jmb

Molecular Epidemiology and Prevalence of Echovirus 30 in Zhejiang Province, China, from 2002 to 2015 [S]

Yin Chen[†], Yi Sun[†], Juying Yan[*], Ziping Miao, Changping Xu, Yanjun Zhang, Haiyan Mao, and Liming Gong

Zhejiang Provincial Center for Disease Control and Prevention, Hangzhou, Zhejiang 310051, P.R. China

Echovirus serotype 30 (ECHO30) has been responsible for several recent worldwide outbreaks of viral meningitis. In Zhejiang Province, China, ECHO30 has been one of the main causes of viral meningitis for years. This study, using phylogenetic analysis of the VP1 gene, was performed to investigate the general molecular epidemiology and genetic patterns of ECHO30 circulating in Zhejiang Province between the years 2002 and 2015. The nucleotide sequences of ECHO30 VP1 showed that they were 64.8% identical with the prototype strain, Bastianni, while the amino acids were 84.9% identical. Phylogenetic analyses showed that ECHO30 in the Zhejiang area has diverged into two genotypes. Genotype I consists of strains isolated since 2002, whereas genotype II includes strains that were mainly isolated during the 2002 to 2004 outbreak. ECHO30 has been endemically circulating in both humans and the environment for a long period of time. Additionally, we evaluated the significance of recombination presented during the years 2005 to 2007 to demonstrate that recombination plays an important role in the prevalence of ECHO30 in the Zhejiang area.

Keywords: Echovirus 30, VP1, molecular epidemiology, phylogenetics, recombination

Introduction

Viral meningitis is a series of infections of the central nervous system that includes mental disorders and disrupted awareness. Many previous studies have concluded that species of *Enterovirus* were the main pathogens causing this disease. The genus *Enterovirus* consists of 12 species, ranging from *Enterovirus* A to J (except *Enterovirus* I) and *Rhinovirus* A to C, according to the latest edition of the International Committee on the Taxonomy of Viruses (http://www. ictvonline.org). ECHO (enteric cytopathic human orphan) virus, including echovirus serotype 30 (ECHO30), belongs to the species *Enterovirus* B, genus *Enterovirus* of the *Picornaviridae* family [1]. Recent reports and studies have shown that ECHO30 was responsible for aseptic meningitis and was implicated in several outbreaks worldwide [2–4]. Different variants of ECHO30 were identified during two outbreaks in 1997 and 2000 in Clermont-Ferrand, France. In the USA, ECHO30 became a primary cause of meningitis outbreaks in 2003 and 2004 [5–

7]. Therefore, long-term surveillance programs to monitor ECHO30 were established by public health departments in countries such as the USA, Denmark, and France [8].

ECHO30 is a picornavirus, which is a single-stranded, positive-sense RNA virus of approximately 7.5 nt in length. The genome contains a single open reading frame coding P1, P2, and P3 regions. Because the VP1 region, included in P1, has unique antigenic properties, VP1-based sequence analysis has been used as a standard for molecular epidemiologic investigations of ECHO30 [9]. Many previous studies of the molecular epidemiology of ECHO30 meningitis outbreaks and sporadic cases based on the VP1 region have been reported worldwide [10, 11]. Phylogenetic analyses of VP1 evidenced the co-circulation of six distinct ECHO30 lineages responsible for the 2005 aseptic meningitis outbreak in France, of which three had co-circulated in California, USA. Recombination events have contributed to the evolutionary history of ECHO30, according to analyses of the viral genome by Mriand *et al.* [7] and Lévêque *et al.* [12].

Several ECHO30 outbreaks have been reported in

Taiwan, Jiangsu, and Shandong provinces, China [13–15], but few dynamic surveillance studies have been reported. Since human enterovirus surveillance based on human specimens is limited and has mainly focused on testing of specimens collected through surveillance of acute flaccid paralysis surveillance and hand-foot-mouth disease [15], we established a surveillance program for general enteroviruses in Zhejiang Province in 2002 and have continued to monitor for them for more than 13 years. Our previous study on the characterization of enterovirus-associated meningitis in Zhejiang from 2002 to 2012 indicated that the dominant serotype ECHO30 had diverged into two genotypes, and the positive detection ratios for these two genotypes were significantly different [16]. Unfortunately, we were not able to identify ECHO30 in the years 2005 and 2007, which may have several possible explanations according to the results from the previous study [16]. Therefore, to build on previous research, our goals in this study were (i) to determine the significance of the genetic patterns involved in the emergence of the different lineages in the evolution of ECHO30; and (ii) to seek reasonable possibilities for the absence for detection of ECHO30 in 2005 and 2007. To reach our goals, we carried out a comprehensive study combined with epidemiological investigations and conducted phylogenetic and recombination analyses to show the prevalence of ECHO30 in Zhejiang at the molecular level during the period 2002−2015.

Materials and Methods

Surveillance Site and Samples

This study was approved by the ethics committee of the Zhejiang Provincial Center for Disease Control and Prevention, China (Approval No. 2017-017). Sample collection from 2002 to 2012 was described by Zhang *et al.* [16]. Following this method, we continued to collect samples from 2013 to 2015.

Virus Isolation and Identification

The isolation and identification process followed the standard protocol published by WHO [17]. We used RD and HEp-2 cells to isolate the virus. These two cell lines were gifts from the Chinese National CDC. The microneutralization test was used to identify the type of positive result.

VP1 Sequencing

Viral RNA was extracted using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. The VP1 region was amplified by RT-PCR (TaKaRa, Dalian). The self-designed primers were VP1-F (5′-GCATTGTGACACCACCAGAC-3′) and VP1-R (5′-CAATTCTGCCAATCAACGTG-3′). Procedures for the RT-PCR were an initial reverse transcription for 30 min at

50°C, denaturing for 2 min at 94°C, followed by 40 cycles of 0.5 min at 94°C, 0.5 min at 52°C, 2 min of elongation at 72°C, and ending with an 8-min extension at 72°C. Sequencing was performed on an ABI 3730XL automatic DNA analyzer (Life Technologies, USA).

Identification of the Enterovirus by Random Amplification

During continuous surveillance over more than 10 years, ECHO30 virus was the predominant agent responsible for aseptic meningitis. After the maximum isolates obtained in 2004, ECHO30 did not present in the surveillance program between 2005 and 2007. Because of frequent recombination in enteroviruses, 10 isolates with poor serotyping results in neutralization assays during the years of 2005−2007 were used for further amplification and sequencing using random amplification. The harvested RD cell culture in which the virus was positively isolated was centrifuged at 3,000 ×*g* for 10 min. After treatment, 200 µl of the cell-free supernatant was subjected to nucleic acid extraction using the Qiagen RNeasy Mini Kit, and total RNA was dissolved in 50 µl of DEPC-treated water with 4 units of recombinant RNase inhibitor (Promega, USA). Reverse transcription of RNA, second strand synthesis, and subsequent random amplification were done according to the method described by Allander *et al.* [18]. The amplified product was visualized by gel electrophoresis, after which fragments >300 bp in length from an isolate with most abundunt amplicons were recovered from the agarose gel (Invitrogen, USA). The amplicon was ligated to the pGEM-T Easy vector and then the chemically transformed DH10B cells were cultured on IPTG/X-Gal plates. White colonies were cultured and subjected to Sanger sequencing using the M13-F primer. Sequences were tested for identity using the BLAST program.

Complete Genomic Sequence Analysis of the Recombinant Strain

Primers for amplifying the genome of the virus strain were designed according to the sequencing information from random cloning (all primer sequences available upon request). 5′ RACE and 3′ RACE were conducted according to the protocols of the Invitrogen 5′ RACE System and TaKaRa 3′-Full RACE Core Set with PrimeScript RTase, respectively. Gene-specific primers were designed on the basis of obtained viral sequences neighboring both ends. Sequence data were assembled using SeqMan of the DNAStar software. The complete genome was subjected to a recombination test with SimPlot software using the window width and step size of 100 bp and 20 bp, respectively [19].

Data Analysis

We checked the sequences and variant positions in the amino acids using Geneious 8.1.7 (http://www.geneious.com). We then performed multiple sequence alignments with data matrixes of other sequences pertaining to the ECHO30 virus downloaded from the GenBank databases using MUSCLE in Geneious. The prototype strain of ECHO30, Bastianni, which was isolated in New York, USA, in 1958, was considered in this analysis as well. Phylogenetic analyses were performed using the maximum

composite likelihood as a model of nucleotide substitution, and the neighbor-joining method to conduct the phylogenetic tree based on MEGA ver. 7.0.14 [20]. The bootstrap value was set as 1,000 for the statistical method. ZHLJ-89-E25/ZJ/2005 (KX774483) and HN-2-E25/HN/2008 (HM031191) were used as outgroups.

Results

We obtained 206 ECHO30 positives from a total of 1,757 samples obtained between 2002 and 2015. Sequences used in this study have been deposited in GenBank with accession numbers shown in Supplemental Table S1. The average length for VP1 amplification was 877 bp, which codes for 292 amino acids. The nucleotide sequences of the ECHO30 VP1 segment showed that they were 64.8% identical to the VP1 region in the prototype strain of ECHO30, Bastianni, while the amino acid sequences of VP1 were 84.9% identical. Table 1 shows amino acid substitution in the VP1 area from different ECHO30 isolates in Zhejiang and from other locations, such as Fujian Province and France, with the Bastianni prototype. There were 28 substitutions among these representative sequences.

The phylogenetic tree showed that VP1 segments in ECHO30 isolates from Zhejiang Province diverged into two groups (E30_h and E30_i) with high bootstrap values, which was consistent with previous studies [11, 16]. Three of the 28 substitutions (18I/V, 54V/I, and 277 G/D) displaying different amino acids were correlated with the divergence of the two clades in the phylogenetic tree. Isolates from the same area and the same year were distributed in different genotypes (E30_h and E30_i); for example, YQ-50-E30/ZJ/2004 (AY879320) in E30_i and YQ-2-E30/ZJ/2004 (KR231967) in E30_h. On the other hand, isolates from the same area that were obtained from different years clustered into different groups, such as PT-113-E30/ZJ/2004 (KR231966) and PT-5-E30/ZJ/2012 (KR231981). E30_h split into two major clades: Clade 1 and Clade 2. Clade 1 included the isolates from the year of 2008−2015 and Clade 2 included those from 2003 to 2014. Time window overlaps between the two clades within E30_h displayed the similar pattern as E30_h with the E30_i group. Isolates in the region of Rui'an from 2012 were scattered in the two internal clades of group E30_h. The 2009 and 2010 Quzhou isolates were separated into Clade 1 and Clade 2 in E30_h.

Analyses of Random Amplification Results

Twelve white colonies were picked up and subjected to sequencing using the M13-F primer after culturing. Fragments of different length were inserted into all 12 clones, nine of which represented the enterovirus gene, and the other three clones had genes randomly amplified from the human genome. Of the nine enterovirus genes, the length ranged from 188 to 780 bp. The nine genes were dispersed in the enterovirus genome. After BLAST analyses, the genes were mapped to the VP1, VP2, 2C, 3A, and 3D regions of the virus. Six genes had highest identity with the ECHO30 virus and the other three genes with the echovirus

Table 1. Unique amino acid changes observed in ECHO30 isolates detected in 10 samples from Zhejiang Province compared with two earlier published strains.

Strain	Clade	Substitution site in amino acid																												
		5	7	8	9	18	54	56	64	74	78	80	84	87	122	132	133	145	155	156	242	258	263	277	285	287	289	290	291
Bastianni E30	II	G	L	N	K	I	V	F	L	A	H	T	A	D	F	N	R	I	K	G	K	P	K	G	T	N	V	L	T
AFP01-56-E30/FJ/2001	II	S	.	.	R	.	.	Y	I	V	Q	A	V	E	.	T	T	V	R	S	R	.	R	.	.	.	L	S	N
HZ-28-E30/ZJ/2002	II	S	.	.	R	.	.	Y	I	V	Q	A	V	E	.	T	T	V	R	S	.	S	R	.	.	.	L	S	N
LH-63-E30/ZJ/2004	II	S	.	.	R	.	.	Y	I	V	Q	A	V	E	.	T	T	V	R	S	.	.	R	.	.	.	L	S	N
NB-15-E30/ZJ/2003	I	S	.	.	.	V	I	Y	I	V	Q	A	V	E	.	T	T	V	.	S	.	.	R	D	.	.	L	T	N
LC-35-E30/ZJ/2008	I	S	.	.	R	V	I	Y	I	V	Q	A	V	E	L	T	T	V	.	S	.	.	R	D	.	.	L	T	N
QZ-37-E30/ZJ/2010	I	S	I	.	.	V	I	Y	I	V	Q	A	V	E	.	T	T	V	.	S	.	.	R	D	.	.	L	T	N
HY-6-E30/ZJ/2012	I	S	I	.	R	V	I	Y	I	V	Q	A	V	E	L	T	T	V	.	S	.	.	R	D	.	.	L	T	N
PT-5-E30/ZJ/2012	I	S	I	.	R	V	I	Y	I	V	Q	A	V	E	L	T	T	V	.	S	.	.	R	D	.	.	L	T	N
RA-138-E30/ZJ/2012	I	S	I	.	R	V	I	Y	I	V	Q	A	V	E	L	T	T	V	.	S	.	.	R	D	.	.	L	T	N
ZS-2-E30/ZJ/2012	I	S	I	.	R	V	I	Y	I	V	Q	A	V	E	L	T	T	V	.	S	.	.	R	D	.	.	L	T	N
RA-3-E30/ZJ/2013	I	S	I	.	.	V	I	Y	I	V	Q	A	V	E	.	T	T	V	.	S	.	.	R	D	A	T	L	T	N

Table 2. Enterovirus sequences from nine randomly amplified clones and BLAST results.

	Insertion length (bp)	Corresponding gene	With most identity
1	338	2C, 3A	ECHO30
2	780	2C	ECHO30
3	395	3D	ECHO30
5	354	VP2	ECHO25
7	379	2C, 3A	ECHO30
8	188	VP1	ECHO25
9	210	2C	ECHO30
10	539	2C	ECHO30
12	353	VP2	ECHO25

25 serotype (ECHO25). Three fragments corresponded to genes encoding capsid proteins, and six encoded nonstructural proteins. The details are shown in Table 2.

Complete Genome Sequence and Its Recombination Analysis

The complete genome sequences of the ECHO25 and ECHO30 mosaic viruses were obtained from specific amplifications, 5' RACE and 3' RACE. The complete genome consists of approximately 7,429 nucleotides, including a 6,585-nt open reading frame encoding a 2,194-amino acid polyprotein. The complete genome data have been deposited in GenBank (Accession No. KX774483).

Since the highest identity was found with the ECHO25 and ECHO30 viruses, the complete genome of the prototype ECHO25 (JV-4, AY302549) and the local ECHO30 virus genome (Zhejiang/17/03/CSF, DQ246620) were downloaded to detect recombination events. An echovirus serotype 4 (ECHO4) genome (AUS250G, FJ172447) commonly found in meningitis cases was also included. The genome of E25/ZE-wly/Zhejiang/CHN/2005 was subjected to SimPlot analysis against these three genomes. The results of the recombination analysis are shown in Fig. 1. Based on the graphically displayed sequence relationship, E25/ZE-wly/Zhejiang/CHN/2005 is a recombinant of the ECHO25 and ECHO30 viruses. In this recombinant strain, genes encoding capsid proteins are more similar to those of the ECHO25 virus. In the area of 5' and 3' UTRs and genes encoding nonstructural proteins, the virus is more identical to the ECHO30 virus.

Discussion

Echoviruses have been known to cause human diseases, including asymptomatic infections, fever, aseptic meningitis,

and serious diseases in newborns, since the first isolation of an echovirus from fecal material obtained from a patient with an asymptomatic infection [21]. They represent a public health concern, since some infections by echoviruses may lead to serious illness and even death. This study reports on the molecular epidemiology of ECHO30 associated with aseptic meningitis in Zhejiang Province, China, from 2002 to 2015. Additionally, we evaluated the significance of recombination presented during the period of 2005−2007 to demonstrate that recombination plays an important role in the ECHO30 prevalence in the Zhejiang area.

Substitutions in the amino-acid sequence level in ECHO30 viruses were investigated, since they may be responsible for variations in transmissibility, pathogenicity, and drug resistance. Similar substitution studies displayed such consequences. Research on avian influenza-infected humans, for example, showed that Q226L in the HA protein was expected to bind strongly to α-2,6 human-like receptors [22]. In the ECHO30 study, we found the average length for VP1 amplification to be 877 bp, which coded for 292 amino acids. It was reported that F122L, found mainly in the E30_h group, was thought to be one of the conserved positions of the drug-binding pocket [23]. Amino acid position 285, which is located at the end of the C terminal of VP1, was identified as variable (T285A) in our sequences. The biological significance of other identified mutations remains uncertain and requires further study.

We obtained complete ECHO30 VP1 sequences from most locations in Zhejiang from the 2002−2004 outbreak. The meningitis cases observed were caused by two major virus groups (E30_h and E30_i) (Fig. 2), as revealed by phylogenetic analysis, and coincided with the previous study [16]. A similar phenomenon was revealed in other areas, such as in Luoding, Guangdong Province, in that different ECHO30 variants existed in the local meningitis outbreak [24]. Because the score of pairwise identity between E30_h and E30_i on the nucleotide level was 65.2, while the amino acid identity was 83.9%, these two groups therefore could be treated as two genotypes of ECHO30 according to the criteria in picornavirus classification [25, 26]. Isolates obtained in Fujian in 2001 showed high homologies with other E30_i isolates from Zhejiang. Isolates of the E30_h group became prevalent starting in 2003, and then replaced E30_i to become the dominant circulating group in the Zhejiang area. E30_i appears to have disappeared after 2004. Substitutions in the amino acids indicate that only three changes led to divergence between E30_h and E30_i (18I/V, 54V/I, and 277G/D), but more studies are needed to verify the biological functions and to determine
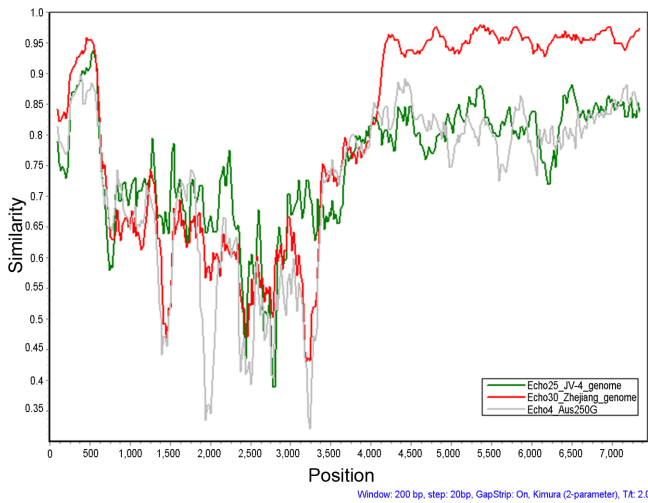
Fig. 1. Recombination analysis results using SimPlot software. The Y axis gives the percentage of identity between E25/ZE-wly/Zhejiang/CHN/2005 and three indicated strains within a sliding window of 100-bp wide centered on the position plotted, with a step size between plots of 20 bp.

the role in the evolution of ECHO30 for these variants. E30_h split into two major clades after it emerged in 2003. Circulating E30_h groups are continuously displaced by new variants and clustered into different clades and subclades in recent years, as indicated by the topology structure of the phylogenetic tree that isolates from the same area might belong to different clusters. Adaptive evolution might occur in human populations because of the possible antigenic drift in susceptible individuals [27, 28]; on the other hand, multiple viral introductions from other China sources and environmental circulations played important roles in endemic co-circulations and transmission in the Zhejiang area [29, 30]. Further study needs to be done to test with these hypotheses.

This study has led to significant advances in understanding the prevalence of the ECHO30 virus in the Zhejiang area over a period of 13 years, with some periods of active and low circulation. There were several outbreaks during the years 2002−2015, and also 0% detection for ECHO30-positive result in the period of 2005−2007. A possible explanation for not finding ECHO30 during 2005 and 2007 may be due to recombination. The strain we identified as ECHO25 is a recombinant of the ECHO25 and ECHO30 viruses, with the ECHO25 VP1 region. This was classified as ECHO25 when using VP1 fragment identification. We therefore propose that recombination may be one of the scenarios for the missing ECHO30 during the period 2005−2007. It is an unusual recombination pattern since usually it
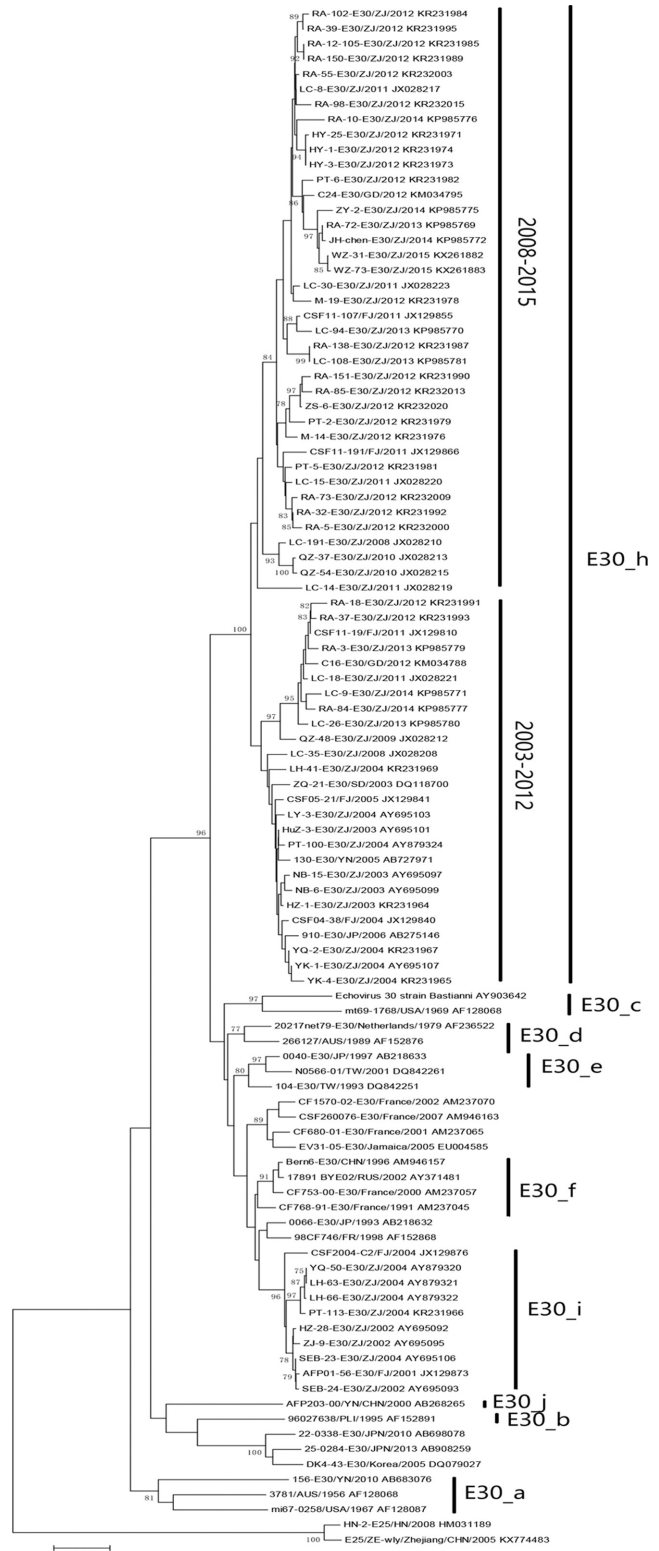

Fig. 2. Maximum likelihood trees of VP1 sequences in ECHO30 in Zhejiang Province during 2002 to 2015.

Bootstrap values based on 1,000 replicates are indicated above the branches.

happens in a nonstructural region [10, 31]. Multiple studies based on VP1 and whole genomes proposed by different authors have found similar phenomena [11, 31]. ECHO30 has been characterized to some extent by repeated cycles of emergence, dominance, and disappearance of individual recombinants over periods of years in China and Spain. Because of recombination, the evolution of ECHO30 is exceptional when compared with other enteroviruses, since recombination may be a key reason for the development of the ECHO30 lineage [31]. Monitoring echovirus circulation both in human populations and the environment is important because individual serotypes may differ, and changes in the predominant type can be accompanied by outbreaks, especially large-scale ones.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Adams MJ, King AM, Carstens EB. 2013. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses. *Arch. Virol.* **158:** 2023-2030.

2. Akiyoshi K, Nakagawa N, Suga T. 2007. An outbreak of aseptic meningitis in a nursery school caused by echovirus type 30 in Kobe, Japan. *Jpn. J. Infect. Dis.* **60:** 66-68.

3. Lukashev AN, Ivanova OE, Eremeeva TP, Gmyl LV. 2008. Analysis of echovirus 30 isolates from Russia and new independent states revealing frequent recombination and reemergence of ancient lineages. *J. Clin. Microbiol.* **46:** 665-670.

4. Choi YJ, Park KS, Baek KA, Jung EH, Nam HS, Kim YB, *et al*. 2010. Molecular characterization of echovirus 30-associated outbreak of aseptic meningitis in Korea in 2008. *J. Microbiol. Biotechnol.* **20:** 643-649.

5. Centers for Disease Control and Prevention. 2003. Outbreaks of aseptic meningitis associated with echoviruses 9 and 30 and preliminary surveillance reports on enterovirus activity - United States, 2003. *MMWR Morb. Mortal. Wkly Rep.* **52:** 761-764.

6. Centers for Disease Control and Prevention. 2006. Enterovirus surveillance - United States, 2002-2004. *MMWR Morb. Mortal. Wkly Rep.* **55:** 153-156.

7. Mriand A, Henquell C, Archimbaud C, Peigu-Lafeuille H, Bailly JL. 2007. Emergence of recent echovirus 30 lineages is marked by serial genetic recombination events. *J. Gen. Virol.* **88:** 166-176.

8. McWilliam Leitch EC, Cabrerizo M, Cardosa J, Harvala H, Ivanova OE, Koike S, *et al*. 2012. The association of recombination events in the founding and emergence of subgenogroup evolutionary lineages of human enterovirus 71. *J. Virol.* **86:** 2676-2685.

9. Oberste MS, Maher K, Kennett ML, Campbell JJ, Carpenter MS, Schnurr D, *et al*. 1999. Molecular epidemiology and genetic diversity of echovirus type 30 (ECHO30): genotypes correlate with temporal dynamics of ECHO30 isolation. *J. Clin. Microbiol.* **37:** 3928–3933.

10. Bailly JL, Brosson D, Archimbaud C, Chambon M, Henquell C, Peigue-Lafeuille H. 2002. Diversity of echovirus 30 during a meningitis outbreak, demonstrated by direct molecular typing from cerebrospinal fluid. *J. Med. Virol.* **68:** 558-567.

11. Yang XH, Yan YS, Weng YW, He AH, Zhang HR, Chen W, *et al*. 2013. Molecular epidemiology of echovirus 30 in Fujian, China between 2001 and 2011. *J. Med. Virol.* **85:** 696-702.

12. Lévêque N, Jacques J, Renois F, Antona D, Abely M, Chomel JJ, *et al*. 2010. Phylogenetic analysis of echovirus 30 isolated during the 2005 outbreak in France reveals existence of multiple lineages and suggests frequent recombination events. *J. Clin. Virol.* **48:** 137-141.

13. Wang JR, Tsai HP, Huang SW, Kuo PH, Kiang D, Liu CC. 2002. Laboratory diagnosis and genetic analysis of an echovirus 30-associated outbreak of aseptic meningitis in Taiwan in 2001. *J. Clin. Microbiol.* **40:** 4439-4444.

14. Zhao YN, Jiang QW, Jiang RJ, Chen L, Perlin DS. 2005. Echovirus 30, Jiangsu Province, China. *Emerg. Infect. Dis.* **11:** 562-567.

15. Tao ZX, Wang HY, Li Y, Liu GF, Xu AQ, Lin XJ, *et al*. 2014. Molecular epidemiology of human enterovirus associated with aseptic meningitis in Shandong province, China, 2006-2012. *PLoS One* **9:** e89766.

16. Zhang L, Yan J, Ojcius DM, Lv H, Miao Z, Chen Y, *et al*. 2013. Novel and predominant pathogen responsible for the enterovirus-associated encephalitis in eastern China. *PLoS One* **8:** e85023.

17. World Health Organization. 2004. *Polio Laboratory Manual*, 4th Ed. Geneva, Switzerland.

18. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc. Natl. Acad. Sci. USA* **102:** 12891-12896.

19. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, *et al*. 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J. Virol.* **73:** 152-160.

20. Kumar S, Stercher G, Tamura K. 2016. Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33:** 1870-1874.

21. Robbins FC, Enders JF, Weller TH, Florentino GL. 1951. Studies on the cultivation of poliomyelitis viruses in tissue culture. V. The direct isolation and serologic identification of virus strains in tissue culture from patients with nonparalytic and paralytic poliomyelitis. *Am. J. Hyg.* **54:** 286-293.

22. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, *et al*. 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. *N. Engl. J. Med.* **368:** 1888-1897.

23. Ke GM, Lin KH, Lu PL, Tung YC, Wang CF, Ke LY, *et al*. 2011. Molecular epidemiology of echovirus 30 in Taiwan, 1988-2008. *Virus Genes* **42:** 178-188.

24. Xiao H, Guan DW, Chen RY, Chen PX, Monagin C, Li W, *et al*. 2013. Molecular characterization of echovirus 30-associated outbreak of aseptic meningitis in Guangdong in 2012. *Virol. J.* **10:** 263-270.

25. Oberste MS, Maher K, Kilpaterick DR, Pallansch MA. 1999. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. *J. Virol.* **73:** 1941-1948.

26. Oberste MS, Haher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. 1999. Typing of human enteroviruses by partial sequencing of VP1. *J. Clin. Microbiol.* **37:** 1288-1293.

27. Palacios G, Casas I, Cisterna D, Trallero G, Tenorio A, Freire C. 2002. Molecular epidemiology of echovirus 30: temporal circulation and prevalence of single lineages. *J. Virol.* **76:** 4940-4949.

28. Nougairede A, Bessaud M, Thiberville SD, Piorkowski G, Ninove L, Zandotti C, *et al*. 2014. Widespread circulation of a new echovirus 30 variant causing aseptic meningitis and non-specific viral illness, South-East France, 2013. *J. Clin. Virol.* **61:** 118-124.

29. Sun Y, Yan JY, Mao HY, Zhang L, Lyu QF, Wu ZH, *et al*. 2013. Characterization of the complete genome of Chikungunya in Zhejiang, China, using a modified virus discovery method based on cDNA-AFLP. *PLoS One* **8:** e83914.

30. Chen D, Wen XH, Sun Y, Mao HY, Zhang YJ, Chen Y, *et al*. 2016. Multiple viral introduction: molecular characterization of influenza B virus in Wenzhozu, Zhejiang, China, from 2011 to 2014 based on hemagglutinin and neuraminidase genes. *Arch. Virol.* **161:** 1005-1013.

31. Cabrerizo M, Echevarria JE, Gonzalez I, Miguel T, Trallero G. 2008. Molecular epidemiological study of HEV-B enteroviruses involved in the increase in meningitis cases occurred in Spain during 2006. *J. Med. Virol.* **80:** 1018-1024.