Molecular epidemiological study of enteroviruses associated with encephalitis in children from Hangzhou, China

Li Wei, MM, Zhang Qiong, MS, Shen Xiao-ting, MS, Liu Yu-jie, MS, Mao Jian-hua, MD, Shu Qiang, MD, Shang Shi-qiang, MS*

Abstract
Enterovirus (EV) has over 100 serotypes of species A–D, which can cause various symptoms in infants. Enterovirus encephalitis (EVE) is serve disease with high morbidity and mortality in children. To well define the epidemiology of EVE, we wanted to know more about EV and EV molecular typing by conducting this study in Hangzhou.

Cerebrospinal fluid samples were collected from children with diagnosis of encephalitis. Meanwhile, one-step real-time RT-PCR was used for the detection of EV, and we also identified the serotypes of EV by using gene sequencing of VP1 or 5’UTR region. A total of 126 CSF specimens were tested and EV was detected in 26 specimens (20.6%). The molecular typing results showed different types of EV strains including Coxsackievirus B2, Coxsackievirus B3, Echovirus 5, Echovirus 16, Echovirus 18, Echovirus 30, and all EV isolates belonging to the human EV species B.

According to the sequence of VP1 and 5’UTR region, E30 may be major cause of children’s EVE in Hangzhou, China.

Abbreviations: CSF = cerebrospinal fluid, EV = enterovirus, EVE = enterovirus encephalitis, GLU = glucose, RT-PCR = reverse transcription-polymerase chain reaction.

Keywords: children, enterovirus, enterovirus encephalitis, PCR

1. Introduction
Enteroviruses (EVs), a genus of the Picornaviridae family comprising more than 100 serotypes, are single-stranded RNA viruses. Viral encephalitis is a common disease with an estimated 200,000 new cases annually around the world, and EVs were the main pathogens which cause viral encephalitis.1 EVs are known to target the central nervous system and are responsible for viral encephalitis.1 Although the prognosis of EV infection in children is favorable, neurological sequelae may be not neglected, such as disturbance of consciousness, paralysis, or even death.1,4 In China, poliomyelitis broke out once because the vaccine-derived polioviruses could recombine with human enterovirus C (HEVC) species and led to paralytic disease.1,3 Chou et al.13 has confirmed that attention deficit hyperactivity disorder (ADHD) was related to enterovirus encephalitis (EVE) in children. In Cangzhou city of China, the major pathogens of viral encephalitis were EVs with a positive rate of 27.8%.15 And in Zhejiang province, the positive rate of viral encephalitis caused by EVs in cerebrospinal fluid (CSF) samples between 2002 and 2012 was 13.9%.16 So in China, viral encephalitis caused by enteroviruses is worthy of attention because of high morbidity and mortality. Since the EV is circulating worldwide and causing a great variety of diseases, such as hand foot mouth disease (HFMD), herpangina, and EVE, particularly in infants and young children,17–19 it is necessary to systematically survey the outbreak of EV during epidemic seasons.

As we all know, many EV serotypes have been reported in different parts of the world, such as Coxsackievirus A9, B1-B5, Echovirus 4, 6, 9, 11, 19, 25, 30, and EV 71, 75, 76.20–23 Due to the large number of EV serotypes and their diverse clinical presentations, we think that it is necessary to find the major serotype of the EV timely and take step immediately to interrupt transmission. Above all, we aim to know more about the clinical characteristics of EVE patients and find out which serotype of EV plays a major role in EVE infection in this study.

2. Subjects and methods
2.1. Subjects
The Children’s Hospital of Zhejiang University School of Medicine, located in Hangzhou, a city in east China, is the largest comprehensive center for pediatric health care in Zhejiang province. As a leading children’s hospital in China, our hospital accepts outpatients more than 1,000,000 every year, and inpatients nearly 30,000. This was a retrospective study conducted over a period of 4 months between May 2015 and
August 2015 in the hospital. Patients who met the following criteria were recruited in the present study: age above 1 month and less than 14 years old, the children who visited our hospital during the study period, and primarily diagnosed with encephalitis by pediatric neurologists as our previous report. 

This study was approved by the medical ethics committee of the Children’s Hospital of Zhejiang University School of Medicine, and informed consent was obtained from patients’ parents or legal guardians.

2.2. Cerebrospinal fluid biochemical analysis

CSF biochemical analysis was performed in Beckman coulter AU5800 (Beckman, CA). The items contain: adenosine deaminase, lactate dehydrogenase, creatine kinase, glucose (GLU), chloridion, and micrototal protein.

2.3. Detection of enterovirus

CSF samples from patients were collected from children with symptom of viral encephalitis. A total of 200μL CSF was took for virus RNA extraction by using magnetic beads. RNA was extracted from CSF samples by using nucleic acid automatic extraction instrument (Zhi-jiang Company, Shanghai, China) according to the manufacturer’s instructions. The detection of EVs in ABI Stepone plus system was performed by using commercial one-step real-time RT-PCR assay kit (Zhi-jiang company).

The real time RT-PCR was conducted under these conditions: 15 minutes at 50°C, 5 minutes at 95°C, and then followed by 40 cycles of 15 seconds at 94°C and 45 seconds at 55°C. Samples with CT value less than 33.0 were identified positive.

2.4. Gene sequencing

All primers used in VP1 gene amplification were based on previous study. RT-PCR kit (Invitrogen, Shanghai, China) was used to synthesize cDNA. According to the provided protocol, the total volume per reverse transcription reaction was 10μL which included 2μL buffer (5×), 0.4μL dNTP (10mM), 1μL DTT, 0.2μL primer mix (Zhi-jiang Company), 0.5μL Superscript III, 5μL EV RNA, and 0.4μL RNasin. The reaction was under these conditions: 22°C, 10 minutes; 45°C, 45 minutes; and 95°C, 5 minutes. The 1st round of the VP1 gene PCR was carried out in a mixture with a total volume of 50μL that including 10μL of the RT-PCR product, 0.5μL DSC Taq (Enzymatics), and 2.5μL outer primers (Zhi-jiang Company). The amplification was under the following conditions: 95°C, 5 minutes; 40 cycles× (95°C, 30 seconds; 42°C, 30 seconds; and 60°C, 45 seconds); 72°C, 10 minutes. The 2nd round PCR was carried out in a mixture with a total volume of 50μL that included 1μL of the 1st round PCR product, 0.5μL DSC Taq (Enzymatics), and 2.5μL outer primers (Zhi-jiang Company). The amplification was under follow conditions: 95°C, 6 minutes; 40 cycles× (95°C, 30 seconds; 60°C, 20 seconds; 72°C, 25 seconds); 72°C, 10 minutes. The PCR product was sequencing in Majorbio (Shanghai, China). For VP1 gene amplification negative samples, we used 5’UTR gene PCR to amplify and identify the subtype of EV. The 1st round primers were as follows: forward primer: TCAAGCCTCTGTYWCCGA; reverse primer: GCTGTCACCATAAGCAGCCA. The 2nd round primers were as follows: forward primer: GTAGTACCCTTCCGCGGT; reverse primer: AGAAGTGATCGGTTCCCGGT. The cDNA synthesis primer is 1st round forward primer. The protocol is same to VP1 gene PCR.

2.5. Phylogenetic analysis

VP1 or 5’UTR gene DNA sequences of the EV isolates were compared to the National Center for Biotechnology Information (NCBI) database through BLAST. Based on the sequences of the VP1 or 5’UTR gene, phylogenetic analysis was done by using the Mega 5.10 software. The tree was constructed by using the neighbor-joining method. Significance of phylogenies was investigated by bootstrap analysis with 1000 pseudoreplicate datasets. Bootstrap values of are indicated on the tree.

3. Results

During the study period, a total of 126 patients visited our inpatient department who were primarily diagnosed with encephalitis. Twenty six CSF samples were tested positive for EV with a positive rate of 20.6%. The EV positive samples from encephalitis patients comprised 1 isolate (3.8%) in May, 12 isolates (46.2%) in June, 13 isolates (50.0%) in July, and 0 isolate (0%) in August, while only 3 children were confirmed EVE in other months of 2015. As shown in Table 1, among EVE isolates, 19 were boys and 7 were girls. Above 85% of EV positive children had the symptoms of twitch and chills. Most of confirmed EVE cases occurred among children above 3 years (92.3%, 24/26) with high positive rate in 7 to 9 years (30.8%).

All EV positive CSF samples were also performed with CSF white blood cells counts and biochemical analysis. The white blood cells are abnormal in all samples, and the average level of adenosine deaminase, lactate dehydrogenase, creatine kinase, GLU, chloridion, and micrototal protein were 0.98U/L, 26.3U/L, 1.7U/L, 3.86mmol/L, 124.5mmol/L, and 168.2mg/L, respectively. Except 1 CSF sample was a little high of GLU (4.92mmol/L). All of CSF samples were normal by CSF biochemical analysis (Table 2).

To confirm serotypes of EV, 26 clinical samples were collected and the VP1 or 5’UTR region of the EV gene was amplified by the conventional RT-PCR. The amplification products were purified, sequenced, and then used for phylogenetic analysis. After comparing with the VP1 or 5’UTR region from different reference EV strains, respectively, the homologous ranged from 94.7% to 98.8%, which met the serotype identification criteria for homologous serotypes. The molecular typing results of VP1 PCR showed that 24 EV isolates were belonged to the human

| Table 1 |
| Clinical characteristics of children with enterovirus encephalitis. |
| Characteristic | n=26 |
| Sex | Male 19 (73.1%) Female 7 (26.9%) |
| Clinical symptoms | Fever 23 (88.5%) Headache 25 (96.2%) Vomiting 24 (92.3%) Twitch 3 (11.5%) Chills 2 (7.7%) |
| Age group | <1 year 1 (3.8%) 1–3 years 1 (3.8%) 3–5 years 3 (11.5%) 5–7 years 6 (23.1%) 7–9 years 8 (30.8%) >9 years 6 (23.1%) |

0 points for this question.
EVs are significant causes of childhood encephalitis, a disease of high morbidity and mortality. And most hospitals have applied real-time RT-PCR method to detect EVs rapidly, such as EV71, CA16. In our previous surveillance, we found that EV71 and CA16 were decreasing and other types of EVs were increasing in Hangzhou after 2010, which were the same with Guangdong province. In this study, we found that a total of 26 children were diagnosed with EVE in the summer of 2015. The major

**Table 2**

|                      | EVE (average) | Normal range |
|----------------------|---------------|--------------|
| ADA, U/L             | 0.98 (0.7–1.3) | 0.0–5.0      |
| LDH, U/L             | 26.3 (11–39)  | 3–50         |
| CK, U/L              | 1.7 (0–12)    | 0–22         |
| GLU, mmol/L          | 3.86 (3.17–4.92) | 2.78–4.50 |
| Cl, mmol/L           | 124.5 (119.6–130.3) | 115–132   |
| MTP, mg/L            | 168.2 (98.6–368.6) | <450      |
| White blood cells    | 48 (16–125)   | <10          |

ADA = adenosine deaminase, CK = creatine kinase, Cl = chloride, CSF = cerebrospinal fluid, EVE = enterovirus encephalitis, GLU = glucose, LDH = lactate dehydrogenase, MTP = micrototal protein.

Based on the sequence of VP1 or 5'UTR region, phylogenetic analysis for the EVs of this study was done by comparison with all available the sequences of VP1 region from the Genbank. From the constructed phylogenetic tree (Fig. 1), we find out that all clinical isolates from Hangzhou belonged to the human EVB species. And Echovirus 30 stains derived from the isolates were most closely related to the stains from previous isolates from Zhejiang in 2012.

### 4. Discussion

EVs are significant causes of childhood encephalitis, a disease of high morbidity and mortality. And most hospitals have applied real-time RT-PCR method to detect EVs rapidly, such as EV71, CA16. In our previous surveillance, we found that EV71 and CA16 were decreasing and other types of EVs were increasing in Hangzhou after 2010, which were the same with Guangdong province. In this study, we found that a total of 26 children were diagnosed with EVE in the summer of 2015. The major
population of EVE was male and school children (age above 3 years old). At the same time, we also found that herpangina caused by EV broke out in the same place, we doubted that herpangina may be related to the EVE in the summer of 2015, Hangzhou (10,210 children were diagnosed with herpangina, with EV positive rate of 71.1%, data not shown in this article). There may be 2 reasons: one is that EV can spread through the fecal-oral route and these children infected with EV were mostly at school. So, more school activities and person to person communication can often increase the chances of pathogen transmission. The other one is that the changes of EV serotype may influence the occurrence of EVE after 2010. Although EV71 is the main pathogen of hand, foot, and mouth disease/herpangina (HFMD/HA) and can cause encephalitis, Echovirus 30 may invades into the central system in Chinese people more easily. Echovirus 30 was the major pathogen causing encephalitis which was confirmed in many provinces of China.\(^\text{16–18}\) Patients’ information was obtained to confirm clinical characteristics of EVE in children to prevent inappropriate antibiotic treatments. Like viral encephalitis, fever, headache, and vomiting were also related diseases in children (such as encephalitis, herpangina, and HFMD) and confirm whether herpangina is related to EVE.

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