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**Brief communication (Original)**

# Phylogenetic analysis of VP1 region of CA16 isolated from children with severe hand-foot-mouth disease

Yin-Guang Cao[a,b], Zhi-Hui Li[c], Ai-Xia Sun[b], Gui-Qing Yang[b], Le-Xin Wang[d], Xin-Xin Lu[a]
*[a]Clinical Laboratory, Beijing Tongren Hospital, Capital Medical University, Beijing, China, [b]Liaocheng People's Hospital of Taishan Medical University, Shandong, China, [c]Key Laboratory of Rare and Uncommon Diseases, Institute of Basic Medicine Shandong Academy of Medical Sciences, Shandong, China, [d]School of Biomedical Sciences, Charles Sturt University, New South Wales, Australia*

***Background:*** CA16 and enterovirus 71 are two key etiological agents for children's hand-foot-mouth disease (HFMD). Large-scale HFMD outbreaks have taken place every year in Liaocheng City, Shandong Province of China since 2008.
***Objective:*** We investigated the genetic background and phyletic evolution of coxsackie virus A16 (CA16)-related severe HFMD in children from Liaocheng City.
***Method:*** CA16 was screened from throat swab and anus specimens obtained from children with severe HFMD between 2008 and 2010. Specific primers were used to amplify the VP1 region of CA 16 for sequence analysis.
***Result:*** A total of 461 specimens were detected to be enterovirus positive from 2008 to 2010 and 401 specimens were CA16 positive. The nucleotide and amino acid sequences of 16 isolates from children with severe HFMD were compared with the reference sequences, and the nucleotide homology was 91.43%–98.65%, and the amino acid homology was 97.98%–100%. Of the16 isolates, 9 isolates and BJ03-ZDP (AY821798), Shzh00-1 (AY790926), Shzh05-1 (EU262658), and GZ08 (FJ198212) strains isolated from Chinese mainland were located on the same branch; the remaining 7 isolates, the strains isolated from Malaysia, Thailand, Vietnam, Australia and other neighboring countries. AF177911 from Taiwan andshzh01-69 strain (AY895111) from Shenzhen were located on another branch.
***Conclusion:*** CA16 is one of the major pathogens of HFMD and the homology of strains is high.

***Keywords:*** Coxsackie virus A16, enterovirus, hand-foot-mouth disease

Coxsackie virus A16 (CA16), a single, positive-sense stranded RNA virus with no envelope, belongs to the Enterovirus genus of the Picornaviridae family. CA16 and enterovirus 71 are the two main etiological agents for children's hand-foot-mouth disease (HFMD) [1]. EV71 infection can not only cause HFMD, but also serious complications, such as aseptic encephalitis, meningitis, and pulmonary edema [2]. Compared with EV71, CA16 infection usually causes milder HFMD symptoms, with a lower incidence of complications [3]. Recent studies showed that CA16 infection may be associated with the occurrence of lethal complications [4] such as myocarditis, refractory shock, and pneumonia [5]. CA16 genome has a full length of approximately 7410 bp. The VP1 gene has a length of 891 bp, encoding the VP1 protein consisting of 297 amino acids. VP1 protein is the site for the neutralizing antigenic determinant of CA16. It is also the basis for enterovirus serotyping.

Large-scale HFMD outbreaks have taken place every year in Liaocheng City, Shandong Province of China since 2008. This study screened CA16 positive specimens from HFMD children hospitalized in Liaocheng People's Hospital between 2008 and 2010, and used specific primers to amplify its VP1 region for sequence analysis, in order to understand the genetic background and phyletic evolution of CA16-related severe HFMD in Liaocheng region.

***Correspondence to:*** Prof. Xinxin Lu, Clinical Laboratory, Beijing Tongren Hospital, Capital Medical University, Beijing, China. E-mail: luxinxin@trhos.com or Prof Lexin Wang, School of Biomedical Sciences, Charles Sturt University, New South Wales 2678, Australia. E-mail: lwang@csu.edu.au

## Materials and methods

### *Specimen collection*

This study was approved by our institutional review board and informed consent was obtained from all study participants. The throat swab specimens and

anus swab specimens were taken from children with HFMD admitted into the hospital. All of the 472 cases were diagnosed in accordance with *Guidelines for Hand-foot-mouth Disease Diagnosis and Treatment (2010 Edition)* issued by the Ministry of Health of China. For each case of HFMD in our study, a pediatric physician was consulted, and a detailed inquiry regarding the illness and clinical examination was made. In 2008, there were cases in 161 patients, of which 82 were boys and 79 were girls, aged between 1 month and 9 years; in 2009, there were cases in 220 patients, of which 120 were boys and 100 were girls, aged between 8 months to 7 years; and in 2010, there were cases in 91 patients, of which 51 were boys and 40 were girls, aged between 6 months and 10 years. The specimens were stored with virus preservation fluid at –80°C immediately after collection.

***Carrier, enzyme, strains and reagents***

SuperScript II reverse transcriptase was purchased from Invitrogen, pGEM-T Easy Vector System and random primers from Promega, RNA enzyme inhibitor, dNTP, Taq DNA polymerase and restriction enzyme from TaKaRa, QIAamp Viral RNA kit from Qiagen, competent *Escherichia coli* DH5α, IPTG and X-Gal were purchased from the Dingguo Biotechnology.

***RNA extraction***

Viral RNA was directly extracted from clinical specimens according to the manual of QIAamp Viral RNA Mini kit.

***Reverse transcriptase***

With viral RNA as the template, cDNA was synthesized by applying random primers in the presence of reverse transcriptase. The 20 μl reaction mixture included 11 μl viral RNA, random primers and dNTP; the reaction condition was 65°C for 5 min. The reverse transcriptase was added for incubation at 25°C for 10 min, extended at 42°C for 50 min, and denatured at 98°C for 5 min.

***Polymerase chain reaction***

With the corresponding conservative cDNA region of international standard CA16 and EV71 strains and the CA16 and EV71 reference strains of the Asian countries as references, Primer Premier version 5.0 was used to design specific primers, using enterovirus general primers, screening primer sequence, reaction conditions and the reaction system as specified in the literature [6]. General primers (E1/E2) of the 5′ UTR region of enterovirus were first applied to screen enterovirus positive specimens, and then CA16 screening primers (CA16-1/CA16-2) were used to screen CA16 positive specimens. Later the complete VP1 region of some CA16 positive specimens was specifically amplified, with the following primer sequences: VP1-1 5′-AGCCCAGGA CAACTTCAC-3′, VP1-2 5′-GCCGATTCACTACC CTAT-3′.

VP1 region of CA16 was PCR amplified, using the PCR reaction system as above under the following conditions: predenatured at 94°C for 5 min, denaturated at 94°C for 1 min, annealed at 50°C for 1 min, extended at 72°C for 1 min 30 s, 39 cycles, and extended at 72°C for 10 min.

The amplified specific fragments were isolated by 0.8% agarose gel electrophoresis; after the fragments were recovered and purified with a TaKaRa Agarose Gel DNA Purification Kit, they were respectively connected with pGEM-T Easy vector and transformed into competent *E. coli* DH5α. The plasmid was extracted with TaKaRa MiniBEST Plasmid Purification Kit after the positive clones were amplified, and digested by the restriction enzyme.

***Sequencing and phylogenetic analysis***

The fragments were sequenced by the Invitrogen Company, and the software used for fragment assembly and phylogenetic analysis included BioEdit, DNASTAR, ClustalX (1.83), and MEGA4.1. VP1 sequences of 24 CA16 strains of different years and areas were selected from GenBank as reference sequences, and BrCr (EV71) was taken as the outside-group control. The specific reference sequences and their sources are given in **Table 1**.

## Results

***Enterovirus and CA16 detection***

The 161 specimens from 2008, 220 specimens from 2009, and 91 specimens from 2010 were detected with the general primers (E1/E2) of 5′ UTR region of enterovirus; the amplified fragments by PCR with the length of about 435 bp were determined as positive. A total of 461 specimens were detected to be enterovirus positive from 2008 to 2010. Enterovirus positive specimens were then amplified with CA16 screening primers (CA16-1/CA16-2). The fragments amplified by PCR with the length of 882 bp were

determined as CA16 positive. The results showed that 401 CA16 positive specimens were amplified from the specimens of year 2008, 2009 and 2010, and 16 specimens were isolated from children with severe HFMD.

### *Amplification of VP1 region in CA16 positive specimens*

CA16 positive samples were specifically amplified by PCR with specific primers (VP1-1/VP1-2) in VP1 region of CA16. The PCR products had the length of about 1100 bp, and were recovered and purified after 0.8% agarose gel electrophoresis. The purified products were connected and transformed, and plasmid extraction and restriction enzyme digestion were performed. The results are shown in **Figure 1**. The strips with a length of about 1.1 KB were obtained for all of the five samples, which was consistent with our expectation. Homology comparison was conducted on the sequences after being spliced by BioEdit and DNASTAR software. It was confirmed that the acquired sequence covered the full length of VP1 gene.

**Table 1.** Reference sequences for phylogenetic analysis

| Sequence | Year of isolation | Place of isolation | GenBank accession number |
|---|---|---|---|
| G-10 | 1951 | South Africa (SAF) | U05876 |
| MY823-3 | 1997 | Sarawak, Malaysia (SAR) | AM292433 |
| 5079 | 1998 | Taiwan (TWN) | AF177911 |
| S70382 | 1998 | Sarawak, Malaysia (SAR) | AM292461 |
| UM16809 | 1998 | Peninsular Malaysia (MAL) | AM292483 |
| 0001 | 1999 | Perth, Western Australia (AUS) | AM292434 |
| UM12969 | 1999 | Peninsular Malaysia (MAL) | AM292479 |
| SB3512 | 2000 | Sarawak, Malaysia (SAR) | AM292469 |
| TSI-2000 | 2000 | Thailand (THAI) | AM292477 |
| UM17115 | 2000 | Peninsular Malaysia (MAL) | AM292484 |
| Shzh00-1 | 2000 | China (CHN) | AY790926 |
| S33072 | 2001 | Sarawak, Malaysia (SAR) | AM292459 |
| Shzh01-69 | 2001 | China (CHN) | AY895111 |
| EV4-5-HUKM | 2002 | Peninsular Malaysia (MAL) | AM292453 |
| SB7883 | 2002 | Sarawak, Malaysia (SAR) | AM292472 |
| SB13044 | 2003 | Sarawak, Malaysia (SAR) | AM292475 |
| BJ03-ZDP | 2003 | China (CHN) | AY821798 |
| 0033 | 2005 | Perth, Western Australia (AUS) | AM292435 |
| 1018T | 2005 | South Vietnam (VNM) | AM292441 |
| SB16087 | 2005 | Sarawak, Malaysia (SAR) | AM292476 |
| Shzh05-1 | 2005 | China (CHN) | EU262658 |
| CNS68762 | 2006 | Sarawak, Malaysia (SAR) | AM292451 |
| FY18 | 2008 | China (CHN) | EU812514 |
| GZ08 | 2008 | China (CHN) | FJ198212 |
| BrCr | 1970 | United States of America (USA) | U22521 |

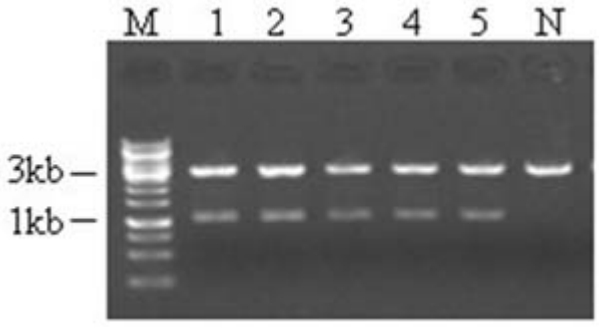


**Figure 1.** Results of the molecular cloning and restriction enzyme analysis of amplified product for CA16 that covers the VP1 region. M: DNA Marker, 1:4232Y, 2:4241Y, 3:4282Y, 4: 5101Y, 5:6041Y, N:pGEM-T Easy Vector

***Homology analysis of nucleotide and amino acid sequences in VP1 region of CA16***

The nucleotide and amino acid sequences of 16 isolates from children with severe HFMD in Liaocheng area were compared, and it was found that the nucleotide homology was 91.43%–98.65%, and the amino acid homology was 97.98%–100%. The amino acid sequence of the VP1 region for 5 isolates was identical. Homology comparison was then performed on the VP1 gene sequences of 16 isolates and those of 24 CA16 isolates from GenBank of different years and areas. The nucleotide and amino acid sequence homology between 16 isolates from children with severe HFMD in Liaocheng area and G-10 international standard strains (EU812514) was lower, which was respectively 78.86%–80.26% and 91.25%–92.26%, and it was also the case for nucleotide and amino acid sequence homology with strains of Fuyang, Anhui (EU812514), which was respectively 78.13%–80.25% and 89.90%–90.91%. Compared with the remaining 22 reference sequences, the nucleotide sequence homology was 88.28%–98.65% and the amino acid sequence homology was 97.31%–100%. **Table 2** shows the results of a comparison of nucleotide and amino acid sequence homology between CA16 isolates from Liaocheng and the reference sequences. It was found that the nucleotide and amino acid sequence homology between the genome sequence of the G-10 international standard strain (U05876) and strains in Fuyang, Anhui (EU812514) from 2008 was respectively 99.82% and 99.64%, and nucleotide and amino acid sequence homology in VP1 region was respectively 99.44% and 98.65%.

***Phylogenetic analysis of CA16 based on VP1 gene***

Phylogenetic analysis was conducted using the neighbor-joining method on 16 CA16 isolates from children with severe HFMD in the Liaocheng area, 24 CA16 strains from GenBank and 1 EV71 international standard strain using MEGA4.1 software. **Figure 2** shows a phylogenetic tree constructed based on the VP1 gene sequence. It can be seen that genetic group A included the G-10 strain (U05876) and the strain from Fuyang, Anhui (EU812514), while the remaining strains belonged to genetic group B. The 16 isolates from children with severe HFMD in the Liaocheng area respectively belonged to two branches on the evolutionary tree. The 9 isolates and BJ03-ZDP (AY821798), Shzh00-1 (AY790926), Shzh05-1 (EU262658), and GZ08 (FJ198212) strains isolated from the Chinese mainland were located on the same branch; the remaining 7 isolates, the strains isolated from Malaysia, Thailand, Vietnam, Australia, and other neighboring countries, AF177911 from Taiwan and shzh01-69 strain (AY895111) from Shenzhen were located on another branch. The SAR-98 strain (AM292461) and SAR-05 strain (AM292476) respectively isolated in 1998 and 2005 from Malaysia were located on two independent branches. The nucleotide sequence homology of the VP1 region between SAR-98 strain (AM292461) and other strains of genetic group B was 88.28%–90.86%, that between SAR-05 strain (AM292476) and other strains of the genetic group B was 89.71%–94.84%; the nucleotide sequence homology of VP1 region between strains of genetic group B excluding these two strains was 91.43%–99.66%.

The variable site analysis of amino acids and nucleotides was conducted on 16 strains isolated from children with severe HFMD, and the results showed that the variations had a scattered distribution, showing no regional clustering.

**Table 2.** Nucleotide and amino acid sequence homology between CA16 isolates from Liaocheng and reference strains

| Reference strains compared with isolates from Liaocheng | Nucleotide sequence homology (%) | Amino acid sequence homology (%) |
|---|---|---|
| G-10 (CA16) | 78.86–80.26 | 91.25–92.26 |
| FY18-08 (CA16) | 78.13–80.25 | 89.90–90.91 |
| Other 22 CA16 reference strains | 88.28–98.65 | 97.31–100 |
| BrCr (EV71) | 80.42–87.50 | 70.03–71.04 |

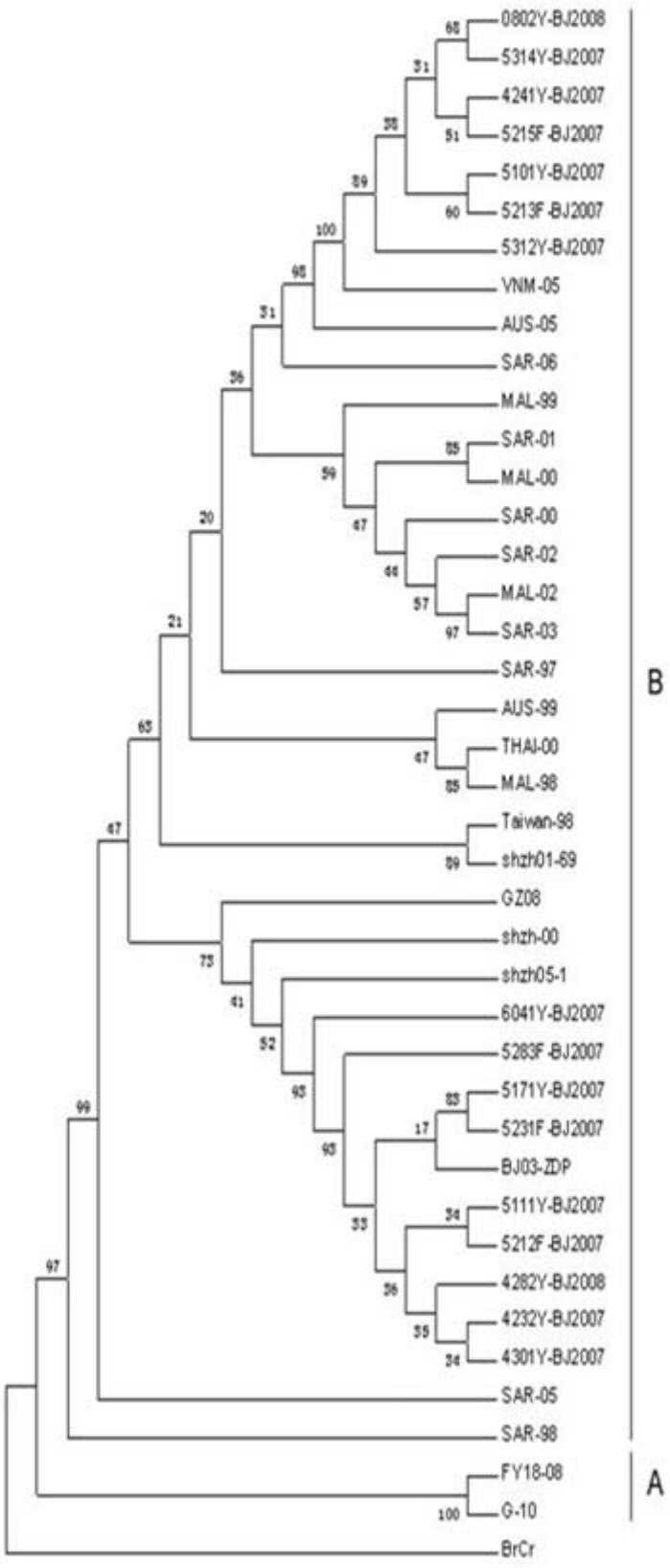


**Figure 2.** The phylogenetic tree of CA16 constructed based on the complete nucleotide sequence of the VP1 gene

## Discussion

Since the 1940s, coxsackie virus has caused hand-foot-mouth disease outbreaks in many countries throughout the world. Research proves that infection by many enteroviruses including CA2, CA4, CA5, CA8, CA10, CA16, CB5, and EV71 will result in hand-foot-mouth disease. Among them, the major etiological agents are CA16 and EV71, which often cause the HFMD outbreaks together, though with variable proportions in each outbreak. CA16 was isolated and identified from HFMD patients in Beijing, Tianjin, Liaoning and other areas in China in the 1980s, which indicates that CA16 is the major etiological agent of HFMD in North China. The HFMD surveys in recent years [6] also show that CA16 and EV71 continue to be the main etiological agents, but there are certain differences in the proportions of the two in the epidemics of HFMD reported by China's provinces and cities.

Compared with EV71, the symptoms caused by CA16 are less severe, and are reported in fewer articles. So far, there are only 6 CA16 strains with complete genome sequences isolated from various countries and regions in GenBank. Recent study has shown that CA16 infection is related to the occurrence of some lethal complications [4, 5]. Therefore, more thorough study of CA16 is warranted.

The standards for phylogenetic analysis of enterovirus vary, usually targeting the VP1 or VP4 gene sequence. In the molecular epidemiology study of EV71, the results obtained from the phylogenetic analysis of VP1 and VP4 sequences were basically consistent [7]. However, when CA16 is submitted to phylogenetic analysis, few VP1 sequences can be used as references, so most of early phylogenetic analysis of CA16 is based on the VP4 sequence. Nevertheless, as the site for the encoding gene of the main neutralizing antigenic determinant, the VP1 region has longer sequence than that of VP4, and contains large amount of information. Thus, it is more suitable for use in researching the variation trend of the virus. In this study, we selected the VP1 sequence for our phylogenetic analysis.

There are currently only a small number of VP1 region sequences of CA16 in GenBank, and most of them are strains isolated from the countries of the Asia-Pacific region. The 24 representative CA16 strains from different countries and times together with an EV71 standard strain BrCr were chosen as the outside-group control, and compared with the VP1 sequences of 16 isolates obtained in this research. It was found through sequence homology analysis that the nucleotide and amino acid sequence homology between 16 CA16 isolates from children with severe HFMD in Liaocheng area was high. However, their homology with the strains isolated from other areas was lower, with certain variations. The nucleotide and amino acid sequence homology with G-10 standard strain was highest; that with strain isolated from Fuyang, Anhui had the next highest homology. The nucleotide and amino acid sequence homology with other reference sequences was even higher. These data indicated that compared with the international standard strains, the CA16 strains have undergone great variations in recent years on both the nucleotide and the amino acid levels, but the homology between the strains in the same area and the same period is higher, indicating that the CA16 variation may be region-dependent.

We constructed a phylogenetic tree based on the nucleotide sequence of the full length of 891 bp of the VP1 region of CA16 (Figure 2). By reference to the standard for EV71 [8] and the standard described by Perera et al. for CA16 [9], which specified that different VP1 genetic groups are indicated at genovariation above 15%. The reference sequence of CA16 listed in **Table 2** and 16 strains isolated from Liaocheng are divided into genetic groups A and B. Genetic group A included the G-10 strain (U05876) and the strain from Fuyang, Anhui (EU812514) in 2008; the other strains were classified into genetic group B. The variation between genetic groups A and B was 19.74%–22.21%, and that between the isolates from genetic group B was lower than 11.7%. It can be seen from the phylogenetic tree in **Figure 2** that 16 CA16 strains isolated from children with severe HFMD in the Liaocheng area were located on two branches with the reference strains. Strains isolated from South and North China provinces and cities such as Guangzhou, Shenzhen, and Beijing were located on one branch; and the strains isolated from Beijing, Australia, Taiwan, Shenzhen, Malaysia, Thailand, Vietnam, and other neighboring countries were located on the other branch. We speculated that the formation of these two clusters on the phylogenetic tree might be related to their phylogenetic source.

Hosoya et al. [10] found that the variation of CA16 is a long process that causes genetic diversity with the passage of time. In one epidemic of HFMD, the strains of a genetic group dominate, while in the

following epidemic, a new genetic group will replace the previous genetic group as the dominant strain. They believed that the repeated outbreaks of HFMD related with CA16 in the Fukushima area of Japan were related to both the accumulated genetic diversity of the virus itself, and the introduction of new genetic groups. The CA16 strains that gathered into clusters on the phylogenetic tree were more closely related to the time of their isolation rather than to the region of their isolation.

In summary, CA16 was a major pathogen of HFMD in Liaocheng City, China, and the homology of strains is high. The strains isolated from children with severe HFMD were classified into genetic group B after comparison with the reference sequences from GenBank. The strains belonged to two branches on the evolutionary tree.

The authors have no conflicts of interest to report.

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