Jundishapur J Microbiol. 2015 December; 8(12): e27113. doi: 10.5812/jjm.27113

Published online 2015 December 26. **Research Article**

Impact of Human Enterovirus 71 Genotypes in Meningoencephalitis in Iran

Pooneh Rahimi,[1,*] Akram Roohandeh,[2] Amir Sohrabi,[3] Ehsan Mostafavi,[4] and Golnaz Bahram Ali[1]

[1]Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, IR Iran
[2]Pharmaceutical Science Branch, Islamic Azad University, Tehran, IR Iran
[3]Department of Molecular Medicine, School of Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran, IR Iran
[4]Department of Epidemiology, Pasteur Institute of Iran, Tehran, IR Iran

*Corresponding author: Pooneh Rahimi, Department of Hepatitis and HIV, Pasteur Institute of Iran, Tehran, IR Iran. Tel: +98-2166969291, Fax: +98-2166969291, E-mail: prahimi@pasteur.ac.ir

Received 2015 January 20; **Revised** 2015 April 29; **Accepted** 2015 May 22.

Abstract

Background: Since the importance of poliovirus has diminished, as a result of its elimination in the majority of countries, non-polioviruses are emerging as causative agents of severe central nervous system (CNS) involvement. Outbreaks of enterovirus 71 (EV71)-associated CNS infections have recently been reported in Asia, Australia, and Europe.
Objectives: This is the first study on genotyping of EV71 in children with meningoencephalitis to be carried out in Iran, and it was conducted in order to obtain an improved understanding of the disease burden of this virus, particularly with regard to CNS involvement.
Patients and Methods: Viral RNA was extracted from 170 cerebrospinal fluid samples obtained from children aged under 8 years with a primary diagnosis of aseptic meningitis. Specific EV71 PCR was conducted to identify the genotype of the detected EV71 viruses.
Results: Human enteroviruses (HEVs) were detected in 89 patients (52.3%). EV71 infection was detected in 19 (21.3%) of the 89 EV71-positive patients, and the C genotype was identified in 15 isolates.
Conclusions: The C genotype should be considered as the prevalent EV71 circulating genotype in Iran, particularly in cases of aseptic meningitis.

Keywords: Human Enterovirus 71, Direct Genotyping, Aseptic Meningitis

1. Background

Poliovirus is gradually diminishing in importance, as a result of the elimination of its wild-type in the majority of countries (1-5). Although herpes simplex virus (HSV) types 1 and 2, as well as HSV 6, are well-known in viral meningitis, the emergence of enterovirus 71 (EV71) as a viral cause of neurological diseases, such as encephalitis and hand-foot and mouth disease (HFMD) in Eastern Europe, Malaysia, Taiwan, Japan, Australia, and Singapore, has drawn attention to non-polio enteroviruses (2-15). It is known that EVs are the major cause of aseptic meningitis (13-15), and have been detected at rates of 46.2 and 52.63% in various studies of children with viral meningitis in Iran (15-20). However, there are no available data regarding the role of EV71 in aseptic meningitis, and its circulating genotype(s) in that country.

2. Objectives

We conducted this study to assess the distribution of the different genotype(s) of EV71 infection in aseptic meningitis and encephalitis for the first time in young children in Iran.

3. Patients and Methods

3.1. Patients

Between 2009 and 2011, 170 children aged under 8 years were admitted to the children's hospitals in Tehran, Iran, with a primary diagnosis of viral meningitis, based on clinical symptoms and laboratory results, such as the sudden onset of fever, headache, a stiff neck, and other signs of meningeal inflammation, a normal glucose concentration in cerebrospinal fluid (CSF), a white blood cell count of greater than 20 cells/mL (mostly mild lymphocytic pleocytosis), a negative CSF culture for bacteria (e.g. tuberculosis), and fungi and tumor etiology. None of the children was immunocompromised, and there was no history of HFMD. Children who had received antibiotics prior to CSF examination were excluded. The informed consent form was signed by the children's parents or guardians, and this study was approved by the ethical committee of the Pasteur institute of Iran. CSF samples were collected by the physicians and transferred on ice to the department of virology, Pasteur institute of Iran, and stored at −80°C for further processing.

Viral RNA was extracted using the high pure viral nucleic acid kit (Roche diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. cDNA synthesis and detection of human enteroviruses (HEVs) was performed as previously described (15-18).

3.2. Genotyping of EV71

To avoid any probable contamination, each PCR posi-

tive sample was separately amplified using specific primers targeting the VP1/VP3 junction region (19, 20). Touch down PCR was carried out in a 25 μL reaction mixture consisting of 2.5 μL 10x PCR buffer (Fermentas Thermo Scientific, Lithuania), 0.75 μL $MgCl_2$ 50 mM (Fermentas, Thermo Scientific, Lithuania), 0.5 μL 10 mM dNTPs (Fermentas, Thermo Scientific, Lithuania), 0.5 μL of 0.04 μmol forward primer, 5'-GCA GCC CAA AAG AAC TTC AC-3', 0.5 μL 0.04 μmol of reverse primer, 5'-ATT TCA GCA GCT TGG AGT GC-3', 0.5 μL Taq DNA polymerase 100 U (Fermentas, Thermo Scientific, Lithuania), 1 μL DMSO, and 2 μL of the EV-positive cDNA (18, 19). The amplification reactions were as follows: initial denaturation at 95°C for 5 minutes, followed by 15 cycles comprising denaturation at 95°C for 35 seconds, annealing at 50°C for 16 seconds, and extension at 72°C for 35 seconds, followed by 40 cycles consisting of denaturation at 95°C for 35 seconds, annealing at 48°C for 16 seconds, and extension at 72°C for 35 seconds, and final extension at 72°C for 10 minutes. The PCR product underwent electrophoresis on a 1.5 % agarose gel containing 0.5 μg/mL ethidium bromide (Cina Gen, Iran) using a 50-bp DNA ladder as a molecular marker to detect a product size of 226bp. Diethylpyrocarbonate water and the EV71, which was kindly provided by Dr. Qibin Leng, were used as negative and positive controls, respectively.

The product was purified using a QIAquich gel extraction kit (Qiagen, Inc. GmbH Hilden, Germany) according to the manufacturer's instructions. It was then sent to a dealer company in Iran, which sent it on to a laboratory in South Korea for sequencing in both directions using a BigDye terminator cycle sequencing kit (Perkin Elmer-Applied Biosystems, Inc. CA), and specific primers on an ABI Prism 3730/3100 DNA analyzer (Perkin Elmer-Applied Biosystems, Inc.). The sequence data were analyzed using BioEdit software (version 7.0.5). Phylogenic analysis was conducted using the molecular evolutionary genetics analysis software package, version 5.0, and the neighbor-joining method with 1000 bootstrap repetitions (Figure 1).

The polio virus type 1 was used as an out-group. The tree was elaborated by the neighbor-joining method using molecular evolutionary genetics analysis 4, and the bootstrap (1,000 replicates) supporting each node is indicated. The scale bar corresponds to 0.1 substitutions/site references and GenBank accession numbers for the sequences used in VP1 comparisons were as follows: EV71B4: gi [AF376067.1] (Australia), EV71B3: gi [AF376117.1] (Australia), EV71B2: gi [AF009530.1] (USA), EV71B1: gi [AF135886.1] (USA), EV71A: gi [AB204852.1] (Japan), EV71A: gi [AB204853.1] (Japan), EV71C3: gi [DQ341356.1] (Malaysia), EV71C1: gi [AF376098.1] (Australia), EV71C1: gi [AF376080.1] (Australia), EV71C4: gi [FJ606447.1] (China), EV71C4: gi [FJ606448.1] (China), EV71C4: gi [FJ606450.1] (China), EV71C4: gi [HQ129932.1] (China), EV71C4: gi [AF302996.1] (China), EV71C4: gi [HQ188292.1] (China). The types of EV 71 analyzed in this study are indicated by diamond with their genotypes.

These EV71 sequences have been deposited in the GenBank database under GenBank accession numbers: gi/JX495150/EV71, to gi/JX495164/EV71.

Statistical analysis was carried out using the chi-square test, with statistical package for the social sciences software, version 19.0. $P < 0.05$ was considered as the level of statistical significance.

4. Results

In total, there were 117 males and 53 females. HEVs-specific primers (5'-UTR) allowed the identification of EV infection in 89 (52.3%) of 170 patients with 38.8% being males and 13.5% being females. EV71 infection was detected in 19 (21.3%) of the 89 EV-positive patients, with 15 being males and four being females. HEV and EV71 infection were both primarily identified in children of 0 - 2 years old (38.2% and 13.5% respectively), with no significant difference between the age groups. EV71 infection was detected throughout the entire duration of this study. Although the difference in infection rate was not significant, it was at its highest during fall and at its lowest in spring.

The genotype of 15 specimens was successfully identified as being genotype C (four specimens were inadequate following the first step) (Figure 1).

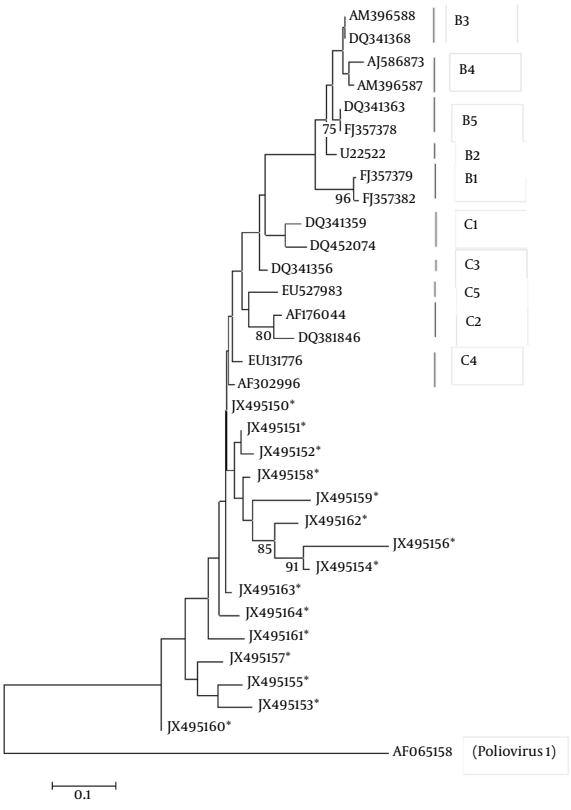

Figure 1. A Phylogenetic Tree Constructed From the VP1 Nucleotide Sequence of the Isolated EV71s From Iran and the Reference EV71 Strains

5. Discussion

Following eradication of the poliovirus, EV71, which frequently causes aseptic meningitis, is considered to be the most dangerous EV (14, 21-24). Features of HFMD are also usually present, but, in a small proportion of affected individuals, neurological complications are the only presentation (23-26). Epidemiological data have shown that males have greater exposure than females to EVs infection (5, 7, 10, 11, 14-25). HEVs-specific primers (5'-UTR) allowed identification of EV in 89 of 170 patients (52.3%), with 38.8% being males and 13.5% being females, and EV71 infection was detected in 19 of the 89 HEV-positive patients (21.3%), with 15 being male and four being female. The prevalence of EV71 infection did not differ significantly between genders (P = 0.591). EV71 infection was primarily identified in children aged under 2 years (13.5%), with no significant difference observed between age groups.

Previous studies have suggested that the far higher population densities in Asia result in a lower average age at infection and more severe symptoms than observed in older European countries, that our results are in agreement with these findings (27-31). Several outbreaks of EV71-related HFMD and CNS involvement have been reported, primarily from Asia (20, 24, 25, 29-33). EV71 infection results in HFMD, with or without neurological manifestations, due to different genotypes of EV71 (20, 24, 28-35). However, further studies are needed to support this hypothesis.

EV71 is classified into three genotypes, A, B, and C, based on molecular analysis using VP1 and/or VP4 gene sequences, with further sub-genotypes, B1-B5 and C1-C5, being present within the B and C genotypes (20, 23, 26, 27). In the present study, the genotype of 15 specimens was successfully identified as being genotype C (four specimens were inadequate following the first step) (Figure 1), and polio virus type 1 was used as an out-group for analysis of the VP1 sequence data. It has recently been proposed that sub-genotype C4 strains represent the new genotype D (26, 27). In addition, a novel sub- genotype, B0, which was circulating in the Netherlands from 1963 to 1967, has been proposed (25).

The VP1 protein contains numerous neutralization sites and corresponds to the virus serotyping; therefore, it was from this that most of the EV71 phylogenies were derived (15-17, 31, 36). It might be possible that two sub-genotypes, B3 and C4, are recombinants of EV-71 and prototype CV-A16/G10 (27, 31, 36, 37). In one study, which considered EV71 isolates from Singapore, Sarawak (Malaysian Borneo), and Perth, Western Australia from 1997 - 2001, it was determined that these recent strains were from genogroups B and C (25, 28, 29, 31-33, 35, 36).

In the present study, only genotype C was identified in 15 specimens, perhaps because of the degradation of the viral genome, due to the presence of RNases in clinical specimens, or a low viral load (15-17). This type has previously been identified in many cases of CNS involvement (31, 32, 38). In 2004 in Austria, EV71 C4 was detected in two patients with severe aseptic meningitis that was not associated with HFMD (14). In contrast, HFMD was associated with an outbreak of the disease in China (14, 18, 20, 27, 31-33). The C1 genotype was also isolated from children with aseptic meningitis without HFMD in Norway (14, 31, 39, 40). According to the phylogenetic tree analysis, these 15 isolates from the C genotype are related more to the C4 sub-genotype than to the other C sub-genotypes. However, further studies to identify the full-length sequence of VP1 and/or VP4 from Iranian isolates and comparison with other isolates are required.

Unfortunately, we could not gain access to patients with HFMD, in order to assess their specimens in the present study, because most of these individuals were out-patients. No data regarding the frequency of EV71 and its circulating genotype(s) are available in Iran; therefore we had nothing with which to compare our findings. In accordance with other studies of EVs in aseptic meningitis in Iran, the present study revealed that, although there were no fatal neurological complications, EV71 infection should be considered as one of the major causes of the disease, especially in very young children in Iran.

Acknowledgments

We wish to thank professor Qibin Leng (key laboratory of molecular virology and immunology, institute Pasteur of Shanghai, Shanghai institutes for biological sciences, Chinese academy of sciences, Shanghai 200031, China) for his material support and for providing us with the positive control of EV71. We also wish to thank Dr. K. Azadmanesh for his general support of this study and the staff of the department of virology, Pasteur institute of Iran.

Footnotes

Authors' Contribution: Study concept and design: Pooneh Rahimi; acquisition of data: Akram Rouhandeh and Amir Sohrabi; analysis and interpretation of data: Pooneh Rahimi, Ehsan Mostafavi, and Golnaz Bahram Ali; drafting of the manuscript: Pooneh Rahimi; critical revision of the manuscript for important intellectual content: Pooneh Rahimi; Administrative, technical, and material support: Pooneh Rahimi; study supervision: Pooneh Rahimi.

Funding/Support: This project was financially supported by the Pasteur institute of Iran under project number 567.

References

1. Schmidt NJ, Lennette EH, Ho HH. An apparently new enterovirus isolated from patients with disease of the central nervous system. *J Infect Dis.* 1974;**129**(3):304–9. [PubMed: 4361245]
2. Azadfar S, Cheraghali F, Moradi A, Javid N, Tabarraei A. Herpes simplex virus meningitis in children in South East of caspian sea, iran. *Jundishapur J Microbiol.* 2014;**7**(1):e8599. doi: 10.5812/jjm.8599. [PubMed: 25147651]
3. Yavarian J, Gavvami N, Mamishi S. Detection of human herpesvirus 6 in cerebrospinal fluid of children with possible encephalitis. *Jundishapur J Microbiol.* 2014;**7**(9):e11821. doi: 10.5812/jjm.11821. [PubMed: 25485059]

4. Deibel R, Gross LL, Collins DN. Isolation of a new enterovirus (38506). *Proc Soc Exp Biol Med.* 1975;**148**(1):203–7. [PubMed: 165523]
5. Wong KT. Enterovirus-associated neurological disease with special reference to enterovirus 71. *Neurol J Southeast Asia.* 2000;**5**:47–9.
6. Wong KT. Emerging and re-emerging epidemic encephalitis: a tale of two viruses. *Neuropathol Appl Neurobiol.* 2000;**26**(4):313–8. [PubMed: 10931364]
7. Wong KT, Lum LC, Lam SK. Enterovirus 71 infection and neurologic complications. *N Engl J Med.* 2000;**342**(5):356–8. doi: 10.1056/NEJM200002033420514. [PubMed: 10660401]
8. Kennett ML, Birch CJ, Lewis FA, Yung AP, Locarnini SA, Gust ID. Enterovirus type 71 infection in Melbourne. *Bull World Health Organ.* 1974;**51**(6):609–15. [PubMed: 4377551]
9. Komatsu H, Shimizu Y, Takeuchi Y, Ishiko H, Takada H. Outbreak of severe neurologic involvement associated with Enterovirus 71 infection. *Pediatr Neurol.* 1999;**20**(1):17–23. [PubMed: 10029254]
10. Bible JM, Iturriza-Gomara M, Megson B, Brown D, Pantelidis P, Earl P, et al. Molecular epidemiology of human enterovirus 71 in the United Kingdom from 1998 to 2006. *J Clin Microbiol.* 2008;**46**(10):3192–200. doi: 10.1128/JCM.00628-08. [PubMed: 18650362]
11. Lum LC, Wong KT, Lam SK, Chua KB, Goh AY, Lim WL, et al. Fatal enterovirus 71 encephalomyelitis. *J Pediatr.* 1998;**133**(6):795–8. [PubMed: 9842048]
12. Chu PY, Lin KH, Hwang KP, Chou LC, Wang CF, Shih SR, et al. Molecular epidemiology of enterovirus 71 in Taiwan. *Arch Virol.* 2001;**146**(3):589–600. [PubMed: 11338392]
13. Dong XN, Ying J, Chen YH. Molecular epidemiology and evolution of worldwide enterovirus 71 strains isolated from 1970 to 2004. *Chinese Sci Bull.* 2007;**52**(11):1484–90. doi: 10.1007/s11434-007-0215-z.
14. Ortner B, Huang CW, Schmid D, Mutz I, Wewalka G, Allerberger F, et al. Epidemiology of enterovirus types causing neurological disease in Austria 1999-2007: detection of clusters of echovirus 30 and enterovirus 71 and analysis of prevalent genotypes. *J Med Virol.* 2009;**81**(2):317–24. doi: 10.1002/jmv.21374. [PubMed: 19107980]
15. 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**(8):2698–704. doi: 10.1128/JCM.00542-06. [PubMed: 16891480]
16. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. *J Virol.* 1999;**73**(3):1941–8. [PubMed: 9971773]
17. Casas I, Palacios GF, Trallero G, Cisterna D, Freire MC, Tenorio A. Molecular characterization of human enteroviruses in clinical samples: comparison between VP2, VP1, and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. *J Med Virol.* 2001;**65**(1):138–48. [PubMed: 11505456]
18. Roohandeh A, Rahimi P, Sohrabi A, Mobasheri M, Azadmanesh K, Shahosseini Z, et al. Frequency of human enterovirus 71 in children under 8 years old with aseptic menengitis in Tehran. *Clin Lab.* 2013;**59**(7-8):915–20. [PubMed: 24133924]
19. Mamishi S, Rahimi P, Sohrabi A, Sabuni F, Edalat R, Mostafavi E, et al. Direct Serotyping of Enteroviruses in Cerebrospinal Fluid of Children With Aseptic Meningitis. *Jundishapur J Microbiol.* 2013;**6**(9):ee7852. doi: 10.5812/jjm.7852.
20. Liu MY, Liu W, Luo J, Liu Y, Zhu Y, Berman H, et al. Characterization of an outbreak of hand, foot, and mouth disease in Nanchang, China in 2010. *PLoS One.* 2011;**6**(9):e25287. doi: 10.1371/journal.pone.0025287. [PubMed: 22216085]
21. Han JF, Cao RY, Tian X, Yu M, Qin ED, Qin CF. Producing infectious enterovirus type 71 in a rapid strategy. *Virol J.* 2010;**7**:116. doi: 10.1186/1743-422X-7-116. [PubMed: 20525351]
22. Huang CC, Liu CC, Chang YC, Chen CY, Wang ST, Yeh TF. Neurologic complications in children with enterovirus 71 infection. *N Engl J Med.* 1999;**341**(13):936–42. doi: 10.1056/NEJM199909233411302. [PubMed: 10498488]
23. McMinn PC. An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiol Rev.* 2002;**26**(1):91–107. [PubMed: 12007645]
24. Hagiwara A, Tagaya I, Yoneyama T. Epidemic of hand, foot and mouth disease associated with enterovirus 71 infection. *Intervirology.* 1978;**9**(1):60–3. [PubMed: 202573]
25. van der Sanden S, Koopmans M, Uslu G, van der Avoort H, Dutch Working Group for Clinical V. Epidemiology of enterovirus 71 in the Netherlands, 1963 to 2008. *J Clin Microbiol.* 2009;**47**(9):2826–33. doi: 10.1128/JCM.00507-09. [PubMed: 19625480]
26. Yip CC, Lau SK, Lo JY, Chan KH, Woo PC, Yuen KY. Genetic characterization of EV71 isolates from 2004 to 2010 reveals predominance and persistent circulation of the newly proposed genotype D and recent emergence of a distinct lineage of subgenotype C2 in Hong Kong. *Virol J.* 2013;**10**:222. doi: 10.1186/1743-422X-10-222. [PubMed: 23822185]
27. Yip CC, Lau SK, Zhou B, Zhang MX, Tsoi HW, Chan KH, et al. Emergence of enterovirus 71 "double-recombinant" strains belonging to a novel genotype D originating from southern China: first evidence for combination of intratypic and intertypic recombination events in EV71. *Arch Virol.* 2010;**155**(9):1413–24. doi: 10.1007/s00705-010-0722-0. [PubMed: 20549263]
28. Deibel R, Flanagan TD. Central nervous system infections. Etiologic and epidemiologic observations in New York State, 1976-1977. *N Y State J Med.* 1979;**79**(5):689–95. [PubMed: 286166]
29. Blomberg J, Lycke E, Ahlfors K, Johnsson T, Wolontis S, von Zeipel G. Letter: New enterovirus type associated with epidemic of aseptic meningitis and-or hand, foot, and mouth disease. *Lancet.* 1974;**2**(7872):112. [PubMed: 4136956]
30. Ishimaru Y, Nakano S, Yamaoka K, Takami S. Outbreaks of hand, foot, and mouth disease by enterovirus 71. High incidence of complication disorders of central nervous system. *Arch Dis Child.* 1980;**55**(8):583–8. [PubMed: 6254449]
31. Cardosa MJ, Perera D, Brown BA, Cheon D, Chan HM, Chan KP, et al. Molecular epidemiology of human enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: comparative analysis of the VP1 and VP4 genes. *Emerg Infect Dis.* 2003;**9**(4):461–8. doi: 10.3201/eid0904.020395. [PubMed: 12702227]
32. Ooi MH, Wong SC, Podin Y, Akin W, del Sel S, Mohan A, et al. Human enterovirus 71 disease in Sarawak, Malaysia: a prospective clinical, virological, and molecular epidemiological study. *Clin Infect Dis.* 2007;**44**(5):646–56. doi: 10.1086/511073. [PubMed: 17278054]
33. Ooi MH, Wong SC, Mohan A, Podin Y, Perera D, Clear D, et al. Identification and validation of clinical predictors for the risk of neurological involvement in children with hand, foot, and mouth disease in Sarawak. *BMC Infect Dis.* 2009;**9**:3. doi: 10.1186/1471-2334-9-3. [PubMed: 19152683]
34. McMinn P, Stratov I, Nagarajan L, Davis S. Neurological manifestations of enterovirus 71 infection in children during an outbreak of hand, foot, and mouth disease in Western Australia. *Clin Infect Dis.* 2001;**32**(2):236–42. doi: 10.1086/318454. [PubMed: 11170913]
35. Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH. Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect Dis.* 2010;**10**(11):778–90. doi: 10.1016/s1473-3099(10)70194-8. [PubMed: 20961813]
36. Perera D, Podin Y, Akin W, Tan CS, Cardosa MJ. Incorrect identification of recent Asian strains of Coxsackievirus A16 as human enterovirus 71: improved primers for the specific detection of human enterovirus 71 by RT PCR. *BMC Infect Dis.* 2004;**4**:11. doi: 10.1186/1471-2334-4-11. [PubMed: 15122971]
37. Chan YF, AbuBakar S. Human enterovirus 71 subgenotype B3 lacks coxsackievirus A16-like neurovirulence in mice infection. *Virol J.* 2005;**2**:74. doi: 10.1186/1743-422x-2-74. [PubMed: 16122396]
38. Tseng FC, Huang HC, Chi CY, Lin TL, Liu CC, Jian JW, et al. Epidemiological survey of enterovirus infections occurring in Taiwan between 2000 and 2005: analysis of sentinel physician surveillance data. *J Med Virol.* 2007;**79**(12):1850–60. doi: 10.1002/jmv.21006. [PubMed: 17935170]
39. Witso E, Palacios G, Ronningen KS, Cinek O, Janowitz D, Rewers M, et al. Asymptomatic circulation of HEV71 in Norway. *Virus Res.* 2007;**123**(1):19–29. doi: 10.1016/j.virusres.2006.07.015. [PubMed: 16965832]
40. Huemer HP, Ortner B, Huang CW, Schmid D, Mutz I, Wewalka G, et al. Isolating Asian enterovirus 71 subgenogroup C4 in two Austrian clinical samples from 2004. *Euro Surveill.* 2008;**13**(28). [PubMed: 18761929]