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Genetic Analysis of the P1 Region of Human Enterovirus 71 Strains and Expression of the 55 F StrainVP1 Protein*

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Abstract: Enterovirus 71 (EV71) is a member of the Entero-virus genus of the Picornaviridae family and is the major cause of Hand, foot, and mouth disease (HFMD) in children. Different strains from Gansu were cloned and the P1 protein was sequenced and analysed. Results indicate that there are three kinds of EV71 infections prevalent in Gansu. The VP1 protein from one of these strains, 55F, was expressed. The recombinant protein was expressed with high level and reacted specifically with the EV71 patient antibody, the recombinant protein was also applied to raise antiserum in rabbits and after the fourth injection a high titer of antiserum was detected by ELISA assay. These data are useful for further clarification of prevalent EV71 strains in the north of China at the molecular level and provide a basis for EV71 diagnosis.

Key words: EV71; Genetic analysis; P1 region; Expression; VP1 protein

Enterovirus 71 (EV71) is a member of the Entero-virus genus of the Picornaviridae family and is the major cause of Hand, foot, and mouth disease (HFMD) in children. EV71 infection can lead a wide spectrum of clinical disease ranging from mild febrile illness to more severe conditions, such as HFMD, poliomyelitis–like paralysis and neurogenic-pulmonary oedema[14]. It was first reported in California in 1969. Later, many countries reported the EV71 epidemic in different areas and was first reported in Wuhan, China in 1995[8, 9]. In 2008, a large HFMD epidemic caused by EV71 infection, occurred in China resulting in about 25 000 cases and 34 deaths. From 2009 to 2010, the number of EV71 infection cases and deaths has increased gradually and the EV71 outbreaks have become an important public health issue in China.

EV71 is a single stranded, positive-sense RNA virus with a genome approximately 7.5 kB in length, has untranslated regions at both the 5' and 3'ends and a single polyprotein that divided into three different genomic regions. The P1 gene region encodes for the structural proteins VP1 to VP4. The P2 and P3 genomic regions encode for non-structural proteins

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2A, 2B, 2C, 3A, 3B, 3C and 3D[3]. The VP1 is the most external and is immunodominant among the viral capsid proteins, it is located mainly on the virion surface and is the source of many major neutralization sites. Several studies have indicated the potential of the VP1 protein to act as an antigen in both diagnosis and subunit vaccine development against EV71[5,15]. Based on the VP1 nucleotide sequence the enterovirus 71 is divided into three genogroups A, B, and C. Most EV71 isolates belong to either genogroups B or C, which are each further divided into five genotypes or subgenogroups, B1–B5 and C1–C5. EV71 genogroup C has become dominant in Europe in recent years with genotypes C1 and C2 occurring in United Kingdom, and genotypes C1 and C4 occuring in Austria and Germany[1,2,6,11]. The BrCr isolate from USA was believed to be the only A subtype, but recently additional strains belonging to the A subgenogroup have been isolated in China[17]; this is also the first time that the other subtypes have been reported in China. Although many isolates have been cloned, there have been no reports about P1 sequences from Gansu province. In this study the P1 sequence of different strains from Gansu were cloned. For 55F, one of the strains the VP1 protein was expressed. These data are useful for further clarification of prevalent EV71 strains in the north of China at the molecular level and provide a basis for EV71 diagnosis.

MATERIALS AND METHODS

Viruses and Cells

The HFMD specimens were collected from 11 children in Gansu province, China, and were detected positive using RT-PCR[7]. Vero cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, USA) containing 10% fetal calf serum (GIBCO, USA) and 5% CO2 in air. Specimens were cultured after 2 generations in Vero cell and were plaque-purified two times. The isolated clones were passaged in vero cell for 2 generations and named 11X, 11Y, 46F, 47F, 51F, 55F, 901, 902, 903 and 917 respectively. Among these strains, 11X and 11Y were from fatal cases with encephalitis, the others were from children with mild symptoms, CoA16 detection for all the specimens were negative.

RNA Extraction

Total RNA was isolated from infected cell samples using a RNA extraction kit (Qiagen, Germany) following the manufacturer's instructions.

RT-PCR

The RT-PCR amplifications were carried out using the following primer set (P1S: ttaaaacaccctgtgggttg, P1R:agggtggtcacagacttcaa) and an RT-PCR amplification Kit (Toyobo, Japan) according to the manufacturer's instructions.

Cloning and sequencing

RT-PCR products were purified using a Gel Extraction Mini Kit (TaKaRa) and were ligated to the pMD18-T plasmid (TaKaRa). Competent *E.coli* JM109 were transformed with the recombinant vector. Positive clones were sequenced by the Sunbio Biotechnology (Beijing) Co. Ltd.

Phylogenetic analysis

Phylogenetic analysis was performed for the EV71 strains to compared with other strains retrieved from GenBank. The sequencing data were aligned using the ClustalW method (Lasergene program version 7.1, DNASTAR Inc. Madison, WI). Phylogenetic trees were constructed for the *P1* genes using the bootstrap

N-J method in the DNASTAR program using 1000 bootstraps. The following sequences, taken from GenBank, were included in the analysis: BJ393 (hm53670), shzh03 (AY465356), shanghai036-2009 (FJ713137), chongqing1-09-China (GQ994989), anhui-09(GQ994988), 001-Luan (CHN)-08 (GQ117124), 142-GZ-CHN-2008 (GU1901 75), 373-GZ-CHN-2009(GU190179), 521-18S-SD-CHN-07(EU753375), 542-Anhui-08(FJ765416), 566-Anhui-08(FJ765421), 0709F-NM-CHN-07(EU910862),0718F-NM-CHN-07 (EU910868), 1901-Luan(CHN)-08(GQ117127), 1906-Luan (CHN)-08(GQ117128), 05488-SD-CHN-2005 (GQ253421),06282-SD-CHN-2006(GQ253423), BJ08-Z025-5(FJ606450),BJ4211(EU024958), BJCDC01-08 (FJ765422), CC01-08(FJ765424), Chongqing3-09-China (GQ994991), CY11-BJ-CHN-2008(FJ469153), CY15-BJ-CHN-2008(FJ469154), CY44-BJ-CHN-2008 (FJ469161), EV71-CQ03-1(AY547501), Fuyang 49 (EU913471), EV71-Hubei-09-China(GU434678), EV71 - SHH02-6(AY547499), Fuyang.Anhui. P.R.C-17.08-1 (EU703812), Fuyang.Anhui.P.R.C-17.08-3(EU703814), H1261F-SD–CHN-2008(GQ253399), Henan2-09-China (GQ994992), JN94F-SD-CHN-2009 (GQ253408), kunming29-08(FJ765426), Lianyungang-JS13-CHN09 (GU353083), Lianyungang -JS24-CHN09(GU353090), LW0011F-SD-CHN-2009(GQ253413), Nanjing-JS06-CHN09(GU353080), Nantong-JS29-CHN09(GU353091), RZ0010F-SD-CHN-2009(GQ253417), shzh01-4 (AY895134), shzh02-62(AY895136), shzh03-106 (AY895138), shzh04-2(AY895139), SHZH98(AF302996), Suqian-JS22-CHN09(GU353088),Suqian-JS34-CHN09 (GU353093), Suzhou-JS30-CHN09(GU353092), SZ-C-08(FJ765429), SZ-H-08(FJ765430), Taizhou- JS18 -CHN09(GU353087),TC24F-SD-CHN-07(EU753418), WH-8-08(FJ765434), Xinhui-9(EU999179), Yangzhou-JS35-CHN09 (GU353094), Yangzhou-JS36-CHN09 (GU353095), Zhenjiang-JS41-CHN09(GU353096), Zhenjiang-JS60-CHN09(GU353106), ZH -ETC368 -GD-CHN-09(GQ487673), ZH-ETC385-GD-CHN-09 (GQ487686),ZH-ETC-153-GD-CHN-09(GQ487672), ZH-JC334-GD-CHN-09(GQ487688), ZH-JC426-GD-CHN-09 (GQ487689), ZJ001(FJ594956)，Zhuhai171 (EU999174), Zhuhai-JC498(EU999172), S11051/SAR/98(AF376081, C1), 2644-ASU-95 (AF135949, C2), KOR-EV71-09 (AY125973,C3), 2258 -CA-79 (AF135880, B1), 2222-IA-88 (AF009540,B2), 26M/AUS/2/99 (AF376101, B3), CN04104/SAR/00 (AF37 6067,B4), S19841 - SAR-03 (B5).

Construction of the expression plasmid

Two PCR primers for the EV71 vp1 protein, which contain specific restriction enzyme digestion sites depending on the multiple cloning sites contained in the expression vector pET32a(+) were used. The sense primer was 5'-CGGGATTCGGAGATAGGGTGGCA GAT-3', the bases underlined are the enzyme digestion site of *Bam*H I; the anti-sense primer was 5'-GC GTGTCGACCAAGAGTGGTGATCGCTGT-3', the bases underlined are the enzyme digestion site of *Sal*I. The PCR product was ligated with the pET32a(+) vector and the ligation product was transformed into BL21 cells. The recombinant was named pET32a-pVP1 and identified with both restriction enzyme digestion and PCR amplification.

SDS-PAGE and Western blotting

The positive recombinant transformant was grown in LB media containing 100 μg/mL Amp while shaking at 220 r/min at 37℃ and then induced with IPTG. Cells were harvested by centrifugation at 12 000 r/min for 1 min. Total cellular pellets were analyzed by 10% SDS-PAGE and Western blotting.

RESULTS AND DISCUSSION

The characterization of EV71 on vero cells and plaque-purification

After second generation blind passages a pathogenic effect (CPE) could be observed when the specimens were passaged in Vero cells, and were visible when cultured for 24 h. Different strains had the almost same CPE time in Vero cells, illustrating that the all strains could grow well in the selected cells. The infectious titers of the strains were all higher than 10^6 CCID50/mL, plaque-purification showed it can form the uniform plaque and there were no significant different on the plaque, but the size of uniform plaque were different, suggested that the stains have different virulence.

PCR and sequence comparisons

The p1 region of ten isolates from Gansu were cloned (Fig. 1), they were all 2586 nucleotide (nt) in length, the same as that of the full-length region of other EV71 strains. It shared 94.1%-99.9% nucleotide homology with reference strains, and 98.6% to 99.8 at the amino acid level(Table 1). Although there were no

Table 1. The nucleotides and amino acids homology of p1 genes compare the isolates from Gansu to other strains

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	25
1		99.9	96.2	96.2	95.9	95.9	95.9	95.6	95.9	95.7	96.4	99.4	95.5	96.2	96.3	96.8	96.2	96.2	94.3	95.7	96.1	95.7	95.5	99.6
2	99.8		96.1	96.1	95.9	95.8	95.9	95.6	95.6	95.7	96.4	99.4	95.5	96.1	96.3	96.7	96.2	96.2	94.3	95.7	96.1	95.6	95.4	99.6
3	99.4	99.2		99.5	97.0	96.9	97.0	96.7	96.9	96.8	97.5	96.3	96.6	97.2	97.4	97.2	97.3	97.3	94.4	96.8	97.1	95.9	95.7	96.1
4	99.8	99.5	99.4		97.0	96.9	97.0	96.7	96.9	96.8	97.6	96.4	96.7	97.2	97.5	97.3	97.3	97.4	94.5	96.8	97.2	96.2	95.8	96.2
5	99.4	99.3	99.1	99.4		99.9	99.7	98.6	99.7	99.8	97.6	95.9	97.5	99.1	98.5	97.3	98.2	98.4	94.5	99.6	97.4	96.2	95.4	95.9
6	99.4	99.3	99.1	99.4	100		99.7	98.5	99.6	99.7	97.6	95.9	97.4	99.0	98.4	97.2	98.1	98.3	94.4	99.5	97.3	96.1	95.3	95.8
7	99.3	99.4	99.0	99.3	99.7	99.7		98.4	100	99.8	97.5	96.0	97.4	98.9	98.3	97.2	98.1	98.3	94.5	99.3	97.3	96.1	95.4	99.9
8	99.5	99.5	99.2	99.5	99.7	99.7	99.5		98.4	98.5	97.3	95.6	97.4	99.0	98.1	96.9	98.0	98.0	94.2	98.4	97.1	95.7	95.0	95.6
9	99.3	99.4	99.0	99.3	99.7	99.7	100	99.5		99.8	97.5	96.0	97.4	98.9	98.3	97.2	98.1	98.2	94.4	99.3	97.3	96.0	95.3	95.9
10	99.1	99.2	98.7	99.1	99.7	99.7	99.8	99.5	99.8		97.4	95.8	97.4	98.9	98.3	97.0	98.1	98.2	94.4	99.3	97.2	95.9	95.2	95.7
11	99.7	99.4	99.3	99.7	99.5	99.5	99.2	99.7	99.2	99.2		96.5	97.4	97.9	98.2	97.8	98.1	98.2	94.9	97.4	97.8	96.4	95.9	96.4
12	99.9	99.9	99.3	99.7	99.3	99.3	99.4	99.7	99.4	99.2	99.5		95.7	96.2	96.5	96.9	96.2	96.3	94.5	95.8	96.2	95.8	95.6	99.5
13	99.2	99.2	98.8	99.2	99.3	99.3	99.4	99.7	99.4	99.4	99.4	99.3		97.7	98.1	96.9	98.0	98.0	94.1	97.3	97.1	95.7	95.3	95.6
14	99.7	99.4	99.3	99.7	99.8	99.8	99.4	99.9	99.4	99.4	99.8	99.5	99.5		98.6	97.4	98.6	98.5	94.6	98.9	97.6	96.3	95.4	96.1
15	99.4	99.4	99.1	99.4	99.5	99.5	99.4	99.9	99.4	99.4	99.5	99.5	99.5	98.8		97.8	99.1	99.0	95.0	98.3	98.0	96.5	95.8	96.4
16	99.4	99.2	99.1	99.4	99.1	99.1	99.0	99.2	99.0	98.7	99.3	99.3	98.8	99.3	99.1		97.6	97.7	95.5	97.1	97.4	96.8	96.2	96.8
17	99.3	99.3	99.0	99.3	99.4	99.4	99.3	99.8	99.3	99.3	99.4	99.4	99.4	99.7	99.7	99.0		98.8	95.1	98.0	97.8	96.4	95.6	96.3
18	99.3	99.3	99.0	99.3	99.4	99.4	99.3	99.8	99.3	99.3	99.4	99.4	99.4	99.7	99.7	99.0	99.5		95.1	98.2	97.9	96.4	95.8	96.2
19	99.2	99.2	98.6	99.0	98.8	98.8	98.7	99.2	98.7	98.7	99.1	99.3	98.8	99.1	99.1	98.6	99.0	99.0		94.5	94.7	94.5	94.5	94.3
20	99.4	99.2	99.1	99.4	99.5	99.5	99.2	99.7	99.2	99.2	99.5	99.3	99.3	99.8	99.5	99.1	99.4	99.4	98.8		97.2	96.0	95.2	95.7
21	99.1	99.1	98.7	99.1	99.0	99.0	98.8	99.3	98.8	98.8	99.2	99.2	99.0	99.2	99.2	98.7	99.1	99.1	98.7	99.0		96.0	95.7	96.1
22	99.9	99.7	99.3	99.7	99.3	99.3	99.2	99.4	99.2	99.0	99.5	99.8	99.1	99.5	99.3	99.3	99.2	99.2	99.1	99.3	99.0		95.5	95.7
23	100	99.8	99.4	99.8	99.4	99.4	99.3	99.5	99.3	99.1	99.7	99.9	99.2	99.7	99.4	99.4	99.3	99.3	99.3	99.4	99.1	99.9		95.4
25	99.7	99.7	99.1	99.4	99.1	99.1	99.2	99.4	99.2	99.0	99.3	99.8	99.1	99.3	99.3	99.1	99.4	99.2	99.1	99.1	99.0	99.5	99.7	

1. 11X, 2. 11Y, 3. 46F, 4. 47F. 5. 51F, 6. 55F, 7. 0902, 8. 0903, 9. 0917，10. 0901, 11. anhui1-09-China, 12. BJ08-Z025-5, 13. BJ393, 14. Fuyang/anhui/17/08/3, 15. GDSG/17/2008, 16. jiansu/07/08/10, 17. SHENZHEN/08, 18. zhejiang/08, 19. SHENZHENG/03, 20. lanzhou 01, 21. shanghai036/2009, 22. henan2-09,23. chongqing1-09, 24. anhui1-09.25. SHENZHEN08 China HFMD.

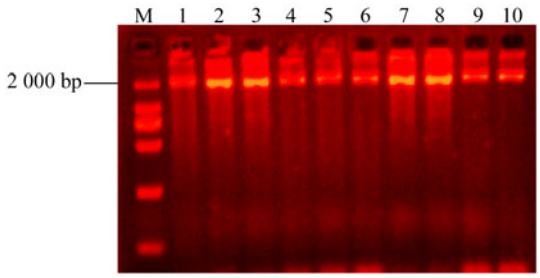

Fig. 1. Amplified product of P1.M: 2000 bp marker, 1-10: different EV71 strains，11X, 11Y, 46F, 47F, 51F, 55F, 901, 902, 903 and 917.

insertions or deletions in the p1 gene of all strains, mutations were found among the different isolates. 11x and 11y, which from the same fatal case, nucleotide and amino acid homologies of p1 were 99.9% and 99.8%, and it shared the highest similarity with strain SHENZHEN08 China HFMD that was also isolated from a fatal case. Strains 51F, 55F, 0901, 0902, 0907 and 0917 that were from the other infected cases shared the highest nucleotide and amino acid homologies with the lanzhou/01 strain, 46F and 47F were highly homologies but had low homologies with other strains.

Phylogenetic and mutation analysis

The p1 nucleotide phylogenetic tree showed that all the strains from Gansu clustered into a separtate group, but in the tree generated from the amino acid sequence, the strains were clustered into 2 subgroups, differentiating strains 11X and 11Y from the others. In order to determine the subtype of the Gansu isolates, a phylogenetic tree based on the vp1 genes was constructed. The resulted showed all isolates from gansu are C4 subtype (Fig. 2). The tree based on the p1 genes also revealed the same result (Fig. 3). Another tree was constucted based on the p1 amino acid sequence and the results showed that all the strains analyzed were divided into three groups with, the isolates from Gansu belonging to group I and II (Fig. 4). The vp1 phylogenetic tree showed that all the strains from Gansu were C4b subtype, but belonged to a different branch. It suggests that the source of infection were from different placelocations, and that at least three kinds of EV71 infections are prevanlent in Gansu. From both the vp1 and p1 nucleotides phylogenetic tree it can be seen that fatal strains 11X and 11Y have a close relationship with fatal strain SHENZHEN08 China HFMD, and we speculate that these strains have the same virulence signal. Amino acids sequence alignment showed there were many mutations among the different strains. The VP1 amino acid A to V mutation at position 170 appears to be a marker for a neurovirulent lineage, and it is possible that this mutation is a virulence determinant[12,13]. However the VP1 at the position of 11x and 11y which from severe neurological fatal cases were also A, so this hypothesis needs to be tested. Alignment of the EV71 p1 gene showed there are 17 nucleotides conserved among the 11x, 11y and SHENZHEN08 China HFMD strains but that are distinct from the other strains, whether these nucleotides are associated with virulence should also be investigated.

Expression of vp1 gene

Recombinant plasmid pET32-VP1 was digested with *Sal*I and *Bam*H I producing two fragments of 5400 bp (pET32a) and 891 bp. PCR identification confirmed the presence of the 891 bp fragment (Fig. 5). To obtain the expressed recombinant protein，the time course expression of recombinant plasmid pET32a-VP1 was induced by IPTG ranging from 0.5-1 mmol at 2, 3, 4, 5 and 6 h respectively. SDS-PAGE revealed that the best conditions were obtained for IPTG with 1 mmol at 6 h. The expressed VP1 protein had a

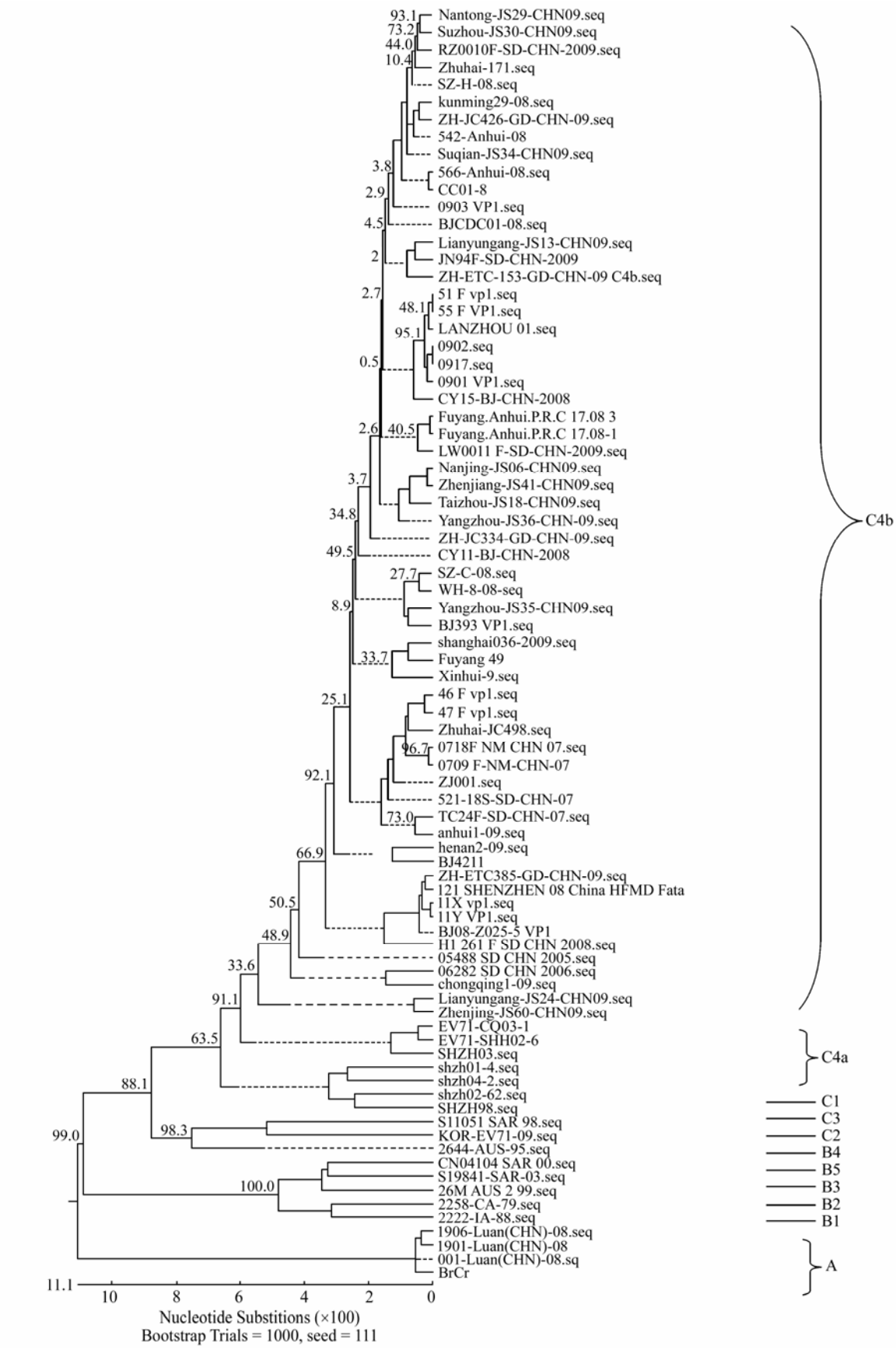

Fig. 2. Phylogenetic tree based on the EV71 vp1 nucleotide sequences.

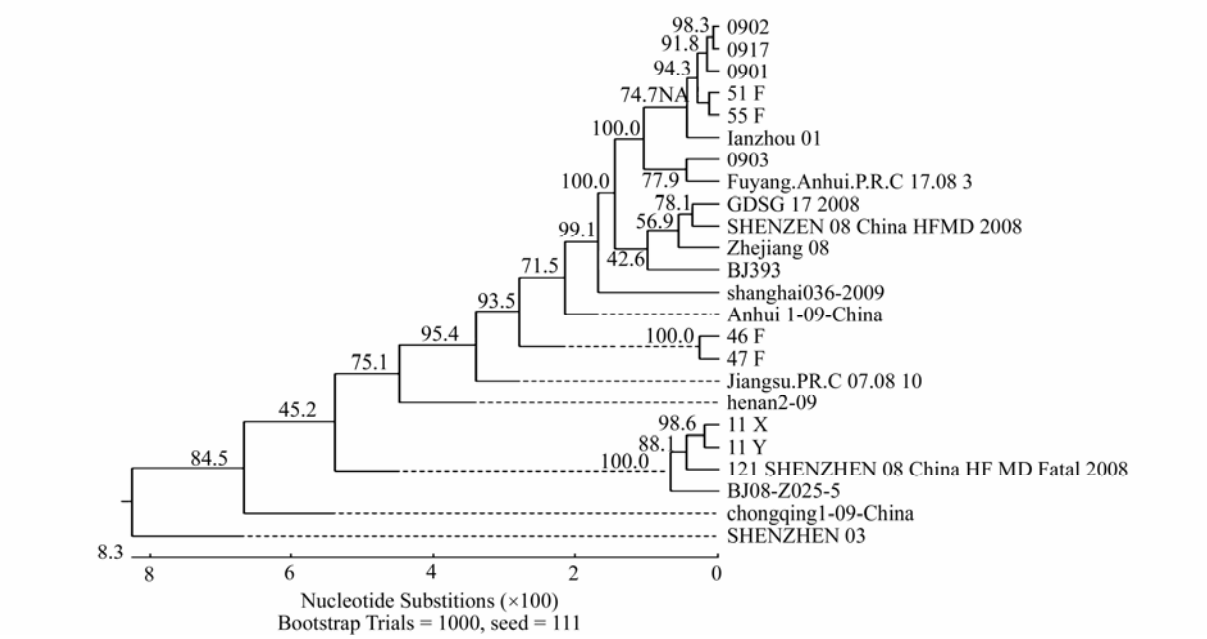

Fig. 3. Phylogenetic tree based on the EV71 p1 nucleotide sequences.

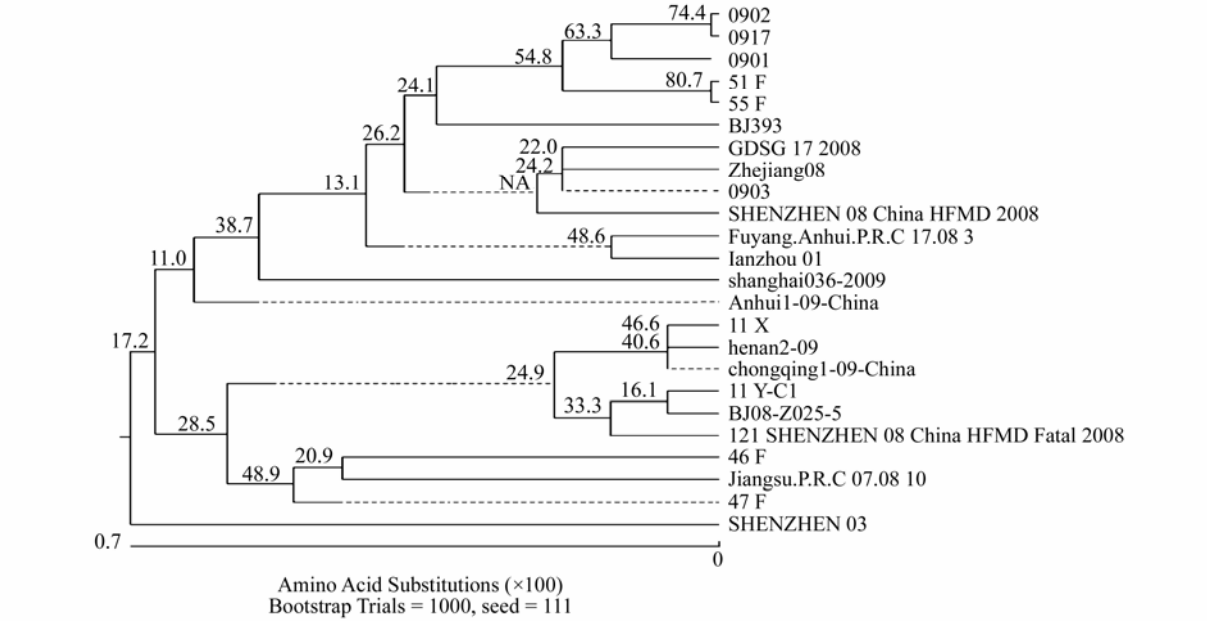

Fig. 4. Phylogenetic tree based on the EV71 p1 amino acid sequence.

molecular weight of 55 KDa as expected (Fig. 6). Western blot showed the expressed protein was able to bind immunologically to anti-EV71 serum (Fig.7). VP1 protein had been expressed with different expression systerm[4,16], if were used as diagnostic antibody, prokaryotic expression would be the best choice because of the high expression levels, but it is difficult to expressed the entire VP1 protein[18]. In this research, the fusion protein with His-tag from pET32a and entire VP1 were expressed, the result revealed recombinant protein was expressed with high levels and reacted specifically with the EV71 patient antibody. The recombinant protein was also applied to raise antiserum in rabbits and after the fourth injection a high titer of antiserum was detected by ELISA assay. This indicated that the protein has a good immunogenicty

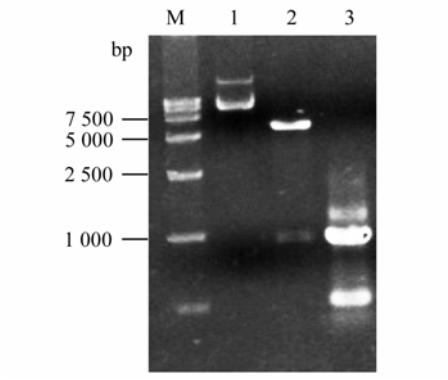

Fig. 5. Identification of recombinant plasmid by PCR and restriction enzyme. M,2500 ladder marker; 1, recombinant plasmid PET32a(+)/VP1; 2, identification of 5400bp fragment by restriction enzyme digestion; 3, identification of 891bp fragment by restriction enzyme digestion.

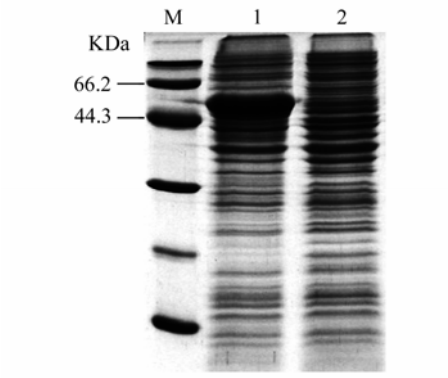

Fig. 6. SDS-PAGE of recombinant VP1 protein. M, low protein marker; 1, after inducing with IPTG; 2, without IPTG treatment.

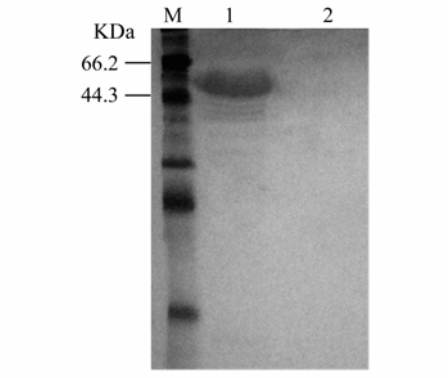

Fig. 7. Western blot of recombinant VP1 protein. M: low protein marker; 1,reaction of the protein with positive serum; 2, reaction of the protein with negative serum.

and would serve as an experiment basis for EV71 diagnosis research.

CONCLUSION

Recently, the number of EV71 infection cases and deaths has increased gradually and the EV71 outbreaks have become an important public health issue in China. In this work we have investigated the properties of the strains isolated in Gansu province at the molecular level. Our findings indicate that there are several distinct strains present in the region. Additionally, we investigated the use of the VP1 protein as a diagnostic tool for EV71. It is hoped that our finding may aid in understanding the diversity of strains and assist in the control of future outbreaks.

References

1. **Bible J M, Iturriza-Gomara M, Megson B, *et al*.** 2006. Molecular epidemiology of human enterovirus71 in the UnitedKingdomfrom1998 to 2006. **J Clin Microbiol**. 46:3192-3200.
2. **Brown B A, Oberste M S, Alexander Jr J P, *et al.*** 1999. Molecular epidemiology and evolution of enterovirus71 strains isolated from1970 to 1998. **J Virol.** 73 (12):9969-9975.
3. **Chan Y F, Sam I-Ch, AbuBaka S.** 2010. Phylogenetic designation of enterovirus 71 genotype sand subgenotype susing complete genome sequences. **Infect Genet Evol,** 10: 404-412.
4. **Chen H L, Huang J Y, Chu T W, *et al*.** 2008. Expression of VP1 protein in the milk of transgenic mice: A potential oral vaccine protects against enterovirus 71 infection. **Vaccine**, 26(23): 2882-2889.
5. **Chumakov M, Voroshilova M, Shindarov L, *et al.*** 1979. Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. **Arch Virol,** 60: 329-340.
6. **Diedrich S, Weinbrecht A, Schreier E.** 2009. Seroprevalence and molecula repidemiology of enterovirus71 in Germany. **Arch Virol,** 154:1139-1142.
7. **Foo D G, Alonso S, Phoon M C, *et al.*** 2007. Identification

of neutralizing linear epitopes from the VP1 capsid protein of Enterovirus 71 using synthetic peptides.**Virus Res,** 125:61-68.

8. **He Y H, Yang F, Li C L, *et al.*** 1999. Identification of EV71 isolates from hand, foot and mouth disease cases in Shenzhen. **Chin J Exp Clin Virol,** 1: 112. (in Chinese)
9. **Li X L, Mao C M, Ma S J, *et al.*** 2009. Generation of neutralizing monoclonal antibodies against Enterovirus71 using syntheticpeptides. **Biochem Biophys Res Commun,** 390:1126-1128.
10. **Moore D M, Cowan K M.** 1978**.** Effect of trypsin and chymotrypsin on the polypeptides of large and small plaque variants of foot-and-mouth disease virus: relationship to specific antigenicity and infectivity. **J Gen Virol,** 41:549-562.
11. **Ortner B, Huang C W, Schmid D, *et al.*** 2009. Epidemiology of entero-virus types causing neurological disease in Austria 1999–2007: detection of clusters of echovirus30 and enterovirus71 and anal-ysis of prevalent genotypes. **J Med Virol**, 81:317-324.
12. **Peter C M.** 2002. An overview of the evolution of enterovirus 71 and its clinical and public health significance.**FEMS Microbiol Rev**. 26: 91-107.
13. **Peter M, Katie L, David P, *et al.*** 2001. Phylogenetic Analysis of Enterovirus 71 Strains Isolated during Linked Epidemics in Malaysia, Singapore, and Western Australia. **J Virol,** 75: 7732-7738.
14. **Sanders S A, Herrero L J, McPhie K, *et al.*** 2006. Molecular epidemiology of enterovirus71 over two decades in an Australian urban community. **Arch Virol**, 151: 1003-1013.
15. **Shih S R, Li Y S, shuen P C, *et al.*** 2000. Expression of capsid protein VP1 for use as antigen for the diagnosis of enterovirus 71 infection. **J Med Virol**, 61:228-34.
16. **Tung W S, Bakar S A, Sekawi Z, *et al.*** 2007. DNA vaccine constructs against enterovirus 71 elicit immune response in mice. **Genet Vaccines Ther**, 5:6.
17. **Yu H Y, Chen W, Chang H Y, *et al.*** 2010. Genetic anylysis of the VP1 region of ecterovirus 71 reveals the emergence of genotype A in central China in 2008. **Virus genes**. 41:1-4.
18. **Zhou S L, Li L L, He Y Q, *et al.*** 2007. Expression of VP1 of Enterovirus 71 in *E. coli***. J Anal Sci**. 23:157-159. (in Chinese)