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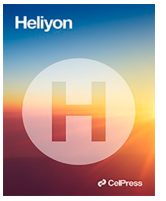

Research article

Metatranscriptomics revealed the molecular characterization of circulating enterovirus strains causing aseptic meningitis in children in Wuxi, China

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ABSTRACT

Enteroviruses are major etiological agents of aseptic meningitis globally, however information on circulating enterovirus types associated with this disease in Wuxi, China is limited. In this study, cerebrospinal fluid samples were collected from 20 pediatric aseptic meningitis cases in a Wuxi hospital in 2020 and subjected to metagenomic analysis to detect pathogens. *Enterovirus B* was detected in 9 cases, including 7 echovirus 18 (E18) and 2 echovirus 11 (E11) strains. The E18 strains exhibited 87.5–98.2% nucleotide identity and phylogenetically clustered with other China E18 strains, while the E11 strains showed 97.59% identity and clustered within the D5 subgroup along with other China E11 strains. One E18 strain was identified as a novel recombinants with a distinct recombination breakpoint within 3D gene. These findings expand knowledge on enteroviruses associated with pediatric aseptic meningitis in Wuxi, and highlight the circulation of genetically diverse E18 and E11 strains, including novel E18 recombinants. Characterization of enterovirus diversity by metagenomic analysis is important for molecular diagnosis and epidemiological tracking of aseptic meningitis cases. Continued surveillance of circulating enterovirus strains in Wuxi that may cause future outbreaks is warranted.

1. Introduction

Enteroviruses (EVs), belonging to the family *Picornaviridae*, are a diverse group of non-enveloped viruses with a positive single-stranded RNA genome. To date, they are classified into 15 species (*Enterovirus A-L*, and *Rhinovirus A-C*) comprising over 300 types. These viruses primarily infect humans through the fecal-oral route and can cause a wide range of clinical manifestations affecting various tissues beyond the gastrointestinal tract. While some infections like hand, foot and mouth disease (HFMD) generally result in mild symptoms such as fever and rashes, other enteroviral infections can lead to more severe conditions such as acute flaccid paralysis, myocarditis, neonatal sepsis, acute hemorrhagic conjunctivitis, encephalitis, and meningitis [1].

Aseptic meningitis is defined as meningitis accompanied by CSF pleocytosis, though enterovirus infections may present without

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pleocytosis, without a positive gram stain or culture, and without a parameningeal focus or systemic illness, resulting in a positive clinical outcome [2,3]. In addition to enteroviruses, many other viruses can cause aseptic meningitis, such as herpes simplex virus, west nile virus, varicella-zoster virus, arboviruses, mumps virus, and some respiratory viruses. Overall, enteroviruses account for over half of aseptic meningitis cases worldwide [4]. So far, nearly 50 types of enteroviruses have been identified as causes of aseptic meningitis globally, distributed across four species - Enterovirus A-D [2,5].

Wuxi, an important city in the Yangtze River Delta region, China. The routine test for etiology of this disease in Wuxi Children's hospital depends on antibody tests in serum, which are constrained by the narrow range of target pathogens, resulting in an incomplete understanding of the pathogen spectrum, since the pathogen of many cases were unidentified. Then, there are delays in pathogen identification and interpretation, which consequently affects diagnosis and treatment of this disease. Given the complexity of the spectrum of agents responsible for this disease, more surveillance and description of the circulating viruses causing aseptic meningitis and neurological symptoms in Wuxi is needed.

In this study, we collected CSF samples from 20 diagnosed cases of aseptic meningitis at Wuxi Children's Hospital in 2020 for metatranscriptomic analysis. We identified two enteroviral types, echovirus 18 and 11, never reported before in this region. Through genetic diversity analysis, phylogenetic characterization, and examination of recombination features, we expanded the molecular epidemiological data on aseptic meningitis in this city. These findings will be valuable for future surveillance and control measures against this disease.

2. Material and methods

2.1. Sample collection

Prior to sample collection, we obtained informed consent from the patients and their parents after providing them with relevant study information. The ethics committee of Wuxi Children's Hospital gave its clearance for this research. The CSF samples were collected upon hospital admission, and the patients' comprehensive clinical manifestations during that period were documented.

2.2. Laboratory work

For sample processing, total RNA was first extracted from 300 μL of CSF per sample using the RNeasy Plus Universal Kit (Qiagen) following the manufacturer's protocol. The Trio RNA-seq Kit (Nugen) was then utilized to deplete host ribosomal RNA (rRNA) and construct Illumina-compatible sequencing libraries. *Meta*-transcriptomic sequencing was subsequently performed on the prepared libraries using the Illumina HiSeq X-Ten platform. Based on the following analysis of assembled contigs, for detected viral strains where whole genome sequencing was unsuccessful, the reverse transcription polymerase chain reaction (RT-PCR) would be utilized to fill gaps and obtain complete genome sequences [6].

2.3. Data analysis

We integrated open source programs from sequencing data processing to various further analysis to build our own analytical pipeline. The following is a brief description of different components. The raw sequencing reads were processed using Trimmomatic to remove low-quality bases and adapter sequences [7]. Human originating reads were subtracted by mapping to the human reference genome using Bowtie2 [8]. The resulting clean non-human reads were taxonomically characterized by BLAST search against the NCBI nt database. Viral reads of interest were extracted and aligned to reference genomes with Bowtie2 to assess coverage. De novo assembly of the aligned reads into contigs was carried out using Megahit with default parameters [9]. The assembled contigs were further analyzed by Blast to determine their genetic relationships with other known viral strains.

2.4. Phylogenetic and recombination

To construct phylogenetic trees, sequences of the identified strains were aligned to reference strains using MUSCLE within the MEGA software suite [10]. Maximum likelihood trees were inferred using the phyML program (v3.0), and the branch support was inferred with 1000 bootstraps replicates [11]. The resulting tree files were then imported into MEGA for visualization and annotation using the User-tree editor.

Putative recombination events were further analyzed using the Recombination Detection Program v4 (RDP4), which integrates multiple methods including RDP, GENECONV, BootScan, MaxChi, Chimaera, SiSCan, and PhylPro [12]. Additionally, SimPlot software (v3.1) was used to perform similarity analysis of the complete genome alignment between the potential recombinant strain and closely related or representative strains [13].

3. Results

3.1. Cases and clinical symptoms

In 2020, we collected 20 cases that were diagnosed with aseptic encephalitis but had no identifiable pathogen based on clinical testing, with a male to female ratio of 13:7 and an average age of 5.6 years. The basic information and major clinical characteristics of

all enrolled cases were summarized in Table S1, among which information on the positive cases with pathogens discovered by transmetagenomics was summarized in Table 1. Generally, these cases presented with symptoms such as fever, vomiting, headache, and most of them exhibited abnormalities of some extent in their electroencephalogram (EEG). The lymphocyte ratio among these patients was significantly elevated, ranging from 45% to 84%, with a mean of 66.36%. This is much higher than the normal range of 20–40%. The white blood cell (WBC) count in the CSF was also significantly elevated, ranging from 10 to 760 $\times$ 10^6/L, with a mean of 147 $\times$ 10^6/L. This is well above the normal range of 0–10 $\times$ 10^6/L (shown in Table 1).

3.2. Metatranscriptomics

All the collected CSF samples underwent transmetagenomic analysis. Between 33,238,228 and 137,784,062 clean reads were generated from these pools, with an average of 66,702,193 reads (Fig. 1a). The viral and other microbial components of the sequencing reads were analyzed, with extra focus given to those associated with potential pathogens of meningitis. Reads blast analysis indicated that 9 cases tested positive for *Enterovirus B*, which was further confirmed through analysis of the assembled contigs. By combining the assembled contigs with RT-PCR results, we successfully recovered the complete coding sequences (CDS) of these strains, which were deposited in GenBank under accession numbers OR095789-OR095797. Mapping analysis revealed these genomes sequences were fully covered by the reads with different depth (Fig. 1b ~ j). The mapping characteristics of detected enteroviruses differed between libraries. This variability may indicate distinct replication states of related viruses in each library, specifically the transcriptional dynamic of different genes (such as structural and non-structural genes). Additionally, multiple factors related to library preparation and sample quality could have influenced the result. Moreover, no additional pathogen information, whether viral or bacterial, was found in these specimens, aside from enteroviruses.

3.3. Bioinformatic analysis

Blastn analysis using the recovered genome sequences revealed that seven strains belonged to Echovirus 18 and two strains belonged to Echovirus 11, with varying degrees of nucleotide identity. Among the E18 strains, nucleotide identities ranged from 98.2% to 87.5%, while the two E11 strains shared 97.59% identity.

Indicated by the reads blast results, the reads from different regions of some E18 strains showed different blast hits, we then performed Blastn analysis using the VP1 and 3D genes as queries separately against the nt database. For the VP1 genes, hits of these strains showed similarity with E18 strains circulating in China, with identities ranging from 99.7% to 93.4%. However, for the 3D genes, results were similar except for CSF26. The 3D gene of CSF26 shared 92.86% identity with an Echovirus E6 isolate found in HFMD cases in China. Notably, CSF16 was identical to a recombinant E18 strain PC06 previously reported to be circulating in Wuxi. For the two E11 strains, VP1 and 3D genes exhibited over 98% identity to E11 strains in China.

Phylogenetic analysis was performed to reveal evolutionary relationships with other reference strains. Maximum likelihood trees constructed using the VP1 gene showed the seven E18 strains clustered within the C2 subgroup, along with other E18 strains circulating in China isolated from AM or HFMD cases, without distinct clustering patterns (Fig. 2a). The two E11 strains fell within the D5 subgroup, clustered with other primarily China-circulating E11 strains detected in patients and the environment (Fig. 2b).

The Echovirus B phylogenetic trees constructed using VP1 and 3D gene sequences reveal that Echovirus 18, Echovirus 6, and other types cluster separately on the VP1 tree, but are intermixed on the 3D tree (Fig. 3a ~ b). This suggests frequent recombination occurs among Echovirus B strains. New found strain CHN/WX/CSF26 clearly exhibits significant recombination as well based on its placement in the different trees. To elucidate recombination patterns, Simplot analysis was conducted. Consistent with the Blastn and phylogenetic results, CSF26 showed recombination within the beginning part of 3D gene of the P3 region (Fig. 3c). One parental sequence was a strain closely related to a strain isolated from an adult meningitis CSF sample, while the other requires confirmation.

Table 1
Major clinical features of the cases targeted.

Case ID/Gender/Age	Major clinical symptoms			Some tests results		
	Fever	Headache	Vomiting	Imaging Disorder[a]	Lymphocyte ratio	WBC($\times$ 10^6/L)
CSF07/M/6	Yes	Yes	Yes	moderately	60	35
CSF10/M/7	Yes	Yes	Yes	slightly	60	98
CSF11/F/5	Yes	Yes	Yes	NA[b]	75	79
CSF14/M/8	No	Dizziness	Yes	moderately	60	59
CSF16/M/5	Yes	Yes	Yes	slightly	80	120
CSF18/F/4	Yes	Yes	Yes	NA	45	367
CSF19/F/7	Yes	Yes	Yes	slightly	60	170
CSF20/M/6	Yes	Yes	Yes	slightly	NA	760
CSF26/M/5	Yes	Yes	Yes	moderately	60	50

[a] Refer to the results of electroencephalogram.
[b] No data available.

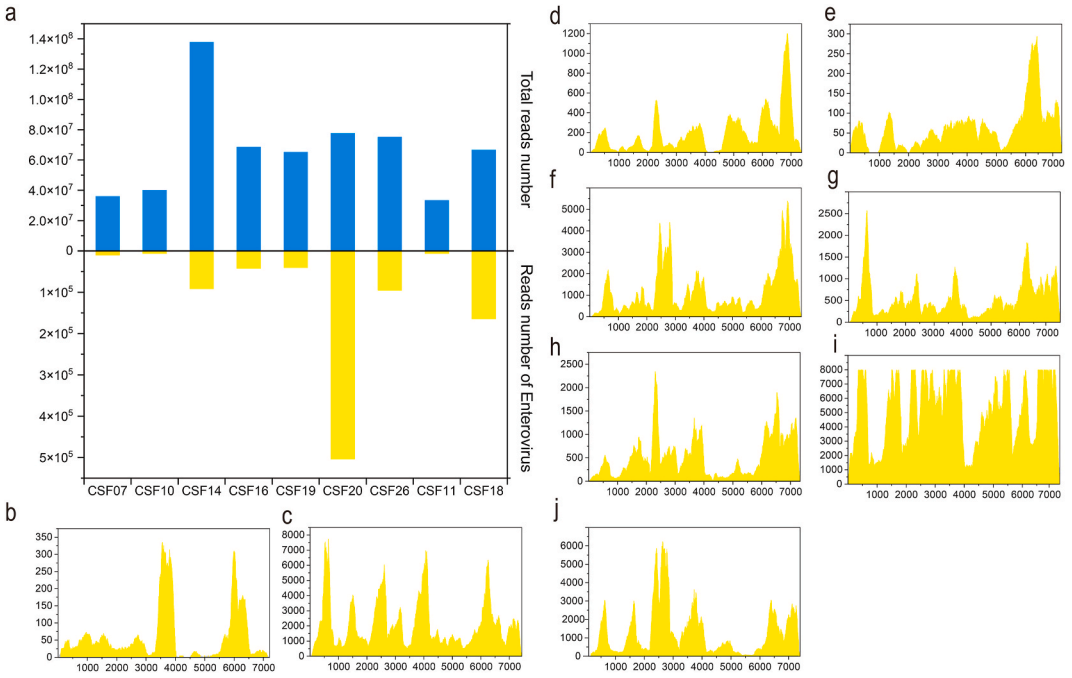

Fig. 1. Overview of deep sequencing data. a: the histogram illustrates the total number of generated reads (black) and enterovirus-associated reads for each sample pool (gray). b ~ j: the genome coverage plots for the detected enterovirus strains. Coverage is shown on the y-axis and genome position is shown on the x-axis.

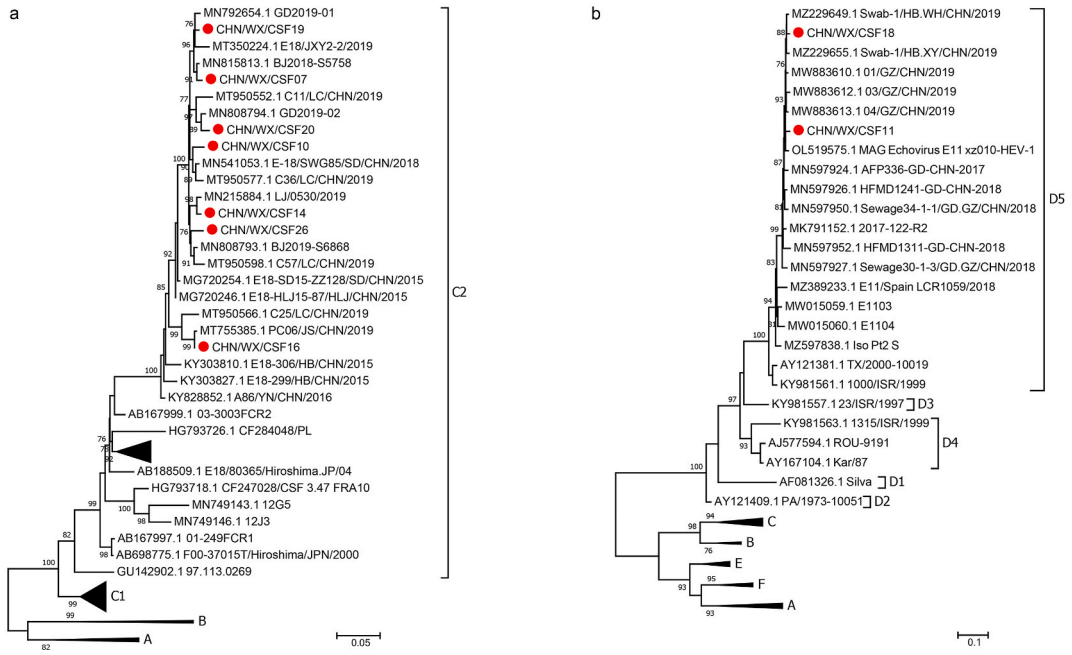

Fig. 2. Maximum likelihood phylogenetic trees based on VP1 gene sequences. a: Phylogenetic relationships of the detected Echovirus 18 strains and reference strains; b: Phylogenetic relationships of the detected Echovirus 11 strains and reference strains. Only the support values larger than 75% were displayed.

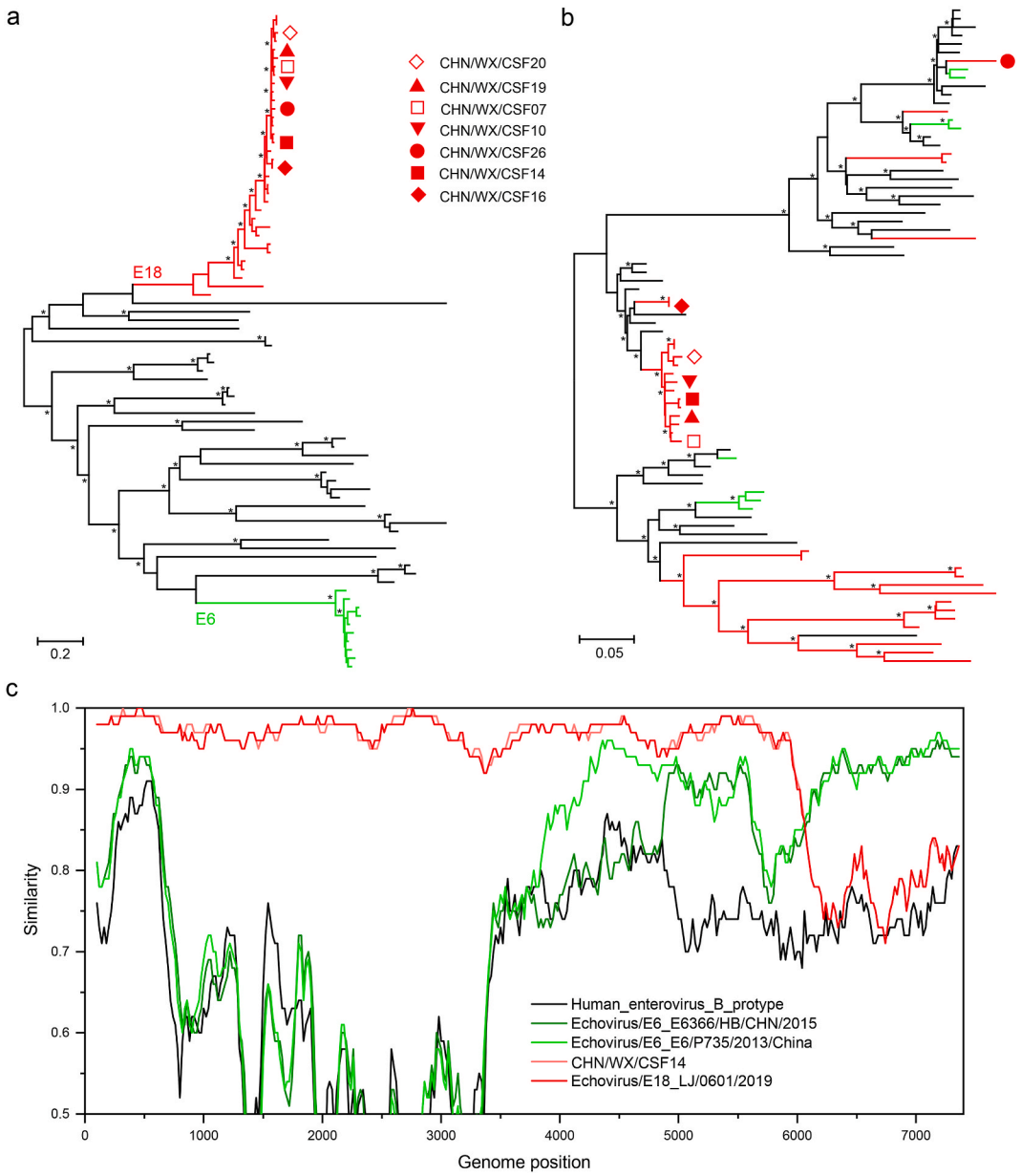

Fig. 3. Phylogenetic and recombination analysis of the detected Echovirus 18 strains. a and b: Maximum likelihood trees showing the phylogenetic relationships of the detected E18 strains and *Enterovirus B* reference sequences based on (a) VP1 and (b) 3D gene alignments. The support values larger than 75% were noted as *; c: Similarity plot of strain CSF26 compared to E18 and E6 reference strains. The y-axis shows nucleotide similarity and the x-axis shows genome position. The full annotated trees of VP1 and 3D can refer to Fig. S1.

4. Discussion

Pediatric encephalitis and meningitis can be triggered by diverse infectious agents, including viruses, bacteria, fungi, and parasites, as well as non-infectious immune-mediated factors [14]. In the past two decades, diagnosis of pediatric neurological infectious diseases like acute meningitis and encephalitis has significantly improved. Molecular detection methods, especially PCR, have proven more accurate than traditional serological tests. However, these methods are limited in throughput and rely on predetermined pathogen spectrum, posing challenges for clinicians. In recent years, unbiased metatranscriptomics has emerged as a novel sequencing approach for pathogen detection and has been widely applied in various clinical cases, such as those of aseptic meningitis or encephalitis [15].

According to internal statistics from the hospital, the Wuxi Children's Hospital admitted over 200 cases of meningitis each year before 2020. After 2020, due to the COVID-19 epidemic, China adopted relevant prevention and control measures. Under this influence, the number of related cases declined, but still remained around 100 cases. In this study, we enrolled 20 pediatric cases

diagnosed with aseptic meningitis. Pathogens were detected in only 9 cases, all identified as enteroviruses. The majority of affected individuals were male, predominantly preschool children. The limited diversity of detected pathogens, in comparison to the etiological spectrum of viral meningitis, may be attributed to the small sample size. For cases without detected pathogens, factors such as the disease status, viral replication efficiency during sampling, or issues related to library construction quality may have influenced the results. Despite the small sample size being a limitation, our findings indicate that, around 2020, enteroviruses, particularly Echovirus 18, were the predominant pathogens causing aseptic meningitis in the Wuxi region.

Enteroviruses are a predominant cause of aseptic encephalitis in children younger than 15 years old in recent years [16]. This type virus spread through direct contact with infected bodily fluids or contaminated surfaces, objects, and water. Once infected, the virus can be shed for weeks through feces, nasal discharge, saliva, and other secretions, facilitating further transmission. Previous studies have shown that over 50 types of enteroviruses distributed across *Enterovirus A-D* are responsible for acute meningitis or encephalitis [5]. E−18 infection manifests from asymptomatic to aseptic meningitis and even fatal outcomes. Aseptic meningitis outbreaks due to E−18 have occurred in China and globally. In 2015, an E18 associated encephalitis/meningitis was first reported in Hebei, China [17]. To date, at least 10 provinces have reported circulating E18, with some documented small-scale outbreaks [18–20]. Generally, circulating E18 strains in China cluster within the C2 subgroup [19]. Although the reported E18 strains all fall within the C2 subgroup, they are dispersed across different branches, suggesting diverse origins.

Recombination, which is very common in enteroviruses like E18, has significant impacts on aspects such as viral evolution, transmission, and virulence [21]. Our detected CHN/WX/CSF14 strain is closely related to LJ/0530/2019, first isolated from an immunocompetent adult with aseptic meningitis [18]. This strain was a recombinant with a 2B gene mosaic from echovirus 30. Another reported recombinant strain was PC06/JS/CHN/2019, which was identical to CHN/WX/CSF16, found in a child with severe encephalitis in Wuxi [22]. This strain had parental origins from E18 and another strain that needs further investigation. CHN/WX/CSF26 is a novel E18 recombinant strain first described in this study. Phylogenetic analysis of the VP1 and 3D genes, along with recombination analysis, confirmed the P1 region of CHN/WX/CSF26 originated from C2 subgenogroup E18 strains circulating in China. Although phylogenetic trees and Blast results demonstrated relatively high nucleotide identity between the 3D gene of CHN/WX/CSF26 and E6 strain E6/P735/2013/China, the identity was only 92.86% [23]. Therefore, the other parental origin of CHN/WX/CSF26 remains undetermined and further investigation is required to fully characterize this novel recombinant strain.

Two decades ago, E11 was suggested to exhibit global prevalence [24]. However, limited E11 sequences have caused consistent underestimation of its spread and importance, with only sporadic HFMD and AE reports [25]. Recently, reported E11 strains in China cluster within D5, with 95–98.4% VP1 identity between strains from Guangdong, Hubei and Jiangsu. Notably, strains circulating in Guangzhou in 2019 triggered an outbreak with 5 pediatric deaths [26]. The two E11 strains found in Wuxi were identical to those within D5, indicating a potential epidemic risk that deserves surveillance in the future.

Some limitations include the small sample size from one hospital and lack of clinical correlation. Nevertheless, this study expands the limited data on enteroviruses associated with pediatric aseptic meningitis in Wuxi. Metagenomic analysis enabled unbiased detection of multiple EV types and discovery of novel E18 recombinants. Our results highlight the genetic diversity of circulating E18 and E11 strains that may cause future outbreaks. Further multi-center studies with increased sampling are warranted to elucidate enterovirus molecular epidemiology in Wuxi and surrounding regions. This will aid in the characterization of viral strains, a crucial aspect in enhancing molecular diagnostics and tracking of aseptic meningitis.

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Ethical standards

This study was approved by the Ethics Committee of Wuxi Children's Hospital (number: 2019-EYLL-001).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Ying Hua: Resources, Formal analysis, Data curation. **Zhenyan Lv:** Resources, Investigation, Formal analysis, Data curation. **Yineng Zhou:** Resources, Formal analysis. **Hongxia Xiang:** Methodology, Formal analysis, Data curation. **Mingxia Sun:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Yan-Jun Kang:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26847.

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