

# Virological investigation of genetic variation of enterovirus type 71 in hand, foot and mouth disease

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**Abstract.** The aim of the present study was to analyze the sequence of the VP1 gene in enterovirus 71 (EV71) isolates and to explore their genetic evolution, so as to provide a scientific basis for the clinical prevention and treatment of hand, foot and mouth disease. The fecal samples of 590 patients with suspected hand, foot and mouth disease treated at Yan'an Hospital (Kunming, China) between January 2015 and December 2016 were collected and EV71 nucleic acid was detected by fluorescence PCR. The viral RNA of EV71-positive samples was extracted, the VP1 gene was amplified by PCR and the products were sequenced. The VP1 gene sequence was analyzed using DNAMAN and MEGA (version 4.0) software and homologous modeling was performed using Pymol software. A total of 50 EV71-positive samples were identified and the detection rate was 8.47% (50/590 cases). All of the 50 EV71 strains were of the C4 subtype. The genetic distance between the strains detected in the present study and EV71 strains detected in Beijing, Anhui and Malaysia was 0.01-0.03, while that between the strains detected in the present study and Australian strains was 2.11. Homologous modeling indicated that the amino acid sequence of the VP1 gene of the detected strains had a H144Y mutation. There was no significant genetic variation in the EV71 strain within the 2-year period. In conclusion, the EV71 strains detected in the present study was similar to that detected in Beijing, Anhui and Malaysia but different to that from Australia. A point mutation was present in the amino acid sequence of the VP1 gene.

## Introduction

Hand, foot and mouth disease (HFMD) is a common infectious disease in children. The clinical symptoms are mainly lesions of varying severity in the palms, feet and mouth, which may be accompanied by fever, sore throat and diarrhea. Enterovirus 71 (EV71) and Coxsackie virus A16 (CA16) are the most common pathogens in pediatric patients with HFMD (1,2). Infection with EV71 frequently results in severe central nervous system complications, including acute encephalitis, paralytic paralysis, encephalomyelitis and other associated neurological complications that may be fatal, particularly in children aged <5 years. Therefore, HFMD currently poses a serious public health threat (3-5).

EV71 belongs to the enterovirus family of picornaviruses and is composed of a single strand of RNA (~7.4 KB). The VP1 gene encodes the peripheral immunogenic protein, which is the major pathogenic factor of the virus. It has an important role in the adsorption and desorption of viral infection (4,6). Furthermore, this protein includes the major neutralization epitope that may be used for virus identification and evolutionary analysis (7). In the present study, the VP1 gene of EV71, the most important pathogen causing HFMD, was analyzed with the aim of enriching the genetic database of HFMD in Yunnan, China. The results of the present study may provide novel clues to elucidate the molecular genetic pathogenesis of HFMD epidemics and provide a scientific basis for the clinical assessment of HFMD risk and prognosis, early clinical intervention and prevention, as well as the development of vaccines or therapeutic targets.

## Materials and methods

**Subjects and specimen collection.** A total of 590 patients with suspected HFMD were recruited at Yan'an Hospital Affiliated to Kunming Medical University (Kunming, China) between January 2015 and December 2016. The patients included 355 males and 235 females aged 0-36 years. A total of 470 patients were ≥5 years old, 118 patients were between 5-18 years old and 2 patients were ≤18 years old. The patients were primarily treated at the dermatology, pediatric and other clinical departments with the first symptoms being lesions in the palms, feet and oral cavity, accompanied by fever and diarrhea. All patients provided oral informed consent. The

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study was approved by Kunming Yan'an Hospital/Kunming Medical University Affiliated Yan'an Hospital Medical Ethics Committee (document no. 2017-035-01). According to the Hand-foot-mouth Disease Prevention and Control Guidelines (2008 edition) issued by the Ministry of Health of the P.R. China (8), fecal specimens were collected within 7 days of disease onset.

**Fluorescence PCR detection of EV71.** EV71 nucleic acid was extracted from the clinical stool samples of suspected cases using a EV71 nucleic acid detection kit (Jiangsu Mole Bioscience Co., Ltd.) based on the centrifuge column method, according to the manufacturer's protocol. Fluorescence PCR was used to detect the EV71 pathogen using an ABI StepOne Plus system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the parameters presented in Table I.

**EV71 viral RNA extraction from EV71-positive specimens.** The EV71 nucleic acid detection kit (Jiangsu Mole Bioscience Co., Ltd.) was used to extract viral RNA from 50 samples tested positive for EV71, according to the manufacturer's protocol.

**Reverse transcription-PCR (RT-PCR).** The extracted EV71 viral RNA was used as the template for RT-PCR. A reverse transcription-PCR kit (Takara Biotechnology Co., Ltd.) was used for RT-PCR according to the manufacturer's instructions. The components of the reaction system are presented in Table II. The reaction mixture was placed in the PCR instrument, reacted at 65°C for 5 min and placed on ice. The components used for the next reaction are presented in Table III. The thermocycling conditions were as follows: 37°C for 15 min, 50°C for 15 min and 65°C for 10 min. The cDNA obtained was used as a template for the PCR amplification of the VP1 gene.

**PCR amplification of the VP1 gene.** VP1 gene primers were designed using Primer Premier software (version 5.0; www.PremierBiosoft.com; Table IV). Primers were synthesized by Beijing Biomed Technology Development Co., Ltd (<http://www.biomed168.com/>). The product cDNA obtained from the above RT-PCR reactions serves as a template for the PCR amplification of VP1 gene. A 50- $\mu$ l PCR mix consisting of 2  $\mu$ l complementary (c)DNA template, 1  $\mu$ l 30 pmol forward and reverse primers, 5  $\mu$ l 10XeasyTaq DNA buffer, 0.5  $\mu$ l 5 U/ $\mu$ l EasyTaq DNA polymerase, 5  $\mu$ l 2.5 mmol/l deoxyribose nucleoside triphosphate mixture and 35.5  $\mu$ l RNA-free pure water. The PCR cycling parameters were as follows: 1 cycle at 94°C for 3 min, 35 cycles at 94°C for 3 min, 56°C for 30 sec and 72°C for 1 min, and 1 cycle at 72°C and 3 min. The products were subjected to 1% agarose gel electrophoresis, stained with Gelview nucleic acid dye (Beijing Biotek Biotechnology Co., Ltd) and observed using a 6000 gel imaging system (Bio-Rad Laboratories, Inc). PCR products were compared with a DL5000 DNA marker (Takara Biotechnology Co., Ltd.) and the presence of target bands at the required length was considered to indicate a positive result.

**VP1 gene sequencing and analysis.** After purification, VP1 gene amplification products were sent to Beijing Biomed Technology Development Co., Ltd (<http://www.biomed168.com/>) for sequence determination using an ABI3730XL automatic DNA sequence analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Sequence comparison was performed using the Basic Local Alignment Search Tool (National Center for Biotechnology Information).

Table I. Cycling parameters of fluorescence PCR detection of EV71.

Step	Temperature (°C)	Time (min:sec)	Cycles (n)
1	45	15:00	1
2	95	02:00	1
3	94	00:10	40
	60	00:40	

Collection of fluorescence signal data was performed at 60°C.

Table II. First step of reverse transcription of EV71.

Component	Volume ( $\mu$ l)
Oligo dT primer (2.5 $\mu$ M)	1
Random 6mers (20 $\mu$ M)	1
Template RNA	2
dNTP mixture (10 mM each)	1
RNase-free H <sub>2</sub> O	5
Total	10

dNTP, deoxyribose nucleoside triphosphate.

Table III. Second step of reverse transcription of EV71.

Component	Volume ( $\mu$ l)
First-step reaction mixture	10.0
PrimeScript reverse transcriptase (200 U/ $\mu$ l)	1.0
5x PrimeScript buffer	4.0
RNase inhibitor (40 U/ $\mu$ l)	0.5
RNase-free H <sub>2</sub> O	4.5
Total	20.0

com/) for sequence determination using an ABI3730XL automatic DNA sequence analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Sequence comparison was performed using the Basic Local Alignment Search Tool (National Center for Biotechnology Information).

**Gene sequence homology comparison and phylogenetic tree analysis.** Gene sequence comparison and homology analysis were performed with DNAMAN software (version 5.1.0.0; Lynnon Biosoft), according to the software instructions. MEGA software (version 4.0; <https://www.megasoftware.net/>) was used to construct phylogenetic trees with neighbor joining and a Kimura 2-parameter model. The bootstrap value was 1,000. All of the reference strain sequences were derived from GeneBank (<https://www.ncbi.nlm.nih.gov/pubmed>).

**Homologous modeling analysis.** The protein structure database (PDB; <http://www.rcsb.org/>) was searched to select

Table IV. PCR primer sequences and product fragment size.

Site	PCR product (bp)	Primer sequence
Enterovirus 71 VP1	1,200	P1: 5'-GGCTGCAATCGTCTGTTACC-3' P2: 5'-AAGTCCCAGAGCTGTCTTCAAATTATGGGAGAAATCGTC-3'

Table V. Pathogen composition of 359 EV nucleic acid-positive cases.

Year	EV nucleic acid-positive cases	EV71	CA16	Other EV types
2015	203	28 (13.8)	32 (15.8)	143 (70.4)
2016	156	22 (14.1)	26 (16.7)	108 (69.2)
Total	359	50 (13.9)	58 (16.2)	251 (69.9)

Data are expressed as n (%). EV, enterovirus; CA 16, Coxsackie virus A16.

appropriate protein structure templates (PDB ID: 3J23). Pymol software (version 2.3; <https://pymol.org/>) was used to simulate the protein spatial structure of the VP1-coding amino acid sequence variation.

## Results

**Clinical features.** Among the 590 patients with suspected HFMD admitted to Yan'an Hospital Affiliated to Kunming Medical University (Kunming, China) between January 2015 and December 2016, the positive rate of an enterovirus nucleic acid etiology was 60.8% (359/590). The pathogen composition of 359 enterovirus nucleic acid-positive cases is shown in Table V. The age and sex composition of 50 EV71-positive cases is shown in Table VI.

**Electrophoresis of VP1 gene amplicon.** The viral RNA was extracted from EV71-positive samples and the VP1 gene was amplified by RT-PCR. The products were identified by agarose gel electrophoresis and the length of the target gene was 1,200 bp (Fig. 1).

**Sequencing analysis of VP1 gene-positive amplification products.** The VP1 gene amplification products of the 50 EV71-positive samples were sent to Beijing Biomed Technology Development Co., Ltd (<http://www.biomed168.com/>) for gene sequencing. 50 sequencing results were compared with the gene sequences of the EV71 reference strain (KU936121.1) in GenBank, and 50 sequence alignment diagrams in GenBank show homology values between 97.96-99.43%. An example of sequence alignment diagrams is shown (Fig. 2).

**Phylogenetic tree analysis of the EV71 VP1 gene.** After sequence alignment, a total of 15 strains with amino acid sequence variation and homology <99.0% were selected from the 50 EV71 strains. The phylogenetic tree was constructed using the adjacency method and Kimura 2-parameter model with MEGA software (version 4.0; Fig. 3).

Table VI. Age and sex composition of 50 EV71-positive cases.

Age	EV71-positive cases	Male	Female
≤5	35	23 (65.7)	12 (34.3)
5-18	13	9 (69.2)	4 (30.8)
≥18	2	0 (0)	2 (100.0)
Total	50	32 (64.0)	18 (36.0)

Data are expressed as n (%). EV, enterovirus.

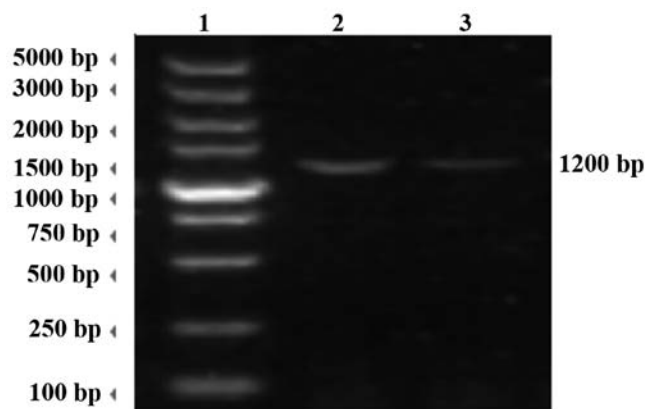


Figure 1. Electrophoresis of the VP1 gene amplification products. Lane 1, DNA marker; lane 2, VP1 amplification product of the positive control EV71; lane 3, VP1 positive amplification product of enterovirus 71.

**Three-dimensional conformation of EV71 virus VP1.** EV71 VP1 contains 297 amino acids and is 32 kD in size. The crystal structure, according to the VP1 type with 'jelly roll'  $\beta$  barrel structure, includes eight  $\beta$  folds arranged in two  $\beta$  patches, each layer containing four  $\beta$  folds (9). VP1 is the major protective neutralization site region of the EV71 virus. Compared with other EV71-associated reference sequences that have been registered in GenBank, an amino acid substitution occurred in

Download ▾ <a href="#">GenBank</a> <a href="#">Graphics</a>				
Enterovirus A71 strain SHAPHC5251/SH/CHN/14, complete genome				
Sequence ID: <a href="#">KU936121.1</a> Length: 7406 Number of Matches: 1				
Range 1: 2228 to 3347 <a href="#">GenBank</a> <a href="#">Graphics</a>			▼ Next Match ▲ Previous Match	
Score	Expect	Identities	Gaps	Strand
1993 bits(1079)	0.0	1110/1123(99%)	10/1123(0%)	Plus/Plus
Query 16	TAGC-AC-CTCATTATAGAGCACATGCCGAGATGGAGTGTGACTACTACACCACAGG	73		
Sbjct 2228	TAGCAACACTCATTATAGAGCACATGCCGAGATGGAGTGTGACTACTACACCACAGG	2287		
Query 74	GTTAGTCAGCATATGGTATCAGACAAATTACGTGGTTCCAAATCGGTGCGCCCAACACAGC	133		
Sbjct 2288	GTTAGTCAGCATATGGTATCAGACAAATTACGTGGTTCCAAATCGGTGCGCCCAACACAGC	2347		
Query 134	CTATATAATAGCACTAGCGGCAGCCAAAAGAAGTTCACTATGAAGTTGTGCAAGGATGC	193		
Sbjct 2348	CTATATAATAGCACTAGCGGCAGCCAAAAGAAGTTCACTATGAAGTTGTGCAAGGATGC	2407		
Query 194	TAGTGATATCTTCAGACGGGACCATCCAGGGAGATAGGGTGGCAGATGTAATTGAAAG	253		
Sbjct 2408	TAGTGATATCTTCAGACGGGACCATCCAGGGAGATAGGGTGGCAGATGTAATTGAAAG	2467		
Query 254	TTCCATAGGAGATAGCGTGAGCAGAGCTCTCACTCGGCTCTACAGCACCCACAGGCCA	313		
Sbjct 2468	TTCCATAGGAGATAGCGTGAGCAGAGCTCTCACTCAAGCTCTACAGCACCCACAGGCCA	2527		
Query 314	GAACACACAGGTGAGCAGTCATCGACTGGATACAGGCAAGGTTCCAGCACTCCAAGCTGC	373		
Sbjct 2528	GAACACACAGGTGAGCAGTCATCGACTGGATACAGGCAAGGTTCCAGCACTCCAAGCTGC	2587		
Query 374	TGAAATGGAGCATCATCAAATGCTAGTGACGAGAGCATGATTGAGACACGCTGTGTCT	433		
Sbjct 2588	TGAAATGGAGCATCATCAAATGCTAGTGACGAGAGCATGATTGAGACACGCTGTGTCT	2647		
Query 434	CAACTCGCATAGTACAGCTGAGACCACTCTTGACAGTTTCTTCAGTAGGGCGGGATTAGT	493		
Sbjct 2648	CAACTCGCATAGTACAGCTGAGACCACTCTTGACAGTTTCTTCAGTAGGGCGGGATTAGT	2707		
Query 494	TGGAGAGATAGATCTCCCTCTTGAGGGCACAACTAACCCTAATGGTATGCCAACTGGGA	553		
Sbjct 2708	TGGAGAGATAGATCTCCCTCTTGAGGGCACAACTAACCCTAATGGTATGCCAACTGGGA	2767		
Query 554	CATAGATATAACAGGTTACGGCAGAAATGCGTAGAAAGGTAGAGCTATTCACCTACATCGG	613		
Sbjct 2768	CATAGATATAACAGGTTACGGCAGAAATGCGTAGAAAGGTAGAGCTATTCACCTACATCGG	2827		
Query 614	CTTTGATGCAGAGTTCACTTTTGTGGGTGCACACCCACCGGGGAAGTTGTCCACAATT	673		
Sbjct 2828	CTTTGATGCAGAGTTCACTTTTGTGGGTGCACACCCACCGGGGAAGTTGTCCACAATT	2887		
Query 674	GCTCCAATATATGTTGTACCTGGAGCCCTAAGCCAGATTCTAGGGAATCTCTTGC	733		
Sbjct 2888	GCTCCAATATATGTTGTACCTGGAGCCCTAAGCCAGATTCTAGGGAATCTCTTGC	2947		
Query 734	ATGGCAAAACGCCACTAACCCTCAGTTTGTGCAAGTTGTGAGACCTCTGCGCAGGT	793		
Sbjct 2948	ATGGCAAAACGCCACTAACCCTCAGTTTGTGCAAGTTGTGAGACCTCTGCGCAGGT	3007		
Query 794	TTCAGTGCCATTTATGTCACTGCGAGTGCTATCAATGGTTTATGACGGATATCCAC	853		
Sbjct 3008	TTCAGTGCCATTTATGTCACTGCGAGTGCTATCAATGGTTTATGACGGATATCCAC	3067		
Query 854	ATTGGGAGAACACAAACAGGAGAAAGATCTTGAATACGGGGCATGTCCTAATAACATGAT	913		
Sbjct 3068	ATTGGGAGAACACAAACAGGAGAAAGATCTTGAATACGGGGCATGTCCTAATAACATGAT	3127		
Query 914	GGGCACGTTCTCGGTGCGGACTGTGGGAACCTCCAAGTCCAAGTATCCTTTAGTGATTAG	973		

Figure 2. Example of the enterovirus 71 VP1 sequence alignment. Sbjct, subject.

the 144th amino acid (histidine → tyrosine) encoded by the VP1 gene of the EV71 virus strain in the present study. As indicated in the three-dimensional conformation presented in Fig. 4, the amino acid mutation site H144Y was located on the surface of the VP1 protein (PDB ID: 3J23 template used for modeling).

## Discussion

HFMD is a common infectious disease in children resulting in skin rashes and lesions on the palms, soles of the feet, oral mucosa, tongue and buttocks, and may be accompanied by



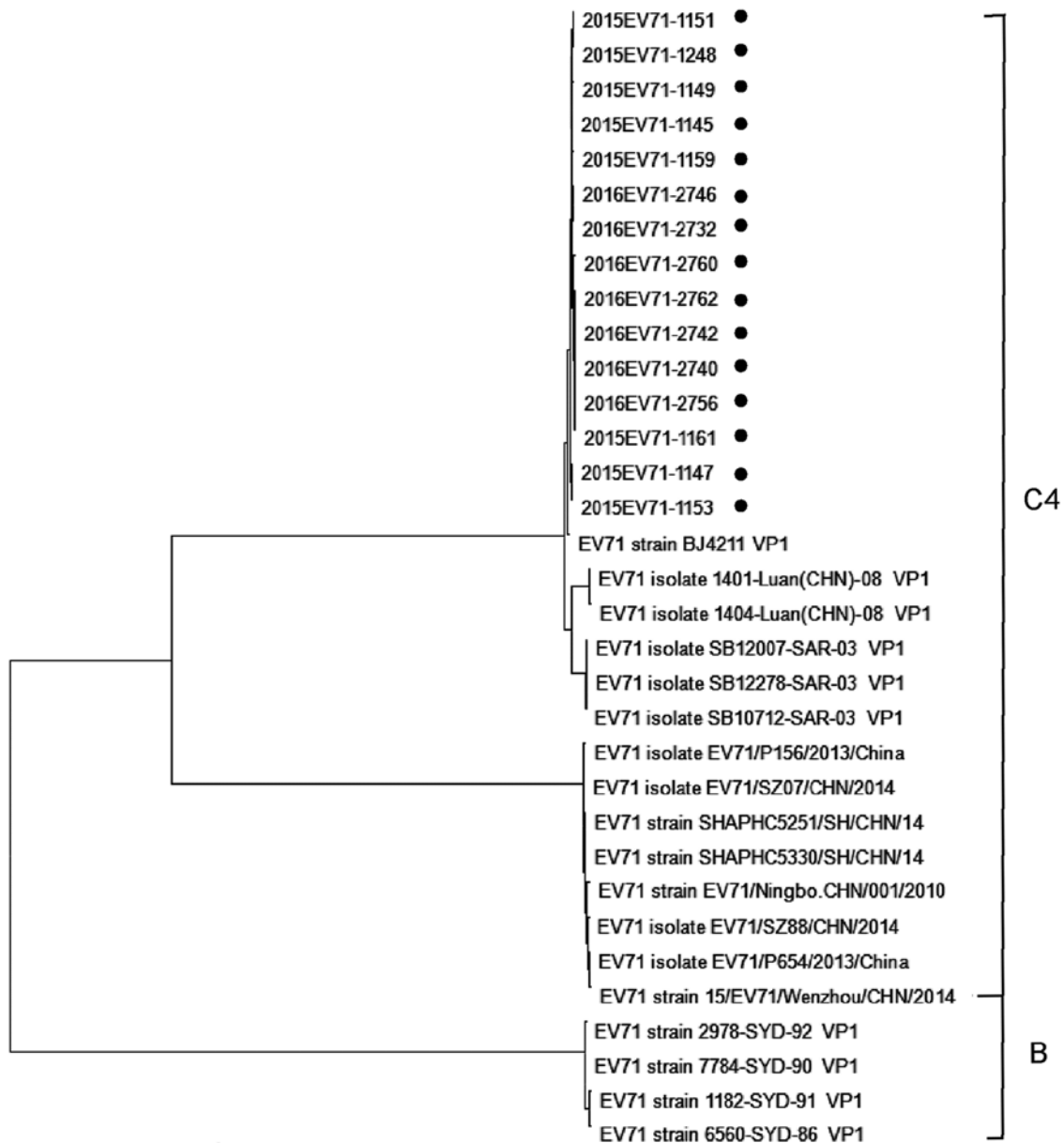


Figure 3. Amino acid evolution tree derived from the enterovirus 71 VP1 gene. ●Indicates strains isolated in the present study.

fever and diarrhea. The disease may be caused by various pathogens, including EV, echovirus and Coxsackievirus, the most common of which are EV71 and CA16. However, other subtypes may also cause HFMD (10), including CA4, CA6, CA10, CA12, CB3, CB5 and echovirus types 4, 19 and 30 (11). While HFMD frequently occurs in preschool children, particularly those aged <5 years, adults may also be infected and the prevalence of HFMD in adults has been exhibiting an increasing trend (12,13). HFMD is prevalent in Southeast Asia, including Taiwan and mainland China, Singapore, Malaysia and Japan. HFMD was first reported in mainland China in 1981, in Shanghai, and associated cases have been since reported in other regions (14,15).

In the present study, a total of 590 fecal samples were obtained from patients with suspected HFMD and a total of 50 EV71-positive patients were identified by fluorescence PCR, with a detection rate of 8.47%. Among the 50 EV71-positive

patients, 32 were male and 18 were female. Furthermore, 2 female patients were adults, which is consistent with the reported low incidence of HFMD in adults and the susceptibility of adult females to viral infection (16). High psychological pressure, physical fatigue, malnutrition, low immunity and close contact with patients with HFMD are important risk factors for HFMD in adults (17). Adults infected with HFMD are prone to cluster infection and infecting family members. Furthermore, adults may be asymptomatic but still highly infectious. In recent years, there have been reports on severe HFMD and serious complications in adults (18,19). Therefore, it is important to prevent and control the prevalence of HFMD in adults.

EV71 is divided into three genotypes, A, B and C, of which B and C may be subdivided into B1-B5 subtypes and C1-C5 subtypes. Previous studies have indicated that the D, E and F subtypes of EV71 have been isolated in India, Hong

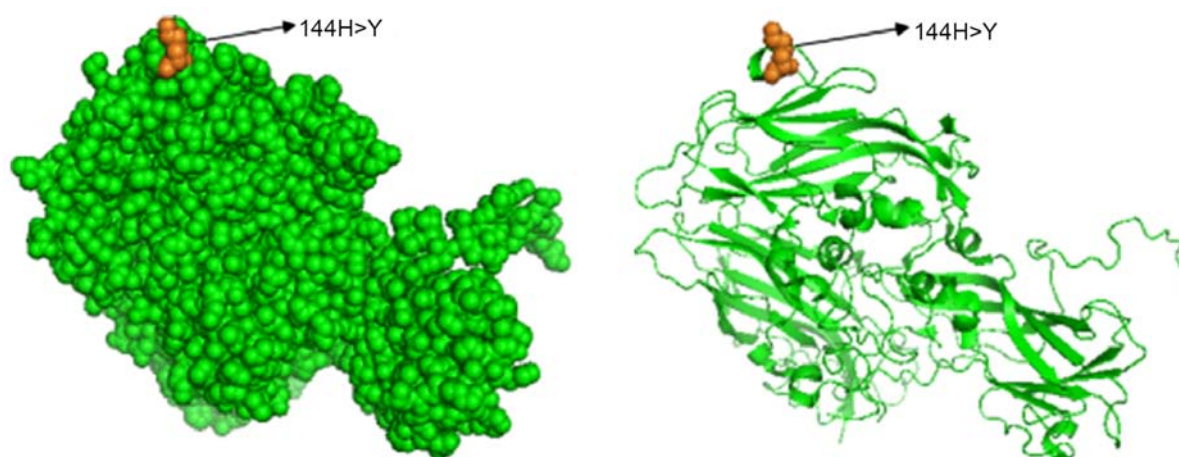


Figure 4. Three-dimensional structure of the enterovirus 71 VP1 amino acid mutation site. The left panel refers to the tertiary structure and right panel refers to the secondary structure.

Kong and Africa (20,21). The VP1 gene sequences of 50 EV71 strains detected in the present study were compared with the EV71 reference strains in GenBank for sequence comparison and homology analysis, and it was confirmed that all of the 50 strains were of the EV71 C4 subtype, which was the same as the EV71 subtype isolated by Fu *et al* (22) in Kunming, Yunnan province in 2011.

In the present study, through sequence comparison analysis, a total of 15 strains with different amino acid sequences were screened out from the 50 EV71 strains, including EV71-VP1-1145-1147, -1149, -1151, -1153, -1159, -1161 and -1248 in 2015 and EV71-VP1-2732, -2740, -2742, -2746, -2756, -2760 and -2762 in 2016. Phylogenetic trees were constructed with MEGA software (version 4.0) and the adjacency method and a Kimura 2-parameter model were used to analyze the genetic origin, variation and association of the virus with other strains in China and in other countries. The viral strains detected in the present study were compared with those in Beijing (EV71 strain BJ4211 VP1), Hefei [EV71 isolate 1401-Luan (CHN)-08 VP1, Anhui, 2008; EV71 isolate 1404-Luan (CHN)-08 VP1] and Sarawak Prefecture, Malaysia [no. EV71 e SB12007-SAR-03 VP1; EV71 isolate 1401-Luan (CHN)-08 VP1]. SB12278-SAR-03 VP1 and EV71 isolate SB10712-SAR-03 VP1 are all on the same branch and the genetic distance is close (0.01-0.03), which indicates that the origin of the virus is similar. The strains isolated from Zhejiang, Ningbo province, in 2010 (EV71 strain EV71/Ningbo. CHN/001/2010), Shanghai in 2014 (EV71 strain SHAPHC5251/SH/CHN/14), Shenzhen in 2014 (EV71 isolate EV71/SZ07/CHN/2014; EV71 isolate EV71/SZ88/CHN/2014), Wenzhou in 2013 and 2014 (EV71 isolate EV71/P156/2013/China; EV71 isolate EV71/P654/2013/China; EV71 strain 15/EV71/Wenzhou/CHN/2014) were in the same evolutionary lineage, and there were certain differences in amino acid sequence, with a genetic distance of 1.52. The genetic distance of strains isolated from Australia in 2006 (EV71 strain 2978-SYD-92 VP1; EV71 strain 7784-SYD-90 VP1; EV71 strain 1182-SYD-91 VP1; EV71 strain 6560-SYD-86 VP1) was 2.11 and the amino acid sequence was quite different (23).

In the present study, 15 strains of variable EV71 strains over two years were selected and subjected to genetic analysis.

No major changes in genetic variation were observed over the 2-year period; however, from an evolutionary perspective, there were still certain differences. The strains detected in the same year had relatively closer genetic distances. This may be due to the small variation of genes and virulence in the region. EV71 strains from the Sarawak region in Malaysia had high homology with strains from the southwest border of China. In recent years, trade and tourism have led to the spread of pathogens, particularly HFMD, and safety monitoring, prevention and control should be implemented to avoid more widespread and serious infection.

The present study revealed that an amino acid substitution occurred in the 144th amino acid of the VP1 protein in the EV71 virus strain in 2015 and 2016. The three-dimensional conformation suggested that the amino acid mutation site H144Y was located on the surface of the VP1 protein. Researchers have used biological software analysis to predict that EV71 epitopes may be distributed between 90-120, 150-170, 200-240 and 230-250 amino acids (24). Through sequence comparison, Li *et al* (25) screened the highly conserved epitopes of EV71 among different gene subtypes as SP55 (amino acid position 163-177) and SP70 (amino acid position 208-222) in the VP1 protein and demonstrated that these epitopes may induce the production of specific neutralizing antibodies against the EV71 virus in mice, which are ideal targets for multi-epitope vaccines. Other studies suggested that EV71 is able to infect white blood cells by binding to specific receptor molecules called P-selectin ligand-1 (PSGL-1) (26). The residue 145 in the capsid protein VP1 of the PSGL-1-binding virus was G or Q (VP1-145G or Q), while that of PSGL-1-unbinding virus was VP1-145e (26). VP1-145 acts as a switch to control the binding of the virus to PSGL-1 by adjusting the exposure of VP1-244K (26). Whether the VP1-144 mutation identified in the present study affects the binding of virus to target cells remains to be further explored.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

YW, XG and HG conceived and designed the study. YW, XYF, YL, YY and CP performed the experiments. YW, YL and YY analyzed the data. YW and YL wrote the manuscript. XG and HG revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The study was approved by Kunming Yan'an Hospital/Kunming Medical University Affiliated Yan'an Hospital Medical Ethics Committee (document no. 2017-035-01). The patients provided oral informed consent and the requirement for written informed consent was waived by the ethics committee.

## Patient consent for publication

Not applicable

## Competing interests

The authors declare that they have no competing interests.

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