Observational Study

Medicine®

OPEN

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.[2] 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.[3,4] In China, poliomyelitis broke out once because the vaccine-derived polioviruses could recombine with human enterovirus C (HEV-C) species and led to paralytic disease.[3] Chou et al[4] 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%.[5] 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%.[6] So in China, viral encephalitis caused by enterovriuses 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,[7–9] 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.[6,10–13] 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

Editor: Seiho Nagafuchi.

Funding/support: This work was supported by Key Projects of the National Science & Technology Pillar Program (2012BAI04B05) and Medical Scientific Projects from Health department of Zhejiang Province (2015KYA119).

The authors have no conflicts of interest to disclose.

Department of Clinical Laboratory, Children's Hospital of Zhejiang University School of Medicine, Hangzhou, PR China.

* Correspondence: Shang Shi-qiang, Department of Clinical Laboratory, The Children's Hospital of Zhejiang University School of Medicine, 3333 Binsheng road, Hangzhou 310052, PR China (e-mail: shangshiqiang33@163.com).

Medicine (2016) 95:40(e4870)

Received: 21 May 2016 / Received in final form: 19 August 2016 / Accepted: 23 August 2016

http://dx.doi.org/10.1097/MD.0000000000004870

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.[13] 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 200 μL 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).[13] 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 35.0 were identified positive.

2.4. Gene sequencing

All primers used in VP1 gene amplification were based on previous study.[14] RT-PCR kit (Invitrogen, Shanghai, China) was used to synthesize cDNA. According to the provided protocol, the total volume per reverse transcription reaction was 10 μL which included 2 μL buffer (5×), 0.4 μL dNTP (10 mM), 1 μL DTT, 0.2 μL primer mix (Zhi-jiang Company), 0.5 μL Superscript III, 5 μL EV RNA, and 0.4 μL 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 50 μL that including 10 μL of the RT-PCR product, 0.5 μL DSC Taq (Enzymatics), and 2.5 μL 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 50 μL that included 1 μL of the 1st round PCR product, 0.5 μL DSC Taq (Enzymatics), and 2.5 μL 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: TCAAGCACTTCTGTYWCCGA; reverse primer: GCTGTCACCATAAGCAGCCA. The 2nd round primers were as follows: forward primer: CGTACTTCG-AGAAGCCYAG; reverse primer: AGAAGTAGTCGGTT-CCGCGT. The cDNA synthesis primer is 1st round forward primer. The protocol is same to VP1 gene PCR.

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%)

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 children, 19 were boys and 7 were girls. Above 85% of EV positive children were accompanied with fever, headache, and vomiting. Lower than 10% 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 0.98 U/L, 26.3 U/L, 1.7 U/L, 3.86 mmol/L, 124.5 mmol/L, and 168.2 mg/L, respectively. Except 1 CSF sample was a little high of GLU (4.92 mmol/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 2

Results of CSF routine analysis in children with enterovirus encephalitis.

	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 (98.0–368.6)	<450
White blood cells	48 (16–125)	<10

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

EVB species, including 1 of Coxsackievirus B2, 3 of Echovirus 5, 3 of Echovirus 16, and 15 of Echovirus 30. VP1 PCR negative samples were performed 5′UTR PCR and sequencing, 4 EV isolates were also belonged to the human EVB species with 2 of Coxsackievirus B3 and 2 of Echovirus 18. According to the part of the VP1 region, the homologous of these isolates were 89.6% to 99.5% by comparing with the same serotypes.

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.[6]

4. Discussion

EVs are significant causes of childhood encephalitis, a disease of high morbidity and mortality.[2] 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,[7] which were the same with Guangdong province.[15] In this study, we found that a total of 26 children were diagnosed with EVE in the summer of 2015. The major

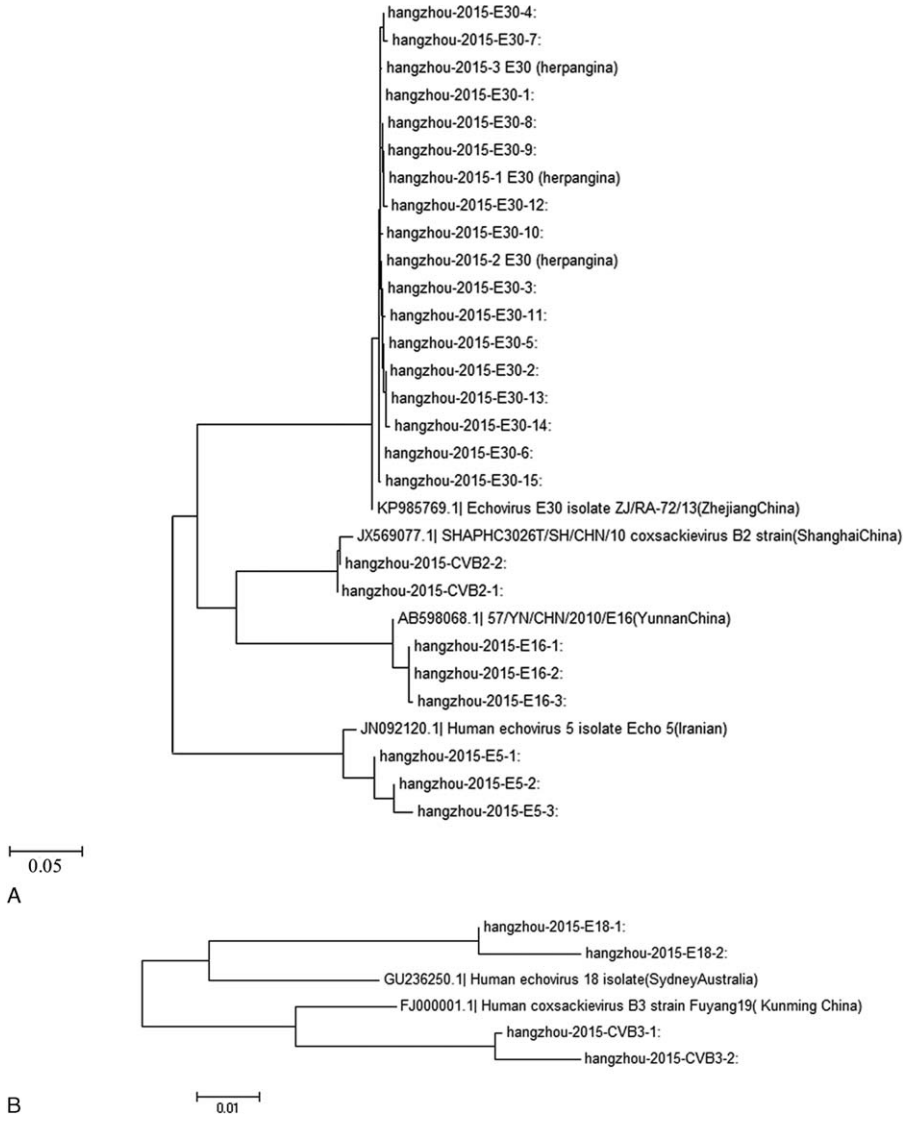

Figure 1. Phylogenetic analysis of the enterovirus isolates based on the VP1 gene (A) or 5′UTR (B) sequences.

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%, 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.[16–18] Patients' information was obtained to confirm clinical characteristics of EVE in children to prevent inappropriately antibiotic treatments. Like viral encephalitis, fever, headache, and vomiting were also common symptoms in children with EVE.[19] Our results also indicated that CSF biochemical analysis may not be useful in identification of EVE in children, and June and July were the main occurring month during the EV epidemic seasons.

In previous studies, many serotypes of EV were reported of causing encephalitis outbreak in children, such as Echovirus 30 in Belgium, 2000,[20] Echovirus 6 in Greece, 2006,[21] Echovirus 4 in Spain, 2008,[22] Echovirus 19 in Uttar Pradesh, India, 2008,[23] Echovirus 9 in Sri Lanka, 2009,[24] and Coxsackievirus B5 in Henan, China[25]; Echovirus 30 is a mostly common serotype in meningitis outbreaks caused by EV around the world.[26] According to the sequence of VP1 or 5′UTR, Echovirus 30 was found to be the major serotype in Hangzhou, 2015. Based on the phylogenetic analysis of the EV isolates through VP1 or 5 ′UTR sequence comparison, it was found that all the isolates from Hangzhou belonged to the human EVB species. The sequences of the VP1 or 5′UTR region were more closely related among the isolates from the near areas. Most importantly, Echovirus 30 was much more closely related to isolates from Zhejiang province. In previous surveillance, Echovirus 30 was reported as major serotype caused EVE in 2002 to 2004 and 2010 to 2012 in Hangzhou (the capital city of Zhejiang province in China).[6] So, Echovirus 30 may be the major EV causing encephalitis in recent years. Interestingly, 3 strains of Echovirus 30 in throat swabs from herpangina children were closely related to Echovirus 30 strains in CSF samples from EVE children. It indicated that Echovirus 30 may be more likely of invading the brain system of children in China.

Epidemiological surveillance plays a crucial role in understanding the relationship between the serotypes of EV infection and diseases. In further surveillance, we will continuously conduct EV-related diseases in children (such as encephalitis, herpangina, and HFMD) and confirm whether herpangina is related to EVE.

References

[1] Xie Y, Tan Y, Chongsuvivatwong V, et al. A population-based acute meningitis and encephalitis syndromes surveillance in Guangxi, China, May 2007–June 2012. PLoS One 2015;10:e0144366.

[2] Fowlkes AL, Honarmand S, Glaser C, et al. Enterovirus-associated encephalitis in the California encephalitis project, 1998–2005. J Infect Dis 2008;198:1685–91.

[3] Kenneth L. Emerging viral infections of the central nervous system: Part 1. Arch Neurol 2009;66:939–48.

[4] Chou IC, Lin CC, Kao CH. Enterovirus encephalitis increases the risk of attention deficit hyperactivity disorder: a Taiwanese population-based case-control study. Medicine (Baltimore) 2015;94:e707.

[5] Kong X, Zhang L, Liu K, et al. Epidemiological features of viral encephalitis in Cangzhou of China with use of multiplex RT-PCR for five RNA viruses. J Virol Methods 2015;222:178–81.

[6] Zhang L, Yan J, Ojcius DM, et al. Novel and predominant pathogen responsible for the enterovirus-associated encephalitis in eastern China. PLoS One 2013;8:e85023.

[7] Li W, Zhang X, Chen X, et al. Epidemiology of childhood enterovirus infections in Hangzhou, China. Virol J 2015;12:58.

[8] Abzug MJ. The enteroviruses: problems in need of treatments. J Infect 2014;68:S108–14.

[9] Menasalvas-Ruiz AI, Salvador-García C, Moreno-Docón A, et al. Enterovirus reverse transcriptase polymerase chain reaction assay incerebrospinal fluid: an essential tool inmeningitis management in childhood. Enferm Infecc Microbiol Clin 2013;31:71–5.

[10] Othman I, Volle R, Elargoubi A, et al. Enterovirus meningitis in Tunisia (Monastir, Mahdia, 2011–2013): identification of virus variants cocirculating in France. Diagn Microbiol Infect Dis 2016;84:116–222.

[11] Kumar A, Shukla D, Kumar R, et al. Molecular epidemiological study of enteroviruses associated with encephalitis in children from India. J Clin Microbiol 2012;50:3509–12.

[12] Lewthwaite P, Perera D, Ooi MH, et al. Enterovirus 75 encephalitis in children, southern India. Emerg Infect Dis 2010;16:1780–2.

[13] Wu T, Fan X, Wang W, et al. Enterovirus infections are associated with white matter damage in neonates. J Paediatr Child Health 2014;50: 817–22.

[14] Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol 2006;44: 2698–704.

[15] Zhou HT, Guo YH, Chen MJ, et al. Changes in enterovirus serotype constituent ratios altered the clinical features of infected children in Guangdong Province, China, from 2010 to 2013. BMC Infect Dis 2016;16:399.

[16] Lu J, Zheng H, Guo X, et al. Elucidation of echovirus 30's origin and transmission during the 2012 aseptic meningitis outbreak in Guangdong, China, through continuing environmental surveillance. Appl Environ Microbiol 2015;81:2311–9.

[17] Yang J, Zhang XL, Tao ZX, et al. Genetic analysis of echovirus 30 isolated from acute meningitis/encephalitis syndrome cases in Linyi city of Shandong province, China. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 2011;25:409–12.

[18] Zhao YN, Jiang QW, Jiang RJ, et al. Sequence analysis of Echovirus type 30 isolated from an aseptic meningitis outbreak in northern Jiangsu province in 2003. Zhonghua Liu Xing Bing Xue Za Zhi 2005;26:282–5.

[19] Glaser CA, Honarmand S, Anderson LJ, et al. Beyond viruses: clinical profiles and etiologies associated with encephalitis. Clin Infect Dis 2006;15:1565–77.

[20] Thoelen I, Lemey P, Van Der Donck I, et al. Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000. J Med Virol 2003; 70:420–9.

[21] Kyriakopoulou Z, Pliaka V, Tsakogiannis D, et al. Genome analysis of two type 6 echovirus (E6) strains recovered from sewage specimens in Greece in 2006. Virus Genes 2012;44:207–16.

[22] Cabrerizo M, Trallero G, Echevarría JE, et al. Molecular characterization of enteroviruses associated with neurological infections in Spain, 2008. J Med Virol 2008;85:1975–7.

[23] Kumar A, Shukla D, Kumar R, et al. An epidemic of encephalitis associated with human enterovirus B in Uttar Pradesh, India, 2008. J Clin Virol 2011;51:142–5.

[24] Danthanarayana N, Williams DT, Williams SH, et al. Acute meningo-encephalitis associated with echovirus 9 infection in Sri Lanka, 2009. J Med Virol 2015;87:2033–9.

[25] Ma H, Huang X, Kang K, et al. Recombination in human coxsackievirus B5 strains that caused an outbreak of viral encephalitis in Henan, China. Arch Virol 2013;158:2169–73.

[26] Österback R, Kalliokoski T, Lähdesmäki T, et al. Echovirus 30 meningitis epidemic followed by an outbreak-specific RT-qPCR. J Clin Virol 2015;69:7–11.