# Molecular epidemiology of human coxsackievirus A16 strains

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**Abstract.** The hand, foot and mouth disease (HFMD) epidemics have mainly been caused by human enterovirus 71 and human coxsackievirus A16 (CA16), which circulated alternatively or together in the epidemic area. The aim of the present study was to provide guidance in the prevention and control of HFMD from CA16 infection. The molecular epidemiology of the human CA16 strains was investigated. Overall, 1,151 specimens (throat swabs) were collected from 1,151 patients with HFMD symptoms. The results of the homology comparison in the VP1 of CA16 strains showed that the CA16 strains belonged to the B1b subgenotype. The difference of the 6 CA16 strains analyzed showed that the most prominent strain was the A genotype, and the most close strains were the B1 gene subtype, particularly the B1b gene subtype. With regards to the amino acids, in addition to the A genotype, the differences of amino acids with other gene subtype was not significant. The present data suggest that more effective and highly targeted intervention mechanisms could be developed for the prevention and control of HFMD.

## Introduction

The hand, foot and mouth disease (HFMD) epidemics have been mainly caused by human enterovirus 71 (EV71) and human coxsackievirus A16 (CA16), which circulated alternatively or together in the epidemic areas (1-5). Studies have mainly focused on EV71 but not CA16, as the most severe or fatal cases were caused by EV71. However, in 1994, CA16 caused the outbreak of the largest HFMD in England (6). Between 1999 and 2006, the leading cause of HFMD was also CA16 in Taiwan (2,579 cases), followed by EV71 (1,760 cases) (http://www.cdc.gov.tw). Surveillance data of Singapore showed that the major circulating virus was CA16 in 2002, 2005 and 2007, while EV71 was only prominent in 2006 (3). Recently, CA16 was also the predominant circulating virus strain of HFMD in mainland China (7,8).

Studies showed that the majority of HFMD with CA16 infections present only mild symptoms (3,4). However, numerous studies reported that the patients with CA16 infections may lead to severe health issues, such as aseptic meningitis, rhombencephalitis, cardiac and pericardial disease, pulmonary complications, spontaneous abortion, and even lethalmyocarditis and pneumonia (3,9-11). CA16 belongs to the enterovirus genus of the Picornaviridae family and is one of the major pathogens associated with human HFMD (1,12). A previous study showed that different genotypes of CA16 may have different neutralizing epitopes or cross-protective capacity (13), suggesting that the confirmation of the CA16 genotype is important to prevent and control HFMD. Retrospective analysis was used in the present study, focusing on the molecular epidemiology of CA16 strains in the Jiujiang region of China in 2012, providing information for HFMD prevention and control in the Jiujiang region.

## Materials and methods

*Patients and clinical specimens*. The patients were identified according to the diagnostic criteria defined by the Ministry of Health (http://www.moh.gov.cn/publicfiles/business/htmlfiles/mohyzs/s3586/201004/46884.htm). Children diagnosed with HFMD, but without serious complications, were classified as having mild HFMD. Patient medical records were reviewed by physicians to collect demographic data, clinical symptoms, clinical diagnoses and outcomes of the in-patient testing. Written informed consent was acquired from parents or guardians of all the participants. The study was approved by the Ethics Review Committee of Jiujiang University (Jiangxi, China). HFMD cases of The Third People's Hospital of Jiujiang in 2012 were counted. Overall, 1,151 specimens (throat swabs) were collected from 1,151 patients with HFMD symptoms in The Third People's Hospital of Jiujiang between January and December 2012, and were tested by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (detection kits for CA16 RNA and EV71 RNA; Daan Gene Co., Ltd., Guangzhou, Guangdong, China). The throat swabs were placed in universal transport medium and stored at

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*Abbreviations:* CA16, human coxsackievirus A16; HFMD, hand, foot and mouth disease

*Key words:* molecular epidemiology, human coxsackievirus A16, Jiujiang region, hand, foot and mouth disease

-80˚C. For RT-qPCR analysis, an ABI7500 Real-Time PCR system (Applied Biosystems, Life Technologies, Waltham, MA, USA) was used as follows: 50˚C for 15 min, followed by 40 cycles of 95˚C for 15 min, 94˚C for 15 sec and 55˚C for 45 sec for EV71 and CA16.

*RNA extraction and RT*. Viral RNA of CA16 was extracted using the viral RNA mini kit (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. RT was performed to synthesize cDNA using an RT kit (Vazyme Biotech Co., Ltd.).

*Classical PCR and direct sequencing for CA16 identification*. To identify the CA16 genotype, the forward primer CVA16VP1 (5'-CATTATTACAATGCCTACCAC-3') and reverse primer VP1 (5'-TCAGTCCCTACTGTCCTAATG-3') were employed to amplify a segment of the VP1 of CVA16 virus, according to the manufacturer's protocol (Vazyme Biotech Co., Ltd.). The reaction product was examined by electrophoresis on a 1.2% agarose gel, with a 100-base pair (bp) DNA ladder serving as a molecular marker, followed by DNA sequencing (Shanghai Sunny Biotech Co., Ltd., Shanghai, China).

*DNA sequencing, nucleotide and amino acid sequences, and phylogenetic analyses*. The sequences obtained from this study were subjected to Basic Local Alignment Search Tool (BLAST) analysis (www.ncbi.nlm.nih.gov/blast), while ClustalW (www.ebi.ac.uk/tools/clustalW2/index.html) was applied for multiple sequence alignment. The sequences were submitted to NCBI (GenBank accession nos. KJ500017, KJ500018, KJ500016, KJ500015, KJ500014 and KJ500013). These sequences were compared using a 378-bp region of VP1 [2785-3162 nucleotide (nt); reference U05876]. Phylogenetic relatedness was evaluated using the Gdula prototype strains and 25 CA16 sequences obtained from other regions of China and worldwide. The sequences were aligned using Lasergene's DNA SeqMan software (version 7.0; DNAStar, Inc., Madison, WI, USA). MEGA 5.0 software (www.megasoftware.net) was used to construct the phylogenetic tree using the neighbor-joining algorithm with 1,000 bootstrap replicates.

## Results

*Epidemiology and genotyping of HFMD*. Fig. 1 shows the number cases of infection of EV71 and CA16 diagnosed by RT-qPCR each month between January and December 2012. Of all the 1,151 samples, 70.5% (811/1,151) of the infections tested positive for the EV71 or CA16 virus by RT-qPCR. EV71 was the most prevalent with 50.8% (585/1,151) in all samples, which peaked in June, followed other enteroviruses in 29.5% (340/1,151) samples, and by CA16 in 19.6% (226/1,151) samples. However, the prevalence of EV71 and CA16 did not differ by geographic location in the Jiujiang region.

*Clinical manifestations of the CA16 infection*. Clinical data were available for patients with CA16 infection. Of all the positive cases, 36.0% (292/811) subjects developed complications. A total of 45 CA16-infected patients (15.4%, 45/292) developed complications such as fever and upper respiratory tract symptoms (cough, runny nose and rapid breathing). The number of females and males was similar. With regards to age group, the highest percentages of CA16-infected patients were children between 1-3 years (89.4%, 202/226).

Table I. Homology of nucleotides and amino acids according to the alignment of VP1 nucleotides of 6 CA16 strains with different representative CA16 strains.

| CA16 genotypes, subtype | Nuclotide homology, % | Amino acid homology, % |
|---|---|---|
| A | 77.8-80.7 | 92.1-93.7 |
| B1a | 91.3-95.8 | 100.0-100.0 |
| B1b | 94.7-97.1 | 99.2-100.0 |
| B2 | 88.4-91.3 | 99.2-100.0 |

CA16, coxsackievirus A16.

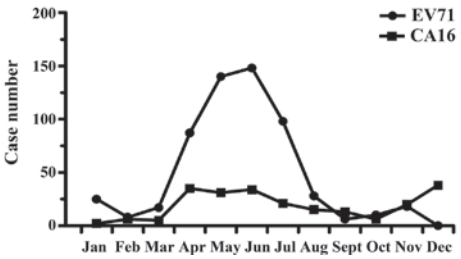


Figure 1. Cases of EV71 and CA16 infection with hand, foot, and mouth disease detected in the Third People's Hospital of Jiujiang City (Jiangxi, China) between January and December 2012. EV71, enterovirus 71; CA16, coxsackievirus A16.

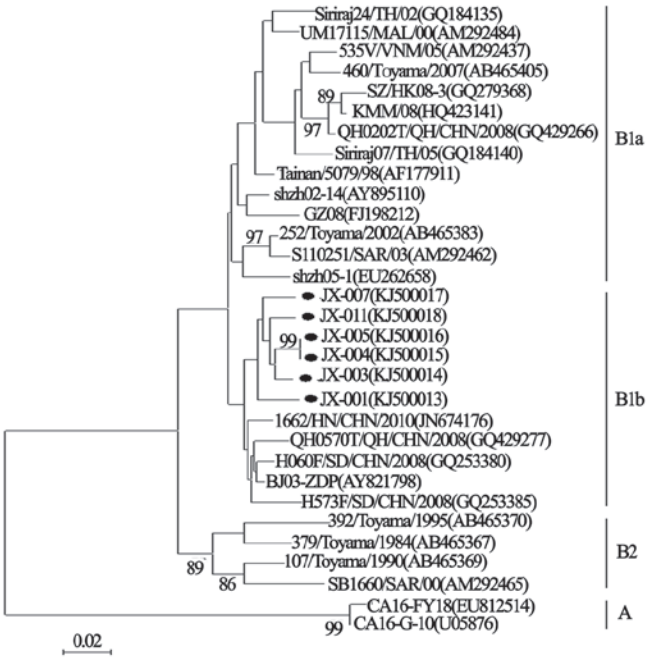


Figure 2. Phylogenetic trees constructed from CA16-positive specimen VP1 nucleotide sequences by the neighbor-joining method with 1,000 bootstrap replicates using MEGA5. The tree was based on a 378-base pair CA16 VP1 sequence (2785-3162 nucleotide, reference U05876). •Strains obtained in the study. CA16, coxsackievirus A16.

*Phylogenetic analysis*. The VP1 gene homology analysis of 6 CA16 strains showed that the nucleotide homology was 96.6-100.0% and the amino acid homology was 100%. Phylogenetic trees were constructed using the 6 sequenced specimens (CA16 VP1, 378 bp, 2785-3162 nt, reference U05876) along with 25 CA16 strains reference sequences downloaded from GenBank. The result of homology comparison in the VP1 of 6 CA16 strains showed that the difference of the 6 nucleotide sequences was largest for the A genotype (77.8-80.7%), and the difference for the B2 gene subtype was large (88.4-91.3%), but the 6 nucleotide sequences were closest in the B1 gene subtype, particularly for the B1b gene subtype (94.7-97.1%) (Table I). With regards to amino acids, in addition to the A genotype (92.1-93.7%), the difference of amino acids with other gene subtype (B1a, B1b and B2) was not large, with differences of 100.0-100.0, 99.2-100.0 and 99.2-100.0%, respectively. The genotype analysis revealed that the CA16 strains of the Jiujiang region belonged to the B1b subgenotype (Fig. 2), commonly found in China. On the basis of this study, the CVA16 strains prevalent in the Jiujiang region in 2012 were genetically similar to the B1b strains of CA16 prevalent in Shandong (GQ253380 and GQ253385), Henan (JN674176), Benjing (GQ429277) and Shenzhen (AY821798).

## Discussion

In the Asia-Pacific region, major epidemics of HFMD are usually caused by EV71 and CA16 (1,12,14,15). The number of HFMD cases has been increasing rapidly in mainland China since 2008, similar to what has been observed in other countries. The major pathogens of HFMD in mainland China are EV71 and CVA16. However, studies have mainly focused on EV71 and not CA16 (6). The present study identified that EV71 is the main etiological agent of HFMD in Jiujiang city of Jiangxi province. However, the exact type of enteroviruses in patients with other types of enteroviruses infection, ~29.5% (340/1,151), could not be determined. While the majority of CA16-associated HFMD infections present only mild symptoms, this study supported this observation. The specimens in the study were collected from patients with mild disease with CA16 infection. Only 45 CA16-infected patients (15.4%, 45/292) developed complications.

The enterovirus VP1 gene has been used extensively in phylogenetic analysis due to the high degree of diversity among virus serotypes (16-18). CA16 strains can be divided into A and B two genotypes. Previous studies showed that the B genotype can be divided into B1 and B2 two subtypes, between 1999 and 2008, and the CA16 strains belong to these two subtypes in mainland China (2,19). Before 2000, the B2 subtype of the CA16 strain was the circulating strain, and subsequently the circulating strain gradually evolved into the B1 subtype (20). In the present study, a 378-bp region of the CA16 VP1 (2785-3162 nt, reference U05876) was used for phylogenetic analysis. The prototype U05876 strain isolated in Finland is evidently different from the strains isolated in the present study. The result of phylogenetic analysis showed that the CA16 strains belong to the B1b genotype in the Jiujiang region of China in 2012. The results of CA16 infection in mice showed that different genotypes of CA16 have different neutralizing epitopes or a cross-protective capacity, indicating a role of the genotype in the prevention and control of HFMD (13). As for the determination of the genotype, analysis of completed genome sequences of CA16 from Jiujiang and other geographic regions could yield a better understanding of enterovirus evolution and genetic variability.

The current phylogenetic analysis showed no substantial sequence difference between certain CA16 strains of Jiujiang and those previously reported in Shandong (GQ253380 and GQ253385), Henan (JN674176), Benjing (GQ429277) and Shenzhen (AY821798). Kilpatrick and Randolph (21) considered that local emergence of a pathogen is commonly driven by changes in human factors. Pathogen invasion results from anthropogenic trade and travel where and when conditions (such as hosts, vectors and climate) are suitable for pathogen drivers, dynamics and control of emerging vector-borne zoonotic diseases, therefore, we consider that similar factors are driving CA16 transmission across the region. Additionally, gathering more sequences from various geographic locations should aid to improve the understanding of CA16 evolutionary associations.

In conclusion, to the best of our knowledge this is the first study to confirm the genotypes of CA16 in Jiujiang city of mainland China. The B1b subgenotype of CA16 was the prevalent agent of HFMD in children of the Jiujiang region in 2012. The study demonstrates that the B1b strains have been circulating in China since 2008, threatening the health of children. The present study provided information for HFMD prevention and control in the Jiujiang region. Therefore, more effective and highly targeted intervention mechanisms could be developed for the prevention and control of HFMD.

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