441–445 (2010)
16. Z. Tao, H. Wang, Y. Li, G. Liu, A. Xu, X. Lin, L. Song, F. Ji, S. Wang, N. Cui, Y. Song, *PLoS ONE* **9**, e89766 (2014)
17. W. Liu, S. Wu, Y. Xiong, T. Li, Z. Wen, M. Yan, K. Qin, Y. Liu, J. Wu, *Co-circulation and genomic recombination of coxsackievirus A16 and enterovirus 71 during a large outbreak of hand, foot, and mouth disease in Central China*, 2014, p. e96051
18. H. Ma, X. Huang, K. Kang, X. Li, X. Tang, Y. Ren, Y. Wang, G. Zhao, B. Xu, *Arch. Virol.* **158**, 2169–2173 (2013)
19. A.N. Lukashev, E.Y. Shumilina, I.S. Belalov, O.E. Ivanova, T.P. Ereemeeva, V.I. Reznik, O.E. Trotsenko, J.F. Drexler, C. Drosten, *J. Gen. Virol.* **95**, 868–873 (2014)
20. A.N. Lukashev, V.A. Lashkevich, O.E. Ivanova, G.A. Koroleva, A.E. Hinkkanen, J. Ilonen, *J. Gen. Virol.* **86**, 3281–3290 (2005)
21. N. Prim, G. Rodriguez, N. Margall, C.M. Del, G. Trallero, N. Rabella, *J. Med. Virol.* **85**, 116–120 (2013)
22. W.A. Nix, M.S. Oberste, M.A. Pallansch, *J. Clin. Microbiol.* **44**, 2698–2704 (2006)
23. A. Kroneman, H. Vennema, K. Deforche, H.V.D. Avoort, S. Penaranda, M.S. Oberste, J. Vinje, M. Koopmans, *J. Clin. Virol.* **51**, 121–125 (2011)
24. H. Li, C.W. Shao, Y. Pan, H.X. Ke, S.H. Ma, *Bing Du Xue Bao* **28**, 108–113 (2012)
25. S.H. Ma, Y. Pan, C.Y. He, J.Y. Chen, H.J. Shi, Q.M. Sun, Q.H. Li, *Zhonghua Liu Xing Bing Xue Za Zhi* **33**, 220–225 (2012)
26. J. Liu, C. Shao, W. Zhao, Y. Zhang, M. Ji, Y. Zhu, Z. Ma, S. Ma, *Zhonghua Liu Xing Bing Xue Za Zhi* **35**, 307–311 (2014)
27. M.S. Oberste, K. Maher, D.R. Kilpatrick, M.A. Pallansch, *J. Virol.* **73**, 1941–1948 (1999)
28. Y.F. Chan, I.C. Sam, S. AbuBakar, *Infect. Genet. Evol.* **10**, 404–412 (2010)
29. J. Santti, H. Harvala, L. Kinnunen, T. Hyypia, *J. Gen. Virol.* **81**, 1361–1372 (2000)