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Sero-molecular epidemiology of enterovirus-associated encephalitis in Zhejiang Province, China, from 2014 to 2017

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ABSTRACT

Background: Recently, both sporadic and outbreak aseptic meningitis caused by enteroviruses have been reported in Zhejiang Province based on a surveillance system.
Methods: This study analysed the epidemiologic features, phylogenetic characteristics and prevalence of enterovirus neutralizing antibodies (nAbs) from 2014 to 2017 in Zhejiang Province.
Results: A total of 584 samples were collected. Males accounted for 66.07% while females accounted for 33.93%. The median age was 6 years (range: 1–15 years). Cases peaked in May and August (81.17%) and 162 cases (28.93%) occurred in June. We detected 15 serotypes, some of which (E6, E9, E18 and E30) were the dominant serotypes prevalent in different years and geographical regions. Phylogenetic results revealed that all of the isolates from this study belonged to the human enterovirus B family. A total of 329 subjects sampled from a healthy population were tested for nAbs against B5, E6 and E30 in Rui'an county in 2015. The seropositive rate of E30 in each age group was significantly higher than that of the other serotypes.
Conclusion: Enterovirus-associated encephalitis pathogens circulating in Zhejiang caused sporadic aseptic meningitis in children. The level of nAbs against human enterovirus reflects the history of previous infections in different age groups. Therefore, additional surveillance sites and more precise seroprevalence studies based on these populations are required to gain better insight into the epidemiology of enterovirus-associated encephalitis in Zhejiang Province.

Introduction

Aseptic meningitis (AM), defined as an infectious disease of the central nervous system (CNS) negative for bacteria, is most frequently caused by a viral infection. AM has gained increased public attention since it is an acute viral infection of the CNS that occurs most frequently in infants and young children (Berlin et al., 1993; Shaker and Abdelhamid, 2015). With the worldwide control of poliovirus and mumps infections, infections with non-polio enteroviruses, including coxsackieviruses (CV) A and B, echoviruses (EV) and new numbered enteroviruses are recognized as the major cause of human AM, although most cases are asymptomatic (Irani, 2008; Nigrovic, 2003).

Epidemics and outbreaks of AM caused by some serotypes of enteroviruses have been reported sporadically (e.g., coxsackievirus B5 [B5] and echovirus 6 [E6]), while some serotypes were endemic over several years (e.g., echovirus 30 [E30]) (Oberste et al., 1999; Chen et al., 2017; Yan et al., 2015; Zhang et al., 2013). Few population-based seroprevalence surveys of enteroviruses have been reported in Zhejiang Province. To our knowledge, an exhaustive seromolecular epidemiology of enterovirus study in this region has not been previously reported. Therefore, to gain comprehensive insight into the seroepidemiology of major prevalent enteroviruses (i.e., B5, E6 and E30) in humans within the Zhejiang region, we conducted a serological survey for neutralising antibodies (nAbs) against endemic strains circulating locally in the community population in 2015 in Zhejiang Province.

A laboratory diagnosis of an enterovirus infection relies on viral isolation in cell cultures, which provides the gold standard for a direct diagnosis. The development of molecular biology and PCR techniques offers potential benefits regarding both the rapidity and efficiency of a viral diagnosis with the application of VP1 gene detection. The VP1 gene encodes the VP1 capsid protein, which is

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the most external and immunodominant protein that also contains neutralisation epitopes. Therefore, VP1 sequences have been found to correlate well with antigenic typing by neutralisation testing and have applications for enterovirus identification and molecular epidemiology (Tao et al., 2014). A surveillance system for enterovirus-associated encephalitis was established during 2002 in Zhejiang Province. As a result of this surveillance system, both sporadic and outbreak AM caused by enteroviruses have been recently reported in Zhejiang Province. Previously, we reported on the molecular epidemiology characterisation of enteroviruses isolated in Zhejiang, China from 2002 to 2013 based on the VP1 region (Yan et al., 2015; Zhang et al., 2013). The present study has extended this research with regards to both the epidemiology and pathogenesis of enterovirus infections from 2014 to 2017 based on the surveillance data in this region. In this study, we retrospectively analysed the epidemiological features of enterovirus-related pathologies diagnosed in surveillance hospitals distributed throughout Zhejiang from 2014 to 2017. Using partial amplification and sequencing of the VP1 gene, we performed a phylogenetic analysis of the enterovirus strains isolated from the cerebrospinal fluid (CSF) and faecal samples of children with enterovirus-related syndromes.

Materials and methods

Surveillance site and samples

This study was approved by the ethics committee of the Zhejiang Provincial Centre for Disease Control and Prevention, China (Approval number: 2016020). All participants provided written informed consent. All methods were carried out in accordance with the principles of the Declaration of Helsinki. Case definition and sample collection were performed as described previously (Zhang et al., 2013). Following these methods, we continued to collect samples from patients under 15 years old during 2014–2017.

To obtain a healthy population, a serosurvey was carried out in Rui'an county of Zhejiang Province in 2015. Participants who had no sign of disease at the time of sample collection were requested to provide a blood sample and to fill out a self-administered questionnaire. Sera were transferred in ice boxes and stored at $-80\,^{\circ}C$ until processed.

The patients' data, including sex, age, place of residence and onset of illness were collected from the Chinese Information System for Disease Control and Prevention. Excel 2010 (Microsoft Inc.) was used to compile the epidemiological data. All statistical tests were conducted with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). For two-sided tests, a P-value less than 0.05 was considered statistically significant.

Laboratory experiments

Viral isolation, serotyping, viral RNA extraction, RT-PCR identification and VP1 gene sequencing were performed as described in our previous study (Zhang et al., 2013). We used RD and HEp-2 cells for viral isolation. These two cell lines were provided as a gift from the Chinese National CDC. Microseroneutralisation tests were performed under biosafety level 2 conditions. The procedure has been described elsewhere in detail (Zhang et al., 2017; Zhu et al., 2018). We used ZJ13-94/E30, ZJ13-100/E6 and ZJ13-15/B5 which were isolated and stored previously by our lab quality nAbs.

Geneious 10.2.2 (www.geneious.com) was used to assemble all VP1 sequences. BLAST was used to identify the different viral serotypes. All sequences obtained were deposited in the GenBank database (**MF467282–MF467371**). Other sequences used in the ML trees were downloaded from GenBank and alignments were performed. Dataset-specific models were selected using the Akaike Information Criterion in Modeltest 3.7 (Posada and Crandall, 1998). A phylogenetic analysis was performed using the General Time Reversible model as a model of nucleotide substitution and the maximum likelihood (ML) method to reconstruct the phylogenetic tree processed with MEGA 7.0.14 (http://www.megasoftware.net/). The statistical significance of the constructed phylogenies was estimated by a bootstrap analysis with 1000 pseudoreplicate data sets.

Results

Cases and epidemiology

A total of 584 samples (including 548 CSF and 36 faeces) were collected from 560 suspected cases of encephalitis from 2014 to 2017. Of the 560 suspected cases, both CSF and faeces were collected for 24 patients. Males accounted for 66.07% (370/560) while females accounted for 33.93% (190/560). The median age was 6 years (range: 1–15 years). The cases peaked in May and August (81.17%), and 162 (28.93%) occurred in June (Figure 1A).

Of the 560 suspected cases, 262 (46.79%) were positive for human enterovirus (HEV) nucleotides, 171 of which were male (46.22%, 171/370) and 91 were female (47.89%, 91/190). There was no statistical significance regarding the difference between males and females ($\chi^2 = 0.142$, $P = 0.706$). There were 254 cases that were VP1 amplification positive. The proportion of HEV-positive results in children aged 6–11 years old was the highest (55.06%), while the lowest proportion was observed in children aged 0–3 years old (14.81%). There was a statistically significant difference regarding the age group ($P = 0.000$). There was a statistically significant positive ratio of HEV detected from different regions ($\chi^2 = 62.38$, $P = 0.000$). The positive ratio of HEV detected from 2014 (55.42%) was higher than that in 2015 (46.88%), 2016 (51.33) and 2017 (28.45), with statistical significance ($\chi^2 = 21.89$, $P = 0.000$). The positive ratio of HEV detected from June (69.13%) was significantly higher than any other month ($\chi^2 = 73.00$, $P = 0.000$) (Tables 1 and 2). We obtained 142 HEVs by virus isolation.

Enterovirus typing and homologous comparison

The HEV isolates were further used for serotyping with a neutralisation assay. There were 15 noted serotypes, including A9, B1, B3, B4, B5, E6, E9, E11, E14, E16, E18, E21, E25, E30 and E33 (Table 2). The dominant serotypes prevailed in different years and regions and were significantly different (Figure 1B). B4, E21 and E33 were only detected in Rui'an, while B1 and E11 were only detected in Haiyan. A9, B5, E6, E9, E18 and E30 were found in all three areas. E30, E18, E6 and E9 were detected frequently during each age group compared to the other serotypes (Figure 1C). HEV was not detected in children aged younger than one year. Different years from 2014 to 2017 displayed different HEV serotypes (Figure 1D). E18 (39.13%) was dominant during 2014, followed by E6 (25.00%). E30 accounted for the majority of the serotypes identified between 2015 (77.97%) and 2016 (58.44%), followed by E6 (8.47%) and E9 (19.48%). E6 (50.0%) was frequently detected during 2017, followed by B5 (8.70%), E9 (8.70%) and E18 (8.70%). Most of the HEV serotypes detected in this study were distributed from May to July, demonstrating a strong periodicity and seasonality (Figure 2).

Phylogenetic analysis

The VP1 sequences obtained in this study were used to construct a phylogenetic tree with seven reference strains from the GenBank database with different serotypes, including CVA1, 16, 24,

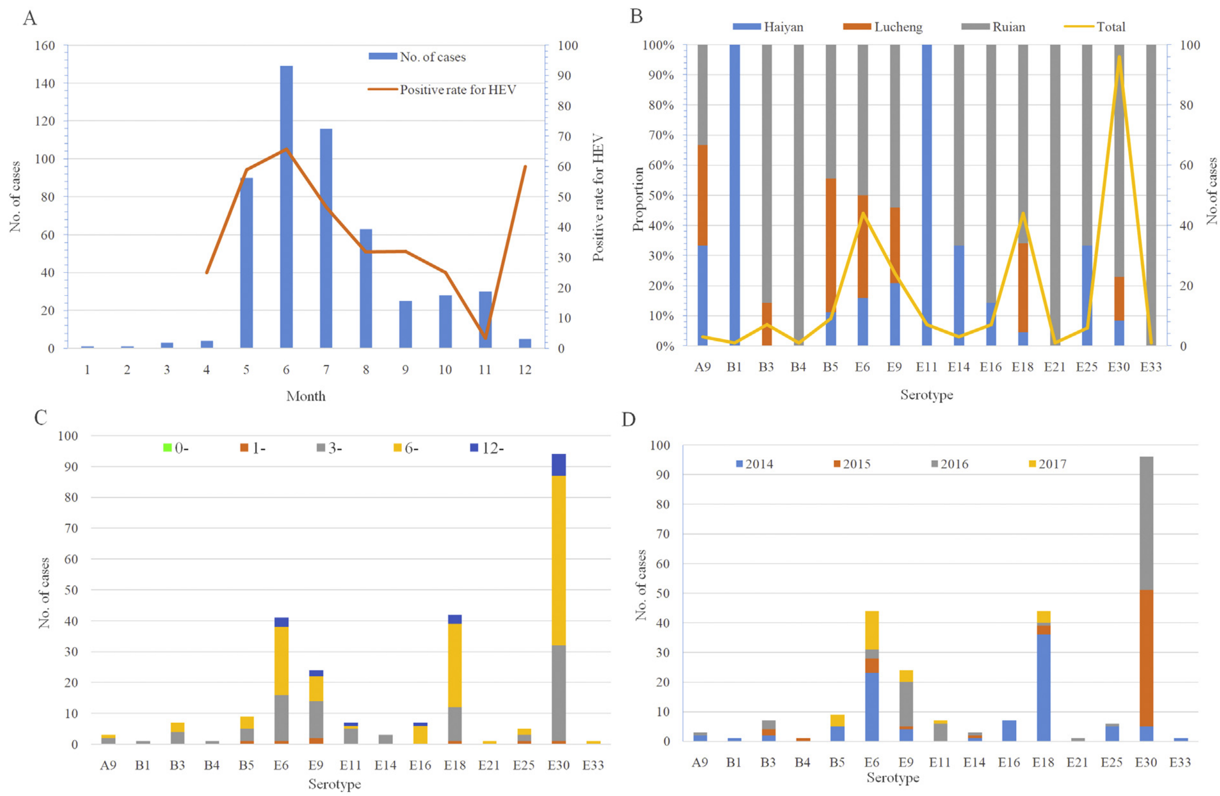

Figure 1. General information regarding the epidemiological features of HEV in Zhejiang Province from 2014 to 2017. (A) Case number and positive rate of HEV in different months. The blue bar represents the number of cases for each month. The orange curve represents the positive rate for each month. (B) Proportion and number of case of infection with different HEV serotypes in different regions. Blue represents isolates from Haiyan. Orange represents isolates from Lucheng. Grey represents isolates from Rui'an. The yellow curve represents the different total number of cases for each serotype. (C) Age distributions for the different HEV serotypes. Green represents the population aged from 0 to 1 years. Orange represents the population aged from 1 to 3 years. Grey represents the population aged from 3 to 6 years. Yellow represents the population aged from 6 to 12 years. Blue represents the population aged from 12 to 15 years. (D) Tendency for the proportion of each HEV serotype from 2014 to 2017. Blue represents the isolates from 2014. Orange represents the isolates from 2015. Grey represents the isolates from 2016. Yellow represents the isolates from 2017.

EV 68, 70, 71 and human rhinovirus A21 (Figure 3). The entire tree was segregated into four clades, including HEV-D, HEV-A, HEV-C and HEV-B, with the exception of rhinoviruses as the outgroup. There were two distinct groups within HEV-B, which constituted all of the VP1 sequences detected in this study. Coxsackievirus serotypes isolated in this study (B3, B4, B5 and A9) were grouped into one monophyly, which was in clade I with E9, E16, E14, E18 and E11. These coxsackieviruses exhibited a phylogenetic distance with the echovirus serotypes. The VP1 sequences from same serotype were clustered into one clade in the ML tree, with a 100-bootstrap value on the node, except for the E6 clade with a 99.9 bootstrap on the node.

Table 1
Information on detection of HEV in Zhejiang Province, during 2014–2017.

Item	N	PCR	%	*CI*
Sex				
Male	370	171	46.22	41.05–51.44
Female	190	91	47.89	40.61–55.25
Age (year)				
0∼	7	0	0	0.00–40.96
1∼	47	8	17.02	7.65–30.81
3∼	175	93	53.14	45.47–60.71
6∼	247	136	55.06	48.63–61.37
12∼	35	15	42.86	26.32–60.65
Unknown	49	10	20.41	10.24–34.34
Area				
Haiyan	49	35	71.43	56.74–83.42
Lucheng	214	56	26.17	20.41–32.60
Rui'an	297	171	57.58	51.73–63.26
Year				
2014	166	92	55.42	47.52–63.13
2015	128	60	46.88	38.00–55.89
2016	150	77	51.33	43.05–59.57
2017	116	33	28.45	20.46–37.57

The geometric mean titre (GMT) of B5, E6 and E30 in Zhejiang Province from 2014 to 2017

A total of 329 subjects aged between new-born and 91 years were tested for nAbs against HEV (Figure 4). The overall seropositive rate of three HEVs was variable and ranged from 42.9% to 77.5%. The seropositive rate for the E30, E6 and B5 serotypes was similar, and increased with age. The seropositive rate of E30 in each age group was higher than that of E6 ($\chi^2 = 31.40$, $P = 0.012$), which was extremely significantly higher than that of B5 ($P = 4.93E-44$). The overall GMTs of the three HEVs were variable and ranged from 4.91 to 21.85. The GMT value of E30 peaked at 75.34, whereas the GMT for B5 peaked in the 7–14 years age group (GMT: 39.40). There was a tendency for the E6 GMT to increase with age, with a value of 14.86 in the group aged 60 years and older (Figure 4).

Discussion

Enteroviruses are the cause of a large number of AM cases, which occur most frequently in infants and young children. We

Table 2
Human enterovirus isolation and serotyping result during the period of 2014–2017.

Year	VP1 positive cases no. (%)	HEV serotypes														
		A9	B1	B3	B4	B5	E6	E9	E11	E14	E16	E18	E21	E25	E30	E33
2014	92 (55.42)	2	1	2		5	23	4		1	7	36		5	5	1
2015	59 (46.09)			2	1		5	1		1		3			46	
2016	77 (51.33)	1		3			3	15	6	1		1	1	1	45	
2017	26 (22.41)					4	13	4	1			4				
Total	254 (45.36)	3	1	7	1	9	44	24	7	3	7	44	1	6	96	1

A: coxsackievirus A; B: coxsackievirus B; E: echovirus.

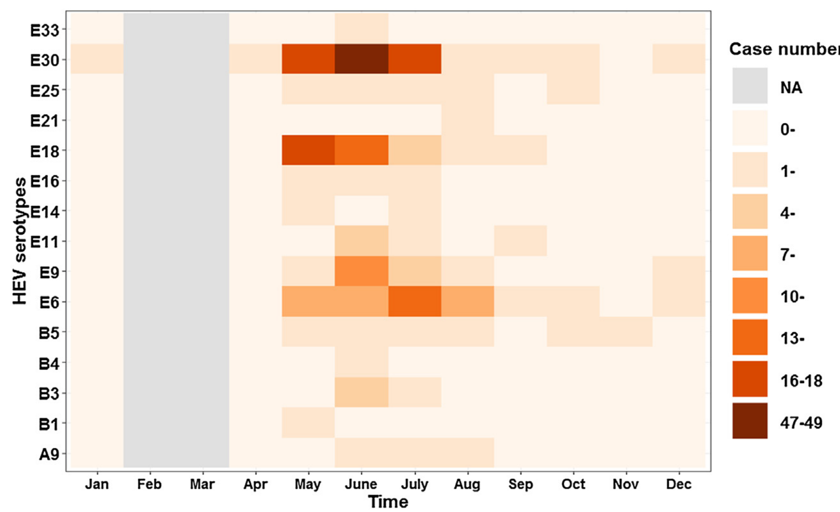

Figure 2. Heatmap of the HEV serotypes for the different months in Zhejiang Province from 2014 to 2017. 'NA' represents the periods in which no sample received; '0' represents that no HEV was detected from the sample.

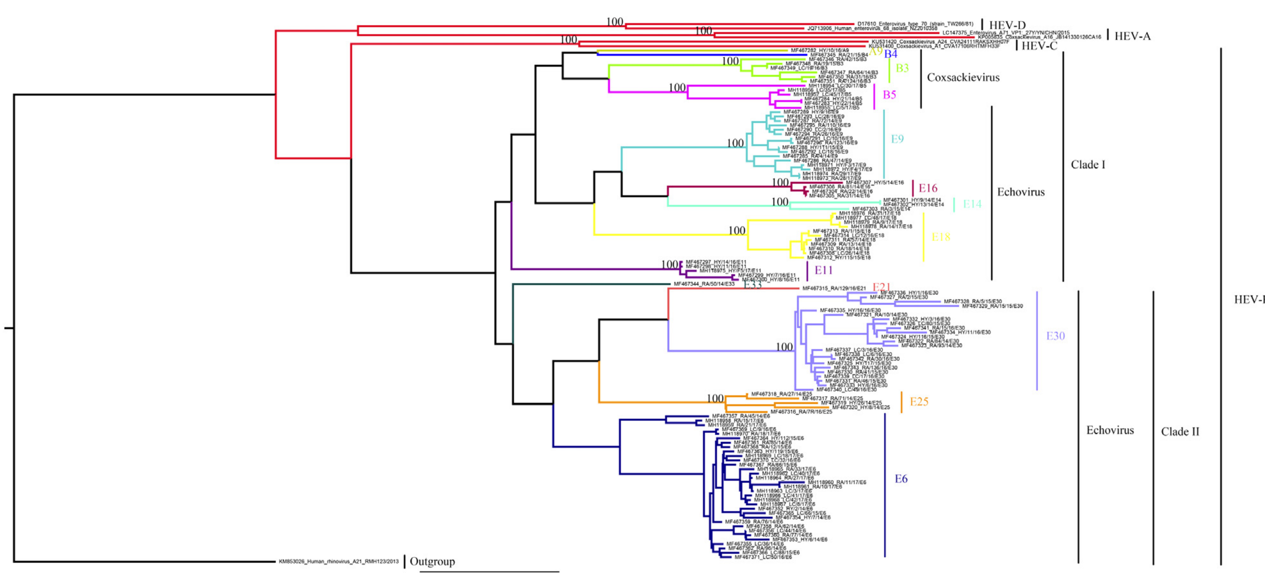

Figure 3. Maximum likelihood tree of VP1 sequences of HEV from Zhejiang Province from 2014 to 2017. Bootstrap values of 100 based on 1000 replicates are indicated above the branches.

previously reported on enterovirus-associated encephalitis in Zhejiang from 2002 to 2013 (Yan et al., 2015; Zhang et al., 2013). The current study extended these findings by investigating both the epidemiologic features and molecular characteristics of samples from confirmed cases between 2014 and 2017 in Zhejiang Province, as well as conducting a serological survey of nAbs against HEVs in healthy individuals. The data from 2014 to 2017 revealed that AM caused by enterovirus infections were confirmed primarily in children younger than 15 years old, especially among those aged 6–11 years, who exhibited the highest rate of

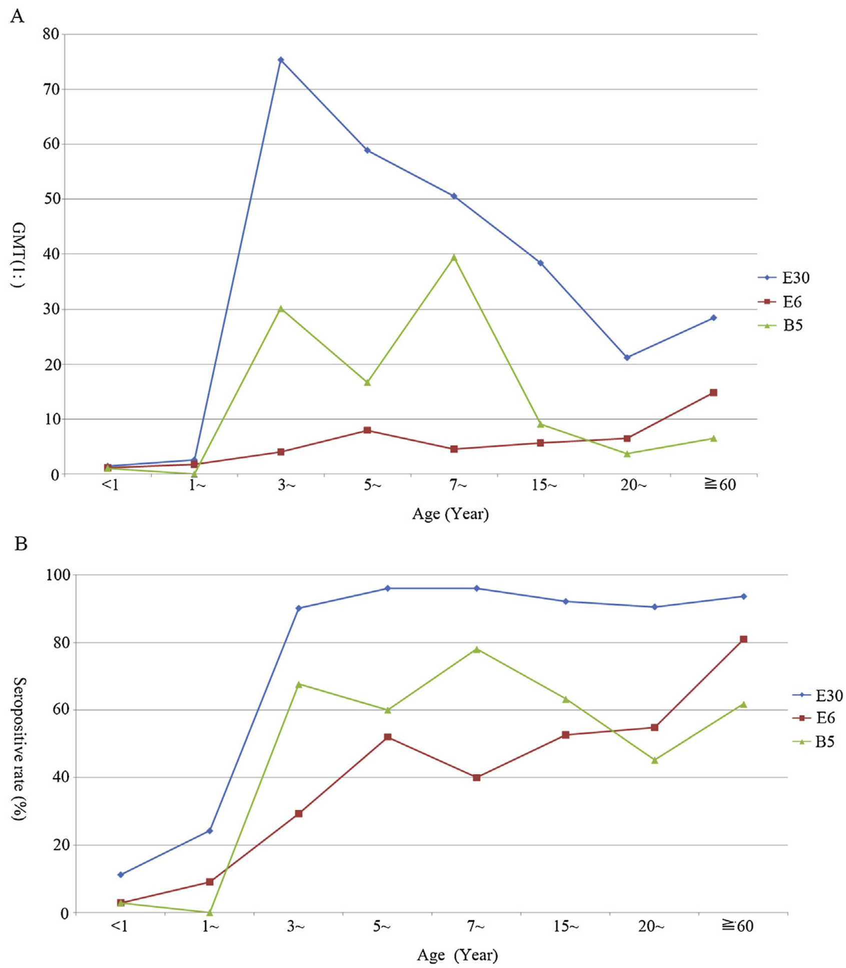

Figure 4. Geometric mean titre (GMT) (A) and seropositive rates (B) of HEV for the different age groups of a healthy population in Rui'an county of Zhejiang Province in 2015.

enterovirus-positive samples. The data from 2014 to 2017 displayed a similar seasonal distribution in prevalence, with these viruses being more prominent during the summer and fall, and less frequent in the winter and spring (Baek et al., 2011). In this study, a total of 15 enterovirus serotypes were detected. Several serotypes (i.e., E11, E16, E18, E21 and E25) were detected for the first time during 2014 to 2017 compared to our previous study. In addition, different years were associated with distinct serotypes with high circulation rates. We previously reported that E30 has been one of the primary causes of AM for years, which was the pathogens that caused an outbreak of suspected AM that occurred in Zhejiang during 2015 (Chen et al., 2017; Sun et al., 2017; Zhang et al., 2013). In accordance with previous studies, we found that E30 was the primary causative agent of AM in Zhejiang from 2015 to 2016, while E18 and E6 replaced E30 as the dominant pathogen in 2014 and 2017, respectively.

Phylogenetic analysis of the VP1 gene in the current study has facilitated the differentiation of circulating serotypes and association of specific HEV genotypes with AM. Some serotypes are associated with global epidemics (e.g., B5, E6 and E30), while others (e.g., A9, B3 and B4) are mainly endemic (Bailly et al., 2011; Fernandez-Garcia et al., 2017; Gao et al., 2018; Smura et al., 2013). E30 was reported to be one of the most frequently detected HEVs in European, American, Asian and African countries responsible for either an epidemic or endemic form of a CNS disease (e.g. AM and sepsis-like illness) (Baek et al., 2011; Bailly et al., 2011; Fernandez-Garcia et al., 2017). In this study, the E30 serotype detected in Zhejiang belonged to Lineage E30_h, which was consistent with our previous research (Chen et al., 2017). The prevalence of E30_h was found to be continuously displaced by new variants and clustered into different clades and sub-clades from 2014 to 2017 in the Zhejiang area, as revealed by the topological structure of the phylogenetic tree. Both E6 and E18 isolated in this study also displayed similar patterns to that of E30 in the tree, with a similar topological structure. E6 and E18 infections can also lead to sporadic cases, outbreaks and epidemic infections. Previous studies have described the transmission of E6 among neonates in neonatal intensive care units and emphasise the potential of enteroviruses to cause widespread illness among this highly susceptible population (Siafakas et al., 2013). A genetic variant of the E18 strain was found to be the cause of an AM outbreak in Taiwan in 2006 (Tsai et al., 2011). In addition, it was reported in 2016 that a complete E18 genome was associated with AM in Hebei and in 2007, and there was also a fatal case of leukoencephalitis in a child infected with E18 reported in France (Brunel et al., 2007; Chen et al., 2016). Thus, more intensive and long-term surveillance of HEVs will be important for monitoring the circulating strains and controlling the risk of various infectious diseases.

Multiple viruses in the *Picornaviridae* family are responsible for causing AM and other diseases (e.g., hand, foot and mouth disease) in individuals of all ages (Baek et al., 2011). Such infections are a concern for both the children found to be infected and exhibit symptoms, as well as a number of healthy adults with unexpected infections, which are primarily asymptomatic (Andreoni and Colton, 2017). Therefore, we conducted a serological survey of the B5, E6 and E30 serotypes in different age groups within a healthy population from Zhejiang to investigate both the seropositive rate and GMTs of these three HEVs. The level of nAbs against HEVs reflects the history of previous infections and specific immunity, which is important for preventing viral infections and reducing the risk of disease occurrence (Zhu et al., 2018). Since there is no effective vaccine and the virus continues to spread, a high level of nAbs indicates a natural infection with no medical intervention. One of the possible reasons for the finding that both the seropositive rate and GMTs were low before the age of three years and subsequently increased dramatically is likely attributed to children living at home before going to kindergarten at the age of three years. Moreover, new-borns and infants aged less than one year have maternally transferred antibodies against HEVs. There were few confirmed cases of AM reported for children aged younger than three years, as indicated by the lower positive rate for HEV detection (Table 1 and Figure 4). It is expected that children will experience one or more natural HEV infections in life after socialising with others more frequently (e.g., kindergarten and school) (Liu et al., 2016). The seropositive rate of all the three enteroviruses, E30, E6 and B5 fluctuated at certain intervals among individuals aged older than three years. The GMT curves were higher for individuals older than 60 years, compared to individuals in their 20s. One possible explanation for this observation is that these three serotypes are frequently asymptomatic infections in adults. Since there are no HEV vaccines, both the seropositive rate and GMT curves represent natural infections in healthy populations. The nAb levels could increase again when the original level of nAbs was too low to be protective following an asymptomatic infection. Low levels of nAbs from previous infections could be boosted following subsequent asymptomatic infections. The results of the present study indicate that the age for this turning point begins when individuals enter their 20s. Moreover, elderly individuals (older than 60 years) have had more opportunities to be infected with HEV compared to younger adults.

To overcome the limitations of the present study and involve a more in-depth analysis in future studies, the following should be considered: (1) additional surveillance sites should be established for HEVs to collect more information regarding the epidemiology of HEVs circulating in Zhejiang Province; and (2) additional healthy populations should be studied and sampling should be expanded to understand the level of nAbs from a more accurate perspective. More detailed information regarding the seroepidemiology of HEV should be included in future studies.

Author contributions

Prepared the first draft of this manuscript: YS, ZPM, JYY. Provided the surveillance data: ZPM, YJC. Provided the virological data: JYY, LMG, YC, HYM, YJZ. Interpreted the surveillance and virological data: YS, ZPM, JYY. All authors reviewed and revised the first and final drafts of this manuscript.

Ethical approval

This study was approved by the ethics committee of the Zhejiang Provincial Centre for Disease Control and Prevention, China (Approval number: 2016020). All participants provided written informed consent. All methods were carried out in accordance with the principles of the Declaration of Helsinki.

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Conflict of interest

No conflict of interest to declare.

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