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Genetic Analysis of the VP1 Region of Human Enterovirus 71 Strains Isolated in Fuyang, China, During 2008*

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Abstract: Enterovirus 71 (EV71) is a common cause of Hand, foot, and mouth disease (HFMD) and may also cause severe neurological diseases, such as encephalitis and poliomyelitis-like paralysis. To examine the genetic diversity of EV71, we determined and analyzed the complete VP1 sequences (891 nucleotides) from nine EV71 strains isolated in Fuyang, China. We found that nine EV71 strains isolated were over 98% homologous at the nucleotide level and 93%-100% homologous to members of the C4 subgenogroup. At the amino acid level, these Fuyang strains were 99% -100% homologous to one another, 97%-100% homologous to members of the C4 subgenogroup, and the histidine(H) at amino acid position 22 was conserved among the Fuyang strains. The results indicate that Fuyang isolates belong to genotype C4, and an H at position 22 appears to be a marker for the Fuyang strains.

Key words: VP1 gene; Genotype C; Enterovirus 71(EV71)

Enterovirus 71 (EV71) is a member of the *Enterovirus* genus of the *Picornaviridae* family and is the major cause of Hand, foot, and mouth disease (HFMD) in children. EV71 infection can be accompanied by a series of syndromes with or without central nervous system involvement, including herpangina, aseptic meningitis, poliomyelitis-like paralysis, and possibly fatal encephalitis (14). Since the initial description of EV71, outbreaks of infection with this virus have been reported periodically in Europe, America, and the Asia-Pacific region. Since 1997, EV71 outbreaks with fatalities have been reported in Asia-Pacific countries. Subsequent smaller EV71-associated epidemics have been reported almost annually. During 2008, a large HFMD epidemic, caused mostly by EV71 infection, occurred in China, resulting in about 25,000 cases and 34 deaths. The EV71 outbreaks have been and continue to be 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. The single long open reading frame (ORF) encodes a polyprotein of about 2194 amino acids. There are four capsid proteins: VP1, VP2, VP3, and VP4. VP1 is the

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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. VP1 has also been used for phylogenetic analyses (13). At present, EV71 can be divided into three distinct genogroups (A, B, and C). Most EV71 isolates belong to genogroups B or C, which are each further divided into five subgenogroups, B1-B5 and C1-C5 (10). The evolution of viral genomes may lead to an altered spectrum of diseases caused by the virus. For the present study, nine EV71 isolates from pediatric patients in Fuyang, China, were collected for determination of the complete VP1 gene sequence. These isolates were then investigated by sequence alignment, molecular phylogenetic analyses, and analysis of neutralizing epitopes.

MATERIALS AND METHODS

Specimens

Clinical specimens were collected from patients with HFMD in Fuyang, China. Basic data for the nine patients are listed in Table 1.

Virus isolation

Vero cells were cultured in five tissue culture flasks in 25 cm^2 of minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS). A 1 mL throat swab sample was inoculated into Vero cells. After contact for 1 h at 37℃, the samples were removed, and MEM supplemented with 2% FCS was added. Within three passages, samples that induced an EV71-specific cytopathic effect (CPE) were considered positive for virus isolates. Samples that did not induce CPE after three passages in Vero cells were considered negative. Supernatants from the inoculated wells showing CPE were harvested and tested by neutralization using the antisera set from the Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College (IMB). The supernatant was extracted and used for the microneutralization test.

RNA extraction, RT-PCR, and Sequencing

Virus RNA was extracted from 140μL of virus culture supernatant using a QIAmp Viral RNA kit (Qiagen, USA) according to the manufacturer's instructions. RT-PCR was performed with the primer pair listed below using an Access Quick ™ RT-PCR System (Promega, USA) in 50-μL reactions. The cycling conditions consisted of an initial denaturation at 45℃ for 45 min, then 94℃ for 5 min followed by 35 cycles of 94℃ for 30 s, 52℃ for 30 s, and 72℃ for 2 min, and a final extension step for 10 min at 72℃ in a thermal cycler. PCR products (910 bp in length) were visualized by agarose gel electrophoresis. These amplified products were gel-purified and then cloned

Table 1. Clinical diagnosis of patients from whom enterovirus 71 was isolated

Isolate	Age/Gender	Specimens	Name of isolates	Patient symptom
1	1.1year/female	throat swab	Fuyang5	Fever
2	1.6 year/male	throat swab	Fuyang22	Fever/exanthema
3	1.7 year/male	throat swab	Fuyang23	Severe disease
4	1 year/male	throat swab	Fuyang24	Fever
5	1.6 year/male	throat swab	Fuyang26	Fever/exanthema
6	3.2 year/male	throat swab	Fuyang29	Fever
7	2 year/female	throat swab	Fuyang31	Fever
8	1.3 year/male	throat swab	Fuyang44	Fever
9	0.6 year/male	throat swab	Fuyang49	Fever/exanthema

into a PMD18-T vector (Takara Dalian Co., Japan) according to standard procedures. Recombinant plasmids that had been confirmed by PCR were sequenced by the Sangon Corporation. Primers were: P1: 5'-CATCCAGGGAGATAGGGTGGCA-3' (2430-2451); P2: 5'-TCCAAATTTCCCAAGAGTGGTG-3' (3319-3341).

Phylogenetic analysis

The sequencing results were analyzed and compared using the MEGA4.1 software package (Rainbow Technologies, INC) to define the genetic variation and the relationships with other strains obtained from GenBank.

The strain/isolate names and GenBank accession numbers

A genotype isolates: BrCr (AB204853), BrCr-TR (AB204852). B genotype isolates: 26M-USA-4-99 (EU364841), 26M-USA-4-99.1 (EU376005), 26MUSA-4-99.CHO (EU376004), 5511-SIN-00 (AY125988), SB-12736-SAR-03 (DQ341362), 5666-SIN-002209 (AF-352027), 5865-SIN-000009 (AF316321). C1 genotype isolates: J115-MAL-01 (DQ341360), 804NO-03 (DQ-452074), 1M-AUS-12-00 (DQ341361). C2 genotype isolates: 6F-AUS-6-99 (DQ381846), 7FAUS-6-99 (DQ341357; Tainan-4643-98 (AF304458), Tainan-6092-98 (AF304459), Tainan-5746-98 (AF- 304457), C3 genotype isolates: 03-KOR-00 (DQ- 341356), 06-KOR-00 (DQ341355). C4 genotype isolates: Fuyang31 (EU913470), Fuyang5 (EU913467), Fuyang23 (EU-8125150), Fuyang22 (EU913466), Fuyang26 (EU-913468), Fuyang49 (EU913471), Fuyang44 (EU-913469), Fuyang.Anhui.P.R.C-17.08-1 (EU703812), Fuyang. Anhui.P.R.C-17.08-1 (EU703814), FY-08 (EU-697901), Fuyang. Anhui.P.R. C-17.08-1 (EU703813), F1-CHN-00 (AB115490), SHZH98 (AF302996), 804-NO-03 (DQ452074), SHZH03 (AY465356), 1431-Yamagata-07 (AB433881), 1571Yamagata-07 (AB43-3883), AnHui-HeFei (EU697903).

RESULTS

Detection of EV71 by RT-PCR and by a neutralization test

After three passages, all nine samples induced the EV71-specific CPE in vero cells and the supernatants from the inoculated wells showing CPE could be neutralized by the IMB anti-EV71 sera set via the cell culture-neutralization test. When amplified using EV71-specific primers via RT-PCR, each sample showed a clear fragment of 910 bp in size. So EV71 was detected in samples from the nine patients.

Nucleotide sequence comparisons

The nucleotide sequences of the amplified fragments revealed that nine EV71 strains isolated were over 98% homologous. The isolates were also 88%-90% homologous to other C subgenogroups (C1, C2, and C3) and 93%-100% homologous to members of the C4 subgenogroup. Dong *et al*. proposed that a level of 15% sequence diversity be used as a cutoff for the delineation of new genotypes and a 9% cutoff for the delineation of subgenogroups (7). So the Fuyang isolates and the other Chinese isolates belonged to genotype C4. SHZH98 had the most divergent VP1 sequences among C4 subgenogroup. The nine EV71 strains with other enteroviruses are displayed in a phylogenetic tree constructed by the neighbor-joining method (Fig.1). Those strains are clustered in three distinct lineages (genotypes), designated A, B, and C. Genotype A contains a single member, BrCr, the EV71 prototype, and differs from all other isolates by 16.4%-19.7%. Genotype B was isolated from 1972 to

1997 in the United States, Australia, Colombia, and Malaysia Genotype C includes viruses from the United States, Australia, China, Canada, and mainland Malaysia. Genotype C is further divided into four subgenogroups. In addition, C at nucleotide position 65 was conserved among the Fuyang strains, while A was conserved within other isolates. Therefore, C at nucleotide position 65 appears to be a marker for Fuyang strains.

Comparison of EV71 VP1 amino acid sequences

The EV71 isolates from patients in Fuyang were 99%-100% homologous to one another at the amino acid level. The isolates were also 96%-97% homologous to other C subgenogroups (C1, C2, and C3) and 97%-100% homologous to members of the C4 subgenogroup. SHZH98 had the lowest homology to C4 (97%), and we found that the deduced sequence of the SHZH98 strain exhibited the largest genetic distance from other C4 members (Fig. 2 and Table 2). In Table 2, we also found that the evolutionary divergence values between sequences of the same genogroups were low, but these values were high between sequences of other genogroups (A and B). This data was identical to the result produced using the neighbor-joining method. The Fuyang isolates were 95%-97% homologous to other genogroups. In addition, the histidine (H) at amino acid position 22 was conserved among the Fuyang strains (Fig. 3), while the lysine (K) at position 43 and the alanine(A) at position 58 were conserved within genogroup C. Therefore, an H at position 22 appears to be a marker for Fuyang strains.

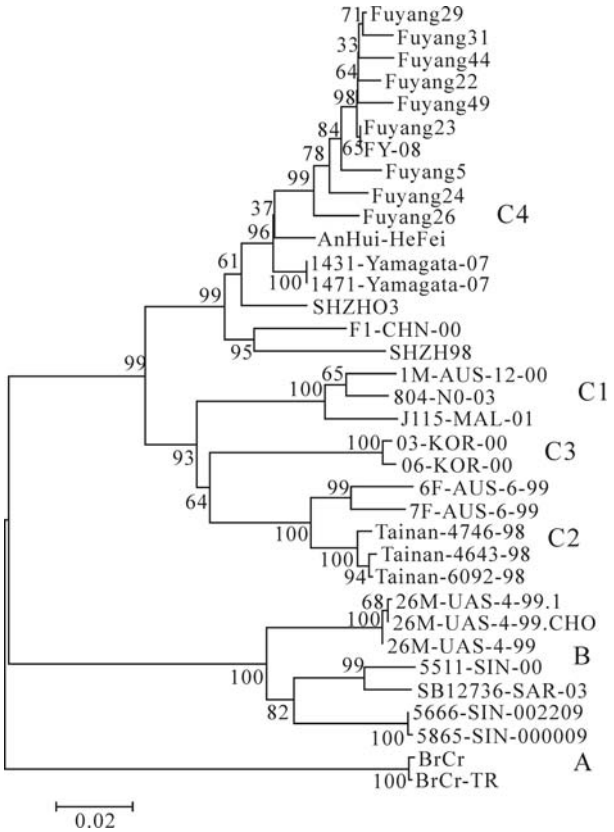

Fig.1. Phylogenetic analysis of 35 EV71 strains found worldwide based on complete VP1 gene sequence.The phylogenetic tree was constructed by the neighbor-joining method with MEGA version 4.1 software, and the reliabilities indicated at the branch nodes were evaluated using 500 bootstrap replications.

The VP1 protein is the major viral neutralization determinant and thus has a high degree of antigenic and genetic diversity that correlates with the viral serotype. Homologous recombination has not been shown to occur within the VP1 gene. Some amino acid substitutions in the VP1 gene were observed between Fuyang strains and other strains. So far, known epitopes recognized by neutralizing antibodies against EV71 are located in the VP1 protein in amino acid regions 91-106 (the BC loop) and 208-222 (SP70) (6, 8). In the deduced VP1 protein sequence of the Fuyang strains (Fig. 3), the BC loop was different from the BC loops of other strains (6F-AUS-6-99, AnHui-HeFei, BrCr, BrCr-TR, F1-CHN-00, Fuyang24, and SHZH98). In contrast, SP70 was identical to those from other isolated strains (data not shown).

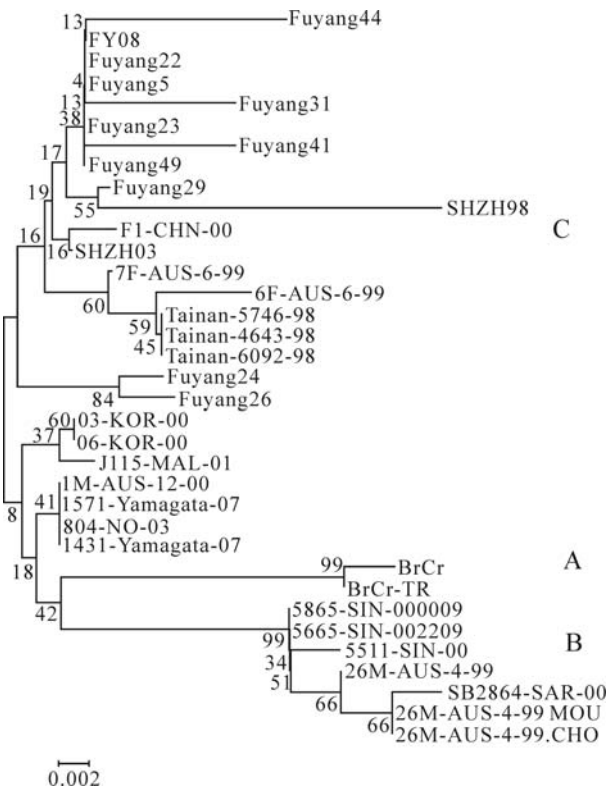

Fig.2. Phylogenetic analysis of 35 EV71 strains found worldwide based on complete VP1 amino acid sequence. The phylogenetic tree was constructed by the neighbor-joining method with MEGA version 4.1 software,and the reliabilities indicated at the branch nodes were evaluated using 500 bootstrap replications.

DISCUSSION

Over the last few years, the incidence of EV71 infection has increased, along with concomitant severe pathophysiologic complications and even death (11). A more rigorous study is required to identify the virulence factor associated with the pathogenicity of EV71. The prototype EV71 strain, BrCr CA-70, a strain isolated in California in 1970, is the only member of genotype A. The strains isolated in the U.S.A. and Australia from 1972 to 1988 and all isolates from a large HFMD outbreak in Malaysia belong to cluster C. Genotype C also includes strains isolated in the U.S.A. and Australia from 1986 to 1995 and isolates collected in 1997 from Sarawak, Malaysia (9). Most strains of genotype B were isolated before 1990. Isolates from a large outbreak in Taiwan in 1998 belong to genotypes B and C3, and fatal cases of EV71 infection were caused by both genotypes B and C3. Subsequent smaller EV71-associated epidemics, including those in 2000 and 2001 with 25 and 26 deaths, respectively, have been reported almost annually (1). In China, EV71 isolates from 2008, some of which caused fatalities, belong to genotype C4. The results show that there is no clear correlation between the severity of the disease and the genetic lineage of the virus because strains of all genogroups have caused severe disease (2).

Scientists have observed that poliovirus strain-specific neurovirulence may be attributed to minor sequence heterogeneity, especially in the 5' UTR and VP1 gene. This observation suggests that only a few critical genetic factors are sufficient to determine the neurovirulence phenotype. (15, 17). The VP1 protein is the major viral neutralization determinant of EV71 and thus has a high degree of antigenic and genetic diversity that correlates with the viral serotype. Because of these characteristics, the whole VP1 gene of the isolated Fuyang strains was sequenced using RT-PCR. The sequences demonstrated that VP1 variability was present in the Fuyang strains when compared to other strains.

Some amino acid substitutions in the VP1 protein were observed between the Fuyang strains and other strains. However, the location of the protective neutralizing epitopes within the VP1 protein has not been established. Although some amino acid substitutions were observed, the BC loop region, which may be important for neutralization, and synthetic peptide SP70, representing a neutralizing linear VP1 epitope of EV71 (data not shown), were conserved. Several

Table 2. Estimates of Evolutionary Divergence between Sequences

Strain	BrCr	03/KOR /00	26M-UAS-4-99	7F-AUS-6-99	804-N0-03	AnHui-HeFei	Fuyang5	Fuyang22	Fuyang23	Fuyang24	Fuyang26	Fuyang29	Fuyang31	Fuyang49	Fuyang44	FY-08	SHZH98	SHZHO3
BrCr		8.01	8.38	7.82	7.60	7.49	7.60	7.62	7.53	7.51	7.39	7.51	7.61	7.55	7.51	7.53	7.61	7.59
03-KOR-00	140.00		8.38	7.29	6.94	7.47	7.70	7.51	7.56	7.71	7.24	7.61	7.59	7.73	7.69	7.56	6.79	7.32
26M-UAS-4-99	129.00	137.00		7.95	7.68	7.89	7.75	7.75	7.80	7.82	7.67	7.82	8.09	7.81	7.91	7.80	7.97	8.21
7F-AUS-6-99	123.00	68.00	137.00		6.71	7.24	7.41	7.36	7.31	7.38	7.23	7.22	7.38	7.35	7.06	7.31	7.10	7.24
804-N0-03	122.00	79.00	125.00	78.00		7.40	7.59	8.06	7.91	7.74	7.40	7.94	8.27	8.00	8.05	7.91	7.19	7.28
AnHui-HeFei	121.00	80.00	115.00	82.00	78.00		4.90	4.70	4.08	4.64	4.32	4.28	5.19	4.65	4.71	4.08	5.84	4.35
Fuyang5	125.00	92.00	122.00	91.00	83.00	27.00		3.98	3.51	3.71	4.36	3.52	4.56	4.39	4.02	3.51	6.38	5.53
Fuyang22	126.00	87.00	120.00	94.00	88.00	26.00	17.00		2.33	3.58	4.19	2.35	3.56	3.34	3.17	2.33	6.44	5.84
Fuyang23	126.00	87.00	118.00	90.00	86.00	20.00	13.00	6.00		3.21	3.51	1.43	2.99	2.73	2.50	0.00	6.20	5.54
Fuyang24	127.00	90.00	124.00	91.00	83.00	27.00	15.00	15.00	11.00		3.74	3.16	4.21	3.92	3.79	3.21	6.37	5.54
Fuyang26	129.00	83.00	120.00	92.00	81.00	24.00	21.00	20.00	14.00	15.00		3.77	4.63	4.47	4.29	3.51	6.13	5.30
Fuyang29	126.00	85.00	120.00	88.00	86.00	22.00	13.00	6.00	2.00	11.00	16.00		2.60	2.79	2.49	1.43	6.16	5.57
Fuyang31	130.00	86.00	125.00	92.00	90.00	29.00	20.00	13.00	9.00	18.00	23.00	7.00		3.85	3.51	2.99	6.71	6.21
Fuyang49	125.00	92.00	121.00	95.00	91.00	27.00	20.00	13.00	9.00	18.00	23.00	9.00	16.00		3.48	2.73	6.46	5.83
Fuyang44	129.00	89.00	122.00	93.00	89.00	27.00	18.00	11.00	7.00	16.00	21.00	7.00	14.00	14.00		2.50	6.51	5.86
FY-08	126.00	87.00	118.00	90.00	86.00	20.00	13.00	6.00	0.00	11.00	14.00	2.00	9.00	9.00	7.00		6.20	5.54
SHZH98	130.00	75.00	129.00	79.00	77.00	42.00	54.00	55.00	51.00	54.00	52.00	49.00	56.00	56.00	56.00	51.00		5.42
SHZHO3	128.00	77.00	118.00	83.00	80.00	22.00	37.00	40.00	36.00	39.00	34.00	36.00	43.00	41.00	41.00	36.00	39.00	

The number of amino acid differences per sequence from analysis between sequences is shown. All results are based on the pairwise analysis of 18 sequences. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). Analyses were conducted in MEGA4.

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Fig.3. Comparison of putative part VP1protein aa 1-120 regions of EV71 Fuyang strains and other isolated strains.

studies showed that the VP1 protein contains neutralizing epitopes independent of other viral capsid proteins and that VP1 can be used as the backbone antigen for developing subunit EV71 vaccines (5, 18). We may infer that the VP1 protein of the Fuyang strains can also be used in the development of subunit EV71 vaccines.

Viruses belonging to genogroup C1 have shown a low level of endemic activity in the Fuyang region over the same time period. Genogroup C2 viruses were strongly linked to severe neurological disease. The increased neurovirulence of these viruses is associated with an A-to-V mutation at amino acid position 170 in VP1. The VP1 A170V mutation appears to be a marker for a neurovirulent lineage, and it is possible that this mutation is a virulence determinant (15,16). However, the Fuyang strains, which do not possess the VP1 A170V substitution, also caused severe neurological disease. Further studies are required to ascertain the role of residue 170 in the neurovirulence of EV71.

The etiology behind the Asia-Pacific neurological epidemics in young children that stem from EV71 outbreaks remains unknown (4). EV71 is constantly changing, with an estimated genomic evolution rate of 1.35×10^2 substitutions per nucleotide (3). Due to the high rate of subclinical infection, a detailed analysis of every wild-type EV71 strain occurring in Fuyang, China, is not possible since the majority of infections are not investigated. As EV71 has the potential to cause widespread epidemics with fatal complications, its epidemiology and molecular evolution, including the possibility of inter-typic and intra-typic recombinations, need to be carefully monitored (12).

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