RESEARCH ARTICLE

Molecular Epidemiology of Echovirus 18 Circulating in Mainland China from 2015 to 2016

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Abstract

Echovirus 18 (E18), a serotype of Enterovirus B (EV-B) species, is an important pathogen in aseptic meningitis. E18 had rarely been detected in mainland China, but became the predominant pathogen associated with viral encephalitis (VE) and meningitis in Hebei province for the first time in 2015. To investigate the molecular epidemiology and genetic characteristics of E18 in mainland China, sixteen E18 strains from patient throat swabs with hand, foot, and mouth disease (HFMD) in six provinces in China collected between 2015 and 2016, and four E18 strains isolated from 18 patient cerebrospinal fluid specimens with VE in Hebei Province in 2015 were obtained and sequenced. Combined with the sequences from the GenBank database, we performed an extensive genetic analysis. Phylogenetic analysis of *VP1* gene sequences revealed that all E18 strains from mainland China after 2015 belonged to subgenotype C2. There were no obvious specific differences in phylogenetic and variation analyses of E18 genome sequences between HFMD and VE/meningitis strains. Potential multiple recombination may have occurred in the 5'-untranslated region and in the P2 and P3 nonstructural protein-encoding regions of E18 strains from China. The current E18 strains were potential multiple-recombinant viruses. Overall, these findings supported that E18 caused HFMD, VE, and meningitis, although there were no significant associations between clinical features and viral genomic characteristics.

Keywords Genetic characteristics · Echovirus 18 (E18) · Genome · Enterovirus · Recombination

Xiangpeng Chen and Tianjiao Ji have contributed equally to this work.

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Introduction

Echovirus 18 (E18), a serotype of species Enterovirus B (EV-B) within the *Enterovirus* genus, was first identified in 1955 in the United States of America (USA). E18 infection is associated with diarrhea, encephalitis, aseptic meningitis, exanthema, acute tonsillitis, and herpangina in children and has morbidity and mortality rates of approximately 5.6% and 1.8%, respectively (Abedi *et al.* 2015). E18 is an important pathogen of aseptic meningitis (Miyamura *et al.* 1990; Turabelidze *et al.* 2009; Wang *et al.* 2011; Krumbholz *et al.* 2016). We previously reported an outbreak of encephalitis/meningitis caused by E18 infection in children in Hebei province, China in 2015 (Chen *et al.* 2017).

E18 is a nonenveloped, single-stranded, positive-sense RNA virus, and the genome contains approximately 7410 nucleotides with a single open-reading frame (ORF). The 5'- and 3'-untranslated regions (UTRs) of the genome are essential for viral replication and expression. The ORF, which contains 6570 nucleotides, encodes a polyprotein of 2189 amino acids composed of three protein precursors:

P1, P2, and P3. The P1 polyprotein precursor is ultimately cleaved into four structural proteins, VP1–VP4, whereas P2 and P3 are processed into seven nonstructural proteins, including 2A–2C and 3A–3D (Andersson *et al.* 2002). Genetic recombination and amino acid mutations play important roles in enterovirus evolution and epidemics (Zhang *et al.* 2011; Zheng *et al.* 2016). However, little information is available concerning the molecular epidemiology of E18 owing to a lack of complete genome sequences.

Accordingly, in this study, we reported 16 *VP1* sequences and six whole genome sequences of E18 strains isolated from children with viral encephalitis (VE) and hand, foot, and mouth disease (HFMD) in China after 2015. Combined with the sequences from the GenBank database, we further performed an extensive genetic analysis to elucidate the molecular epidemiology and genetic characteristics of E18 in mainland China.

Materials and Methods

Sample Collection and Virus Isolation

Sixteen throat swabs from patients with HFMD were collected from different provinces in China between 2015 and 2016. Eighteen cerebrospinal fluid specimens were collected from pediatric patients who were hospitalized with VE or viral meningitis (VM) in 2015. All specimens identified as enterovirus infection by real-time PCR (BioPerfectus Technologies, China; cat. no. JC20101) were cultured in rhabdomyosarcoma cells for virus isolation.

Determination of the *VP1* Gene Sequence and Full Genome Sequence

Viral RNA was extracted using a QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). The genome and *VP1* gene were amplified using segmented primers and sequenced. Primers for amplifying the complete genome of E18 were designed using the published sequence of the E18-314 (KX767786) strain. Positive PCR products were sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at Sinogenomax Company. Primers for PCR amplification and sequencing are listed in Supplementary Table S1.

Sequences from GenBank

Overall, 129 complete *VP1* coding sequences and 18 complete genome sequences of E18 were downloaded from GenBank before January 1, 2018. Eighty-four *VP1* gene sequences and 11 genome sequences (Supplementary

Table S2) were chosen for analysis after rejection of some similar sequences. These 11 genome sequences of E18 from the GenBank database included six from Germany (2010) (Krumbholz *et al.* 2016), one from Korea (2005) (Park *et al.* 2011), one from the USA (1955) (Andersson *et al.* 2002), and three from China (2015, 2016) (Chen *et al.* 2017; Zhang *et al.* 2017). The genome sequences of the EV-B prototypes strains were used as references in the phylogenetic and recombination analysis.

Phylogenetic and Evolutionary Analysis

Sequence assembly was performed using the Sequencher program (version 5.0; Gene Codes Corporation, USA). Alignment of the *VP1* gene and complete genome sequences was performed with the ClustalW package in the MEGA program (version 5.0; Sudhir Kumar, Arizona State University, Tempe, AZ, USA) (Tamura *et al.* 2011).

Phylogenetic trees based on the alignments of the *VP1* gene sequences were constructed with Bayesian Evolutionary Analysis Sampling Trees (BEAST, version 1.7) software using a constant size tree prior and a lognormal relaxed clock (Drummond *et al.* 2012). The evolution rates of Chinese E18 were also determined based on *VP1* gene sequences by BEAST software. A GTR + Γ substitution model was selected as the best-fit model by jModelTest (version 2.1) according to Akaike information criterion (Posada and Crandall 1998). Each Bayesian MCMC analysis was run for 100 million generations. Posterior probabilities were calculated using Tracer software (version 1.5).

Phylogenetic analysis based on the complete genome sequences and mature protein encoding regions was performed using the MEGA program (version 5.0; Sudhir Kumar, Arizona State University, Tempe, AZ, USA), using the Kimura 2-parameter substitution model and neighbor-joining method. Bootstrap testing with 1000 replicates was used to estimate the strength of the phylogenetic trees. Bootstrap values > 80% were considered to be a strong support for the tree topology. The genetic distance and identity matrices were determined by group mean and pairwise estimations of the sequence divergence with MEGA and BioEdit programs, respectively (Thompson *et al.* 1997).

E18 Recombination Analysis

Potential recombination analysis within the complete genome sequences of E18 was performed using the similarity plot and bootscan method (version 3.5.1; Stuart Ray, Johns Hopkins University, Baltimore, MD, USA) (Lole *et al.* 1999). The neighbor-joining method and Kimura 2-parameter substitution model were used in this analysis, with a window size of 500 nucleotides (nt) and a step size

of 20 nt. The value of the permuted trees > 80% suggested the potential recombination events.

Results

Virus Isolation

Totally thirty-four E18 strains were isolated. Eighteen E18 strains were obtained from patients with VE and VM in Hebei Province during 2015. Sixteen E18 strains were obtained from patients with HFMD in Shandong, Hebei, Shaanxi, Heilongjiang, Jiangsu, and Yunnan provinces in China between 2015 and 2016. The *VP1* gene sequences of all E18 strains isolated from patients with HFMD were determined. Two E18 strains isolated from patients with HFMD and four E18 strains isolated from patients with VE were chosen for complete genome sequencing.

VP1 Sequence Analysis

In total, 16 complete *VP1* gene sequences and six genome sequences were acquired and submitted to the GenBank

database (Table 1). In total, 100 complete *VP1* gene sequences from E18 were used for analysis, including 84 retrieved from public databases dated before January 1, 2018. As indicated in our previous study, the phylogenetic tree revealed that E18 strains could be divided into genotypes A, B, and C with the support of the high confidence values (posterior value > 99%). Genotype C can be divided into subgenotypes C1 and C2. All E18 *VP1* sequences obtained from patients in China after 2015 belonged to the subgenotype C2 branch (Chen *et al.* 2017). Except for HeB15-54462, all E18 *VP1* sequences obtained from China after 2015 clustered together. There were no evident differences in phylogenetic analysis of the *VP1* sequences between HFMD and VE/VM strains (Fig. 1).

As shown in Supplementary Table S3, there were 72 amino acid substitutions between the prototype strain (Metcalf) and the clinical strains of subgenotype C in this study. Three amino acid substitutions (M104L, Y215F, and I216V) were only found in Chinese strains. The substitution of M104L occurred only in the Hebei strains, including strains from VE/VM and HFMD cases. For amino acids 6, 10, 262, and 275, almost all Chinese strains were consistent with the prototype strain, whereas most of the strains from

Table 1 Sequences of the *VP1* gene and complete genome of E18 strains in this study.

Strain name	GenBank accession number	Provinces of isolation	Years of isolation	Sequences	Clinical diagnosis
SD15-128	MG720254	Shandong	2015	VP1	HFMD
SD15-129	MG720255	Shandong	2015	VP1	HFMD
HeB15-254	MG720244	Hebei	2015	VP1	HFMD
HeB15-255	MG720243	Hebei	2015	VP1	HFMD
SaX15-4	MG720253	Shaanxi	2015	VP1	HFMD
HLJ15-85	MG720245	Heilongjiang	2015	VP1	HFMD
HLJ15-87	MG720246	Heilongjiang	2015	VP1	HFMD
HLJ15-93	MG720247	Heilongjiang	2015	VP1	HFMD
HLJ15-96	MG720248	Heilongjiang	2015	VP1	HFMD
JS15-82	MG720249	Jiangsu	2015	VP1	HFMD
JS15-83	MG720250	Jiangsu	2015	VP1	HFMD
JS15-86	MG720251	Jiangsu	2015	VP1	HFMD
JS15-113	MG720252	Jiangsu	2015	VP1	HFMD
YN16-F213	MG720242	Yunnan	2016	VP1	HFMD
HeB15-462	MK256761	Hebei	2015	VP1	HFMD
HeB15-498	MK256762	Hebei	2015	VP1	HFMD
HeB15-54462	MG720260	Hebei	2015	Genome	HFMD
HeB15-54498	MG720261	Hebei	2015	Genome	HFMD
E18-221	MG720256	Hebei	2015	Genome	VE
E18-291	MG720257	Hebei	2015	Genome	VE
E18-393	MG720258	Hebei	2015	Genome	VE
E18-398	MG720259	Hebei	2015	Genome	VE

VE viral encephalitis, VM viral meningitis, HFMD hand, foot, and mouth disease.

Fig. 1 Phylogenetic tree (PT) based on the complete *VP1* gene sequences of echovirus 18 (E18). The PT was constructed with BEAST software (100 million generations) using a lognormal relaxed clock, a constant size tree prior, and the GTR + G substitution model. The posterior probabilities from the MrBayes analyses supporting the trees are indicated at the nodes; only values over 95% are shown.

other countries changed (R6K, N10D, V262T/A, and D275E). However, no significant differences between HFMD strains and VE/VM strains were found. Compared with the prototype strain Metcalf, as the E18 strains from VE/VM, one amino acid substitution at residue 84 (R→N/S) in the BC loop was also found in all the strains from HFMD. Further investigations are required to determine whether these amino acid changes significantly affected the prevalence of E18.

Complete Genome Analysis

In total, 17 complete genome sequences of E18 were used for genomic analysis, including six from this study and 11 retrieved from the GenBank database. Phylogenetic

Squares indicate China E18 strains after 2015. The filled squares indicate sequences of E18 isolated from HFMD cases, whereas the hollow squares indicate sequences of E18 isolated from VE/VM cases. Filled circles indicate the E18 prototype strain Metcalf. The scale bar indicates years. The strain name, year of sampling, and GenBank accession numbers are shown.

analysis showed that these isolates were grouped into subgenotypes C1 (Germany) and C2 (one from Korea and nine from China) based on the complete *VP1* region, except the prototype strain Metcalf. Eight of nine Chinese complete genome sequences were clustered together closely, whereas the HeB15-54462 strain had a closer phylogenetic relationship with the kor05-ECV18-054cn strain from Korea (Fig. 1).

No deletions or insertions were observed in the coding regions. Nucleotide substitutions in the clinical E18 strains were scattered all over the genome. For complete genomes of the genotype C strains, pairwise nucleotide sequence identities were 81.6%–99.9% among the 16 isolates and 98.8%–99.9% and 83.5%–99.7% within groups C1 and C2, respectively. A comprehensive comparison of nucleotide

Table 2 Pairwise nucleotide sequence identities based on corresponding regions of 17 complete genomic sequences of E18.

Region	Identities (%)				
	C1	C2	C	Between C1 and C2	Between Metcalf and C
Complete	98.8–99.9	83.5–99.7	81.6–99.9	81.6–85.3	79.3–81.1
5' UTR	99.0–100	89.3–99.5	84.1–100	84.1–85.9	80.3–82.1
P1	98.5–99.9	88.7–99.8	86.5–99.9	86.5–88.6	79.1–80.2
VP4	99.0–100	86.9–100	83.0–100	83.0–90.3	74.8–81.1
VP2	98.4–100	89.3–100	85.3–100	85.3–89.5	79.9–82.5
VP3	98.7–100	89.2–99.8	86.4–100	86.4–88.2	78.0–80.5
VP1	97.7–99.8	87.6–100	86.0–100	86.0–89.9	78.0–79.4
P2	99.1–100	79.5–99.9	78.2–100	78.2–82.1	79.1–81.1
2A	98.8–100	78.8–100	76.4–100	76.4–80.2	77.3–79.7
2B	100	74.7–100	74.7–100	74.7–79.1	76.0–81.4
2C	98.8–100	79.2–99.8	78.7–100	78.7–82.2	79.9–82.8
P3	98.5–99.9	77.3–100	77.3–100	77.4–85.7	77.9–82.2
3A	99.2–100	75.6–100	75.6–100	76.0–89.8	75.2–79.7
3B	98.4–100	74.2–100	74.2–100	75.7–92.4	78.7–98.4
3C	98.3–100	74.6–100	74.6–100	75.0–87.6	76.5–82.3
3D	98.4–100	77.9–100	77.4–100	77.4–84.0	78.7–82.5
3' UTR	97.6–100	72.9–100	71.7–100	71.7–83.5	78.8–91.7

Seventeen complete genomic sequences: six complete genome sequences were from this study and are listed in Table 1; the remaining 11 complete genome sequences were downloaded from GenBank before January 1, 2018 and are listed in Supplementary Material. *UTR* untranslated region.

Fig. 2 Phylogenetic trees (PTs) showing the relationships between the E18 isolates and EV-B prototype strains. These PTs were constructed by the different genomic regions of 16 complete E18 genomic sequences and EV-B prototype sequences. The neighbor-joining trees were reconstructed based on the *P1*, *P2*, and *P3* regions.

Triangles indicate the subgenotype C1. Filled circles indicate the subgenotype C2. Squares indicate the E18 prototype Metcalf strain. The percentages of bootstrap replicates (percentage of 1000 pseudoreplicate datasets) supporting the trees are indicated at the nodes; only values over 80% are shown.

Fig. 3 Similarity plot and results of bootscanning analyses of all C2 genotypes of E18 and other EV-B prototype strains based on the full-length genomes. Six strains in this study were chosen as representative strains. A sliding window of 500 nucleotides moving in 20-nucleotide steps was used in this analysis. **A, B** HeB15-54462; **C, D** HeB15-54498; **E, F** E18-221, E18-291, and E18-398; **G, H** E18-393. **A, C, E, G** Results of similarity plot analyses; **B, D, F, H** results of bootscanning analyses.

sequences based on each gene was also performed (Table 2). The sequences of structural protein-encoding regions (*VP1–4*) were highly conserved, whereas the *P1* gene nucleotide sequence identities within the genotype C were more than 86.5%. Comparatively, nonstructural genes (*2A, 2B, 2C, 3A, 3B, 3C, and 3D*) exhibited much more diversity, particularly in the *2B* region, with the lowest identity of 74.7% (Table 2).

Phylogenetic Analysis of *P1*, *P2*, and *P3* Coding Regions

Phylogenetic relationship analyses based on protein-coding regions were performed with MEGA 5.0 software. The grouping of the strains in phylogenetic dendrograms based on *P1*, *P2*, and *P3* regions was different (Fig. 2), suggesting that potential intratypic recombination had occurred between C1 and C2 strains.

In addition, potential multiple intertypic recombination events between the genome sequences of these E18 strains and other serotypes of species EV-B were also observed. Phylogenetic analyses based on nucleotide sequences of *P1* showed that all 16 sequences exhibited the closest phylogenetic relationship with the E18 prototype strain Metcalf (Fig. 2). However, based on the 5' UTR, 3' UTR, *P2*, and *P3* regions, subgenotype C1 and C2 sequences clustered with different EV-B serotypes, but not near the E18 prototype strain (Fig. 2, Supplementary Figure S1). The different topologies of the phylogenetic trees between *P1* and *P2–3* regions suggested that multiple intertypic recombinations of these circulating E18 strains with other EV-B serotypes may have occurred in noncoding or nonstructural protein-coding regions.

Recombination Analysis

To further depict the recombination events, similarity plots and bootscan analyses were performed using Simplot software v3.5.1 with the EV-B prototype strain as reference sequences. All the strains in this study were analyzed (Fig. 3). The results revealed that multiple recombination events occurred between the genomic sequences of representative strains and other EV-B serotypes. As indicated in the results, these sequences showed highest similarity and

the closest phylogenetic relationship with the E18 prototype strain Metcalf in the *P1* region. However, in some segments of the 5' UTR, *P2*, and *P3* regions, the representative strains showed lower similarity with E18 Metcalf (Fig. 3A, 3C, 3E, and 3G). The bootscanning results further confirmed the phylogenetic relationships (Fig. 3B, 3D, 3F, and 3H). As illustrated in our previous study (Chen *et al.* 2017), two apparent crossing sites in the 5' UTR and *2A* gene regions revealed intertype recombination events that were consistent with the deduction described above. According to the bootscanning results, it was hard to confirm the donor sequences.

Discussion

In a previous study, we reported an outbreak of E18-associated encephalitis/meningitis in children in Hebei province, in the central part of China during 2015. E18 was responsible for about 75% of all the confirmed enterovirus infections among these VE/VM cases and became the predominant virus pathogen of VE/VM in Hebei province for the first time, surpassing E6, E30, and CV-B5 (Chen *et al.* 2017). In this study, the 16 E18 strains were from patients with HFMD, confirming the connections between E18 infections and HFMD. Thus, our findings suggested that attention must be paid to E18 during further HFMD surveillance in China.

In this study, molecular epidemiology analysis of E18 was conducted using the complete *VP1* gene and complete genome sequences. The global distribution and genetic characteristics of E18 genotypes were clarified. As previously reported, all E18 strains could be divided into genotypes A, B, and C except the E18 prototype strain Metcalf, which was a unique strain. The genotype A strains were from China and included two strains from patients with diarrhea and acute flaccid paralysis. The strains of genotype B were all collected from India in 2008 (Chen *et al.* 2017). The subgenotypes C1 and C2 strains were the predominant subgenotypes circulating globally in recent years (Chen *et al.* 2017). All E18 strains obtained after 2015 in China belong to the C2 genotype, and all strains clustered into one branch, except HeB15-54462.

Genetic recombination was evolutionarily important for EVs and could lead to changes in the virulence of enterovirus and trigger serious public health problems (Lukashov *et al.* 2005; Zhang *et al.* 2011; Lukashov *et al.* 2014). The results of this study suggested that the current E18 strains were potential multiple-recombinant viruses containing many other EV-B donor sequences. Recombination was frequently detected in the 5' UTR, *P2*, and *P3* regions, for which it was hard to confirm the donor sequences. However, the sequences of the *P1* region were relatively

conserved because this structural protein is important for proper assembly of the viral capsid by the virus (Oberste *et al.* 2004; Lukashev 2005). The P1 region is also important for receptor recognition, which is associated with the tropism of enterovirus (He *et al.* 2002; Rezaikin *et al.* 2009; Chen *et al.* 2012).

Based on each of the analyzed regions (P1, P2, and P3), phylogenetic analysis also revealed that these nine Chinese E18 strains (seven strains from Hebei province during 2015 and two strains from Yunnan province during 2016) clustered into different branches with other EV-B prototype strains. They exhibited much more diversity with the current European endemic strains in genome sequence identities; however, the recombinational patterns were also different among China E18 strains. Zhang *et al.* (2017) reported that the E18 strains A83 and A86 recombined with the swine vesicular disease virus strain HK70 in the 3B region. In contrast, similar results were not observed among the strains in this study. Thus, these E18 sequences had obviously intra- and intergenotype differences, suggesting that these E18 strains may have evolved independently, through several different transmission chains, in China.

The causes of the outbreak of E18-associated encephalitis/meningitis in children in Hebei province are multifactorial. Changes in the virus are likely to contribute to or exacerbate the outbreak. VP1 protein from enterovirus contains many significant neutralizing antigenic determinants. Mutations at critical sites may cause escape from the neutralization immunity, which then may lead to the prevalence of this strain. In this study, three amino acid substitutions (M104L, Y215F, and I216V) were only found in Chinese strains. However, most of the strains from other countries had changes at amino acids 6 (R → K), 10 (N → D), 262 (V → T/A), and 275 (D → E), and almost all Chinese strains were consistent with the prototype strain Metcalf. Further studies are needed to determine the potential effects of these mutations in China epidemic strains. The B-C loop located at amino acids 78–89 in the E18 VP1 region forms a part of the epitope on the surface of the virion, which is important for the reactivity of type-specific neutralizing antibodies (Reimann *et al.* 1991). There was one amino acid change located at residue 84 (R → N) of the BC loop. The V152S amino acid change in the DE loop structure of the VP1 protein was reported in Taiwan E18 isolates during an associated outbreak of aseptic meningitis in 2006 (Tsai *et al.* 2011). However, this amino acid substitution was not found in this study. Genetic variations in the E18 strains suggested antigenic drift, which may have contributed to this outbreak (Chen *et al.* 2017). Additional studies are required to confirm whether these amino acid changes play significant roles in mediating the prevalence of E18.

In summary, in this study, we found that current China E18 strains were potential multiple-recombinant viruses, which is consistent with previous studies (Chen *et al.* 2017; Zhang *et al.* 2017). Furthermore, these China E18 strains can cause HFMD, VE, and meningitis, although there were no significant associations between clinical features and viral genomic characteristics. Currently, there are not enough E18 genome sequences to elucidate the associations between genetic characteristics and clinical manifestations. Accordingly, more studies are needed to determine the detailed evolutionary and genetic characteristics of E18 strains in mainland China.

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Author Contributions XC, ZX, and WX conceived and designed the experiments; XC, TJ, and JG performed the experiments; XC and WW analyzed the data; XC and ZX contributed to the writing of the manuscript. All authors reviewed and approved the final manuscript for submission.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement The study was approved by the Medical Ethics Committee of Beijing Children's Hospital, Capital Medical University (Permit No. 2015-8). Informed consents were obtained from legal guardians of all patients prior to the collection of CSF and throat swab samples.

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