Laboratory diagnosis and genetic analysis of a family clustering outbreak of aseptic meningitis due to echovirus 30

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Echovirus 30 (E30) is a major pathogen associated with aseptic meningitis. In the summer of 2014, a family clustering aseptic meningitis outbreak occurred in urban–rural fringe of Ningbo city in Zhejiang Province in China. To identify the etiologic agent, specimens were tested by cell culture and reverse transcriptase–polymerase chain reaction. Pathogenic examination confirmed that the outbreak is caused by E30. The first case is a 6-year-old child, who studied in kindergarten in local, suffered from headache and fever. Same symptoms appeared in his parents, aunts, and other six relatives continuously. Meanwhile, vomiting occurred in majority of the patients and diarrhea in parts of them. White blood cells in cerebrospinal fluid (CSF) exceeded normal range in all patients. Protein levels in CSF were above normal range in half of the patients. Glucose levels in CSF were within normal range in all patients. We isolated six strains E30 in the stool specimens of patients, and carried out sequencing analysis to VP1 region. Sequencing results showed that 100% sequence identity was seen in both nucleotide and amino acid levels. Phylogenetic analysis discovered that isolate in this study was grouped into sublineage D2 together with sequences isolated from other areas of China in the 2000s and 2010s. Our study is the first family clustering outbreak of aseptic meningitis caused by E30 in Zhejiang Province in China. It is essential to establish an enterovirus molecular surveillance system in China to prevent mass outbreaks in Zhejiang.

Keywords: Echovirus 30, Aseptic meningitis, Outbreaks, VP1

Introduction

Echovirus 30 (E30), with an ssRNA positive-stranded genome, is a member of the genus Enterovirus in the family Picornaviridae and belongs to human enterovirus subgroup B.^{1,2} It is a major pathogen associated with aseptic meningitis in adults as well as children,^{3,4} which has occurred at high prevalence in recent decades all over the world.^{5–7} In China, outbreaks of aseptic meningitis associated with E30 have been sequentially reported in Jiangsu and Shandong in recent years.^{8,9} In our prior study, we found that E30 was the predominant serotype in enterovirus-associated encephalitis from 2010 to 2012.¹⁰ In July 2014, there was an outbreak of aseptic meningitis caused by E30 in Zhejiang Province, China. All patients belonged to a single family and required hospitalization.

In this study, we report on this outbreak, review the epidemiology and clinical features of viral meningitis associated with E30, and clarify knowledge regarding genetic traits of E30.

Materials and methods

Collection of epidemiological and clinical information

A questionnaire was administered to patients to collect information regarding symptoms and epidemiological characteristics between June 10 and July 20, this period was chosen to include information from 20 days before the onset of the first case of meningitis to 10 days after the onset of the final case of this disease. Clinical data were collected from patients' hospital records. The questionnaire items related to each patient's frequency of attendance at places of worship, prior contact with meningitis patients, attendance at social events, travel, and residence.

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Specimen collection and processing

Specimens consisting of throat swab and stool specimens were collected in viral transport medium. Cerebrospinal fluid (CSF) specimens were directly collected in sterile tubes from inpatients. Nonsterile specimens including throat swab and stool specimens (pretreated with penicillin [500 U/ml], gentamicin [500 µg/ml], and amphotericin B [Fungizone; 10 µg/ml]) were centrifuged at $3000 \times g$ for 20 min at 4 °C before inoculation into cell cultures.

Virus isolation

Isolation of an EV from associated body fluids and stool sample provides the strongest evidence of an enteroviral etiology. RD (Human rhabdomyosarcoma cells) cell lines, which were maintained in MEM medium (Gibco, USA) supplemented with 2% fetal calf serum (FCS) (Gibco), 100 U/ml penicillin (Sigma, USA), and 100 mg/ml streptomycin (Sigma), in an atmosphere containing 5% CO₂ at 37 °C, were used for EV isolation in this study.

Viral RNA extraction

Viral RNA was extracted from these CSF, throat swab, and stool specimens using the RNeasy Mini kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, 200 µL of the specimens were mixed with 600 µL of RLT buffer and 6 µL of β-mercaptoethanol and incubated for at least 1 min at room temperature. After the addition of 600 µL of cold 70% ethanol, the mixture was vortexed and applied to a spin-column. After the washing steps, RNA was eluted in 30 µL of RNase-free water.

Identification of E30

E30 was detected using an approach described in our previous study by Zhang *et al.*¹⁰ Real-time qRT-PCR reactions

were performed using the One-Step Real-time qRT-PCR Kit (TaKaRa, Japan). Each reaction mixture consisted of 10 pmol of the primers F: 5'-CTGYRGCGGAACCGACTAC-3', R: 5'-ATTGTCACCATAAGCAGCCA-3', 5 pmol of the probe P: 5'-FAM-TTGGGTGTCCGTGTTT-MGB-3', and 4 µl of template RNA in a final volume of 50 µl. Real-time qRT-PCR reactions were performed according to the instructions of the manufacturer.

VP1 gene sequencing and phylogenetic analysis

A High Fidelity one-step RT-PCR Kit (TaKaRa, Japan) was used to amplify the target gene segment. According to the provided protocol, the total volume per PCR was 50 µl which included 10 pmol of each of the primers VP1-F: 5'-GCRTGCAATGAYTTCTCWGT-3', VP1-R: 5'-GCICIGAYTGITGCCRAA-3'. The reverse transcription reaction was performed by incubation at 50 °C for 30 min and terminated by incubation at 94 °C for 2 min, then followed by 30 cycles at 94 °C for 30 s, 45 °C for 30 s, 72 °C for 1 min. The products were detected in 1.5% Ethidium Bromide pre-stained agarose gel after electrophoresis. The products were purified and then used for sequencing. VP1 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 gene, phylogenetic analysis was performed using the Mega 5.10 software.

Results

E30 outbreak description

A six-year-old boy with no remarkable past disease history was admitted to Ningbo Beilun People's Hospital on the night of June 30, 2014, with complaints of fever, headache,

Figure 1 Timeline of pertinent exposures and dates of illness onset in family clusters of cases of echovirus 30 infection in China. Notes: CSF: cerebrospinal fluid; E30: echovirus 30; rRT-PCR: real-time reverse transcription-polymerase chain reaction.

and vomiting that had started in the afternoon. He studied in a local kindergarten. The patient had no close friends or relatives with febrile illness before he became sick. There were no clinical manifestations other than mild tachycardia that was presumably associated with the patient's fever. Notably, the patient exhibited no definite neck stiffness. We noted mild leukocytosis ($8.5 \times 10^9/L$) that was within normal limits. The patient's C-reactive protein (CRP) level was elevated slightly (14 mg/L). We suspected meningitis and performed a CSF analysis. The results of this analysis revealed a white blood cell count of 70 cells/ μL (65% leukocyte cells), a protein level of 414.5 mmol/L, and a glucose level of 3.51 mmol/L. No bacterial growth was confirmed in the CSF.

As shown in Fig. 1, the day after Patient 1 was admitted to the hospital, his parents came to our hospital complaining of similar symptoms and were hospitalized immediately. On 3 July 2014, an aunt, who lived with Patient 1 began to experience mild headache symptoms. She did not seek medical attention until her headache became worse on July 6. Patients 5 and 6 were cousins of patient, who arrived at Patient 1 lived from another province on July 2 and July 4, respectively. Patients 5 and 6, who both slept in Patient 1's bed and were exposed to the environment containing the virus, presented with the same symptoms on July 5 and July 10, respectively. The median time from the onset of illness to discharge was 14.5 days (range, 13–16 days). All patients were negative for E30 by throat swab within 10 days after the onset of illness.

Clinical and laboratory features

Symptoms and laboratory data for patients admitted to the hospital are summarized in Table 1. All patients suffered from headache and fever. Five patients experienced vomiting and two patients experienced diarrhea. The number of white blood cells in CSF exceeded the normal range for all patients. Protein levels in the CSF were above the normal range for half of the patients. Glucose levels in the CSF were within the normal range for all patients. White blood cells were slightly elevated in the peripheral blood of several patients, and CRP levels were significantly increased. We conducted electroencephalography and brain imaging studies of several patients, however, no abnormal findings were obtained (not shown).

Phylogenetic analysis of the VP1 gene

E30 strains were isolated from the stool samples of all patients. We sequenced the VP1 region of six E30 strains to analyze the genetic characteristics of E30 in the examined outbreak. We found that these six strains were highly homologous at both the nucleotide and amino acid levels (100%). A Phylogenetic analysis was conducted on complete VP1 nucleotide sequences to investigate the genetic relationships among E30 isolates from Zhejiang and other regions during different periods (Fig. 2). Nucleotide sequences encoding for VP1 were

Table 1 Symptoms and laboratory data for patients in the examined family clustering outbreak of aseptic meningitis due to echovirus 30

	Gender	Age (years)	Symptoms					CSF				Blood	
			Headache	Fever	Vomiting	Diarrhea	Rash	WBC (cells/ μ L)	Protein (mmol/L)	Glucose (mmol/L)	Percentage of PMN (%)	WBC (10^9 /L)	CRP (mg/L)
Patient 1	Male	6	Y	Y	Y	Y	N	70*	414.5	3.51	35	8.5	14*
Patient 2	Male	33	Y	Y	Y	N	N	20*	677.2*	3.22	59	10.1*	44*
Patient 3	Female	27	Y	Y	N	N	N	120*	502.0*	3.37	30	10.5*	12*
Patient 4	Female	26	Y	Y	Y	Y	N	150*	495.4*	4.09	65	5.4	25*
Patient 5	Female	16	Y	Y	Y	N	N	30*	286.0	3.77	37	6.3	31*
Patient 6	Male	12	Y	Y	Y	N	N	15*	269.0	3.46	43	9.4	27*

Notes: CSF: cerebrospinal fluid; WBC: white blood count; PMN: polymorphonuclear; CRP: C-reactive protein. The normal WBC, protein, and glucose ranges for the CSF are 0–10 cells/ μL , 80–430 mmol/L, and 2.5–4.5 mmol/L, respectively. The normal WBC and CRP ranges for blood are $4\text{--}10 \times 10^9/L$ and 0–8 mg/L, respectively.

*Result outside the normal range.

Figure 2 Phylogenetic analysis of the VP1 gene of echovirus 30.

Notes: The tree was constructed using the neighbor-joining method. Significance of phylogenies was investigated by bootstrap analysis with 1000 pseudoreplicate data-sets. Bootstrap values are indicated on the tree. Δ : Echovirus 30 isolated in this investigation. The bracket on the right indicates the echovirus 30 species.

analyzed via the neighbor-joining methods, and trees were constructed using the MEGA software package, version 5.10. E30 can be clustered into 4 lineage groups (A, B, C, and D) based on genetic relationships of the capsid-encoding region VP1. Sequences of isolate in this study were grouped into sublineage D2 together with sequences isolated from other areas of China in the 2000s and 2010s.

Discussions

We verified direct contact in all examined cases, this observation is consistent with the findings of prior research from Italy and Japan.^{11,12} Direct or indirect contact was the main mode of propagation among subjects included in this study. Similarly to other enteroviruses, E30 can be transmitted via the digestive tract through contaminated food, articles in daily use, and contamination water.^{4,13} Although none of the common sources (contaminated food or water) have been implicated to date in the examined outbreak viral meningitis outbreaks are sometimes associated with the recreational use of water, and a contaminated environment is therefore regarded as a potential source of infection in this outbreak. However, no exposure to such an environment has been identified

in the included cases. No specific control measures are available for non-polio enteroviruses.¹³ Good personal and communal hygiene is recommended (thorough hand washing as a routine practice, prompt and safe disposal of waste and faeces, and prompt washing or disposal of the soiled articles in preschool and nursery facilities), as well as disinfection of contaminated surfaces with household cleaning products, and avoidance of shared utensils and drinking containers.¹³

All patients in this outbreak were living in the urban-rural fringe under poor health conditions. In recent years, as economic development has occurred, China's urban health conditions have tremendously improved. Various types of infectious disease are commonly found in the urban-rural fringe due to this region's large population and poor environment. The urban-rural fringe falls into gray areas of public health administration, and the government should strengthen management of this region.

Previous records of E30 meningitis demonstrated that headache and fever were observed in most cases, followed by vomiting (60–90%), diarrhea (11%), and rash (3–9%).^{13–16} One report from Shanghai in China indicated that more than half of E30 meningitis patients developed encephalitis,¹⁷ however, all other case reports described

favorable outcomes. The cases described here were similar to previously described cases, although no patients presented with skin lesions or developed encephalitis. Although polymorphonuclear (PMN) predominance in the CSF appeared to be atypical for viral meningitis, similar findings have been obtained in prior reports.¹² The present cases further suggested that PMN predominance in the CSF does not always indicate bacterial meningitis. Lower cell numbers and protein levels in the CSF tend to be observed in enterovirus meningitis than in herpes virus meningitis.¹⁴ However, these two parameters greatly differed across the case described here (cell count, 15–150/ μ L; protein level, 269.0–677.2 mmol/L). These findings rendered it difficult to speculate regarding causative agents based on CSF pleocytosis or protein increase alone. In addition, no obvious elevation of white blood cells was detected in the patients' blood specimens and patients' CRP levels were only slightly increased. The degree of inflammation in the CSF was not indicative of disease severity.

Homology analysis revealed that the six strains isolated in this study were completely consistent at both the nucleotide and amino acid levels. This finding indicated that the outbreak originated from a single E30 genotype. Based on complete sequences of VP1 genes, sequences of global E30 isolates can be divided into four genetic lineages (A–D) that exhibit at least 15% diversity between clusters.¹⁸ Phylogenetic analysis revealed that the isolate in the described outbreak belonged to the D2 sublineage. An evolutionary tree indicates that E30 isolated in China in recent years belongs to the C group or the D group. The E30 isolate discovered in 2001 in Taiwan belongs to the C1 sublineage, and the E30 isolated in 2004 in Zhejiang belongs to the C2 sublineage. The E30 isolated in 2010 in Linyi, which belongs to the D2 subtype, is highly homologous with the isolate in this study, a finding supported by strong bootstrap results (98%).

In summary, we analyzed a family clustering outbreak of aseptic meningitis using epidemiology, clinical features and etiology. Etiological examination confirmed that this outbreak was caused by E30. This report describes the first family clustering outbreak of aseptic meningitis caused by E30. The outbreak strain identified in our report belongs to the D2 subgroup, a strain from this subgroup was responsible for an outbreak reported in Linyi in 2010. Thus, it is essential to establish an enterovirus molecular surveillance system in China to prevent mass outbreaks in Zhejiang.

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