Original Article

The Association of Non-polio Enteroviruses with Aseptic Meningitis in Children in Iran

***Pooneh Rahimi[1], Hakimeh Mahdian Naser[2], Amir Sohrabi[3]**

[1]Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran;
[2]Department of Microbiology, Science and Research Branch**,** Islamic Azad University, Tehran, Iran;
[3]Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran, Iran.

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Introduction: Viral meningitis is an inflammation of the leptomeninges as a manifestation of central nervous system (CNS) infection, and more than 85% of viral meningitis cases are caused by non-polio enteroviruses. Molecular methods such as polymerase chain reaction (PCR) are increasingly used to detect and type non-polio enteroviruses. Here, we describe the impact of these viruses on aseptic meningitis in young children in Iran. **Methods:** Cerebrospinal fluids were collected from 366 children under 8 years old with primary clinical diagnosis of aseptic meningitis. Real-Time RT-PCR and PCR were performed using specific primers for each human enterovirus (HEV), human parechovirus (HPeV), and enterovirus 71 (EV71). **Results:** Positive results were obtained for HEV, HPeV, and EV71 in 118 (32.2%), 154 (42%), and 94 (25.7%) patients, respectively. HPeV detection rate was higher in both male and female patients with no statistically significant difference. **Conclusion:** Using molecular methods, such as PCR as routine laboratory diagnostic tests for HEVs will result in better understanding of disease burden of these viruses, especially in central nervous system involvements, and will consequently reduce hospitalization and use of antibiotic, which often lead to other complications. *J Med Microbiol Infec Dis, 2014, 2 (2): 56-60.*

Keywords: Non-polio enteroviruses, Human enteroviruses, Parechoviruses, Enterovirus 71, Aseptic meningitis.

INTRODUCTION

Viral aseptic meningitis syndrome is an acute meningeal inflammation with symptoms, such as fever and pleocytosis of the cerebrospinal fluid, and enteroviruses are the main causative agents of this disease [1]. Enteroviral meningitis is commonly considered as a subclinical disease, but its clinical characteristics and cerebrospinal fluid (CSF) findings are similar to those of bacterial or herpetic infections. This similarity results in initiation of treatment before diagnosis and also prolonged hospitalization [1, 2]. However, using molecular methods, such as PCR on CSF could exclude bacterial meningitis [2-4].

Enteroviruses are non-enveloped, positive single-stranded RNA viruses of the Picornaviridae family, which cause a wide spectrum of diseases and clinical presentations including aseptic meningitis, so they should be considered in 85-95% of identified cases [2-4]. Molecular epidemiological data on non-polio enteroviruses in aseptic meningitis need to be improved in our country. During the years 2007-2012 various cross-sectional studies were conducted to provide more epidemiological information on human enteroviruses (HEVs), human parechoviruses (HPeVs), and enterovirus 71 (EV71) and their role in aseptic meningitis in young children [5-7]. This article summarizes the results of those studies to better understand the disease burden of these viruses.

MATERIAL AND METHODS

Patients: CSF samples were collected by physicians (based on statistical sample size calculation that was used to determine the frequency of each virus) from 366 children under 8 years old admitted to children's hospitals in Tehran with primary diagnosis of viral meningitis, which was made based on clinical symptoms and laboratory results, such as neurological symptoms (clinically suspected meningitis, seizures, encephalitis, or paralysis), fever, or hypothermia with signs of circulatory and/or respiratory dysfunction characterized by tachycardia or bradycardia (abnormal heart rate characteristics), vomiting, headache, signs of meningeal inflammation, CSF white blood cell count <10 cells/mm3, CSF protein level <0.35 g/L, glucose level of 2.8-4.4 mmol/L, a negative CSF culture for bacteria (*e.g.*, tuberculosis), and also for tumor etiology. Irritability (as judged by the examining physician), low blood pressure, decreased saturation, and abnormalities were revealed on diagnostic imaging of the brain, as well as symptoms of respiratory infections (rhinorrhea, cough, tachypnea, apnea, wheezing, and/or abnormalities on radiograph of the thorax), and diarrhea and/or vomiting. They also had negative results in blood culture tests for sepsis illness during 2007-2012. Informed consent forms were signed by patients' parent(s) or their official custodian.

***Correspondence:** Pooneh Rahimi
Department of Hepatitis and AIDS, Pasteur Institute of Iran, No. 69, Pasteur Ave., Tehran, Iran, 1316943551.
Email: prahimi@pasteur.ac.ir

Tel/Fax: +98 (21) 66969291

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None of them was immunocompromised. The specimens were transported on ice to the Department of Virology, Pasteur Institute of Iran and stored at -80°C for further processing.

RNA extraction. Viral RNA was extracted using the High Pure Viral Nucleic Acid kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. As a positive control, RNA from oral polio vaccine (OPV) was also extracted at the same time. The extracted RNAs were stored at -80°C.

cDNA synthesis. Reverse transcription was performed in a 20 µL reaction mixture. At first, 8 µL of the extracted RNA from specimens was incubated at 75°C for 5 min. One µL of the extracted RNA from OPV was used as the positive control. Then, the extracted RNAs were chilled on ice for 3 min. The reaction mixture consisted of 4 µl of RevertAid RT buffer (Fermentas Thermo Scientific, Lithuania), 2 µL of 10 mM dNTPs (Fermentas Thermo Scientific, Lithuania), 0.5 µL of random hexamer primer, 0.5 µL of 40 U/µL RNase inhibitor protector (Fermentas Thermo Scientific, Lithuania), 1 µL of 200 U/µL RevertAid RT (Fermentas Thermo Scientific**,** Lithuania), and 4 µL of nuclease-free water. It was incubated at room temperature for 8 min and then at 42°C for 60 min followed by incubation at 75°C for 10 min.

Enteroviruses and Parechoviruses Detection

Real-Time PCR Assay. TaqMan amplification was performed in a 25 µL reaction volume containing 12.5 µL 2x Premix Ex Taq (TaKaRa, Japan), 1 µL of 10 pmol/µL primers and Probes (EV1; 5'-GGCCCTGAATGCGGCT-AAT-3') and EV2; (5'-GGGATTGTCACCATAAGCA-GCC-3'), and 0.5 µL of 10 pmol/µL TaqMan probes **(**FAM 5'-AACCGACTACTTTGGGTGTCCGTGTTTC-3' TAMRA**)**, and 3 µL of the cDNA product. To detect any probable inhibitor and confirm the quality of extraction, 3 µL of an eGFP plasmid was added to each sample during the process of RNA extraction.

The extracted eGFP plasmid was diagnosed using specific primers (eGFP1; 5'-CTGCTGCCCGACAACCA-3', eGFP2; 5'-ACCATGTGATCGCGCTTCTC-3', and probe (HEX 5'-CCAGTCCGCCCTGAGCAAAGA-3' BHQ). The thermocycling profile for real-time PCR was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 61°C for 30 s. Samples were also screened using the HPeV specific primers AN345 and AN344, 0.4 µL AN257 TaqMan probe (Table 1) by real-time PCR as described previously [8, 9].

Detection of EV71. To avoid any probable contamination, each enterovirus PCR-positive sample was amplified separately using specific primers. EV71-VP1 gene was targeted and amplified in a 25 µL reaction mixture consisting of 2.5 µL 10x PCR buffer (Fermentas Thermo Scientific, Lithuania), 0.75 µL of 50 mM $MgCl_2$ (Fermentas Thermo Scientific), 0.5 µL10 mM dNTPs (Fermentas Thermo Scientific, Lithuania), 1.5 µL of 0.04 µmol forward primer, 5'-ATAATAGCAYTRGCGGCAG-CCCA-3', 1.5 µL of 0.04 µmol reverse primer, 5'-AGAGGGAGRTCTACTTCYCC-3', 0.3 µL of 100 U Taq DNA polymerase (Fermentas Thermo Scinetific, Lithuania), and 5 µL of cDNA [7,8].

The amplification reaction was performed under the following conditions: an initial denaturation at 95°C for 5 min, followed by 45 cycles comprising denaturation at 95°C for 35 s, annealing at 55°C for 35 s, and extension at 72°C for 35 s plus a final extension at 72°C for 10 min. Ten µL of amplified products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide, with a 50 bp DNA ladder serving as a molecular marker. The 376 bp product was confirmed in comparison with DEPC water as the negative control and EV71 as the positive control (Figure 1).

Statistical methods. Data were analyzed using SPSS version 19. Prevalence of non-polio enteroviruses infection among age groups, genders, and other variables was compared using χ^2 test, and alpha was set at <0.05.

Table 1. Oligonucleotide sequences of the primers for detecting of human parechoviruses

Primer	Sequenceb	Position
AN345	GTAACASWWGCCTCTGGGSCCAAAAG	421-446c
AN344	GGCCCCWGRTCAGATCCAYAGT	615-594c
AN257a	CCTRYGGGTACCTYCWGGGCATCCTTC	556-582c

a: AN257 is labeled at the 5′end with HEX and at the 3′ end with Black Hole Quencher 1.

b: Ambiguity codes: R, A or G; Y, C or T; W, A or T; and S, C or G

c: Positions are relative to the genome of HPeV1−Harris (GenBank accession number s45208)

RESULTS

A total of 366 patients were studied, in whom 243 (66.4%) were male and 123 (33.6%) were female. Aseptic meningitis was diagnosed more frequently in >1-year old children, and it was statistically significant (p<0.001)

The detection rates for HEV, HPeV, and EV71 have been depicted in Table 2.

The detection rate of HPeV was higher in both male and female patients in comparison with HEV and EV71, but the prevalence of HPeV infection did not differ significantly between two genders (p=0.44).

The total median age for viral infection (HEV, HPeV, and EV71) was 20.12, 26.67, and 21.3 months, respectively. However, no significant difference was found (p=0.15). These non-polio enteroviruses were prevalent in all four major regions of Tehran, with the range of 25-54.3%, and there was no significant relationship between infection with these viruses and region of residency (p=0.85).

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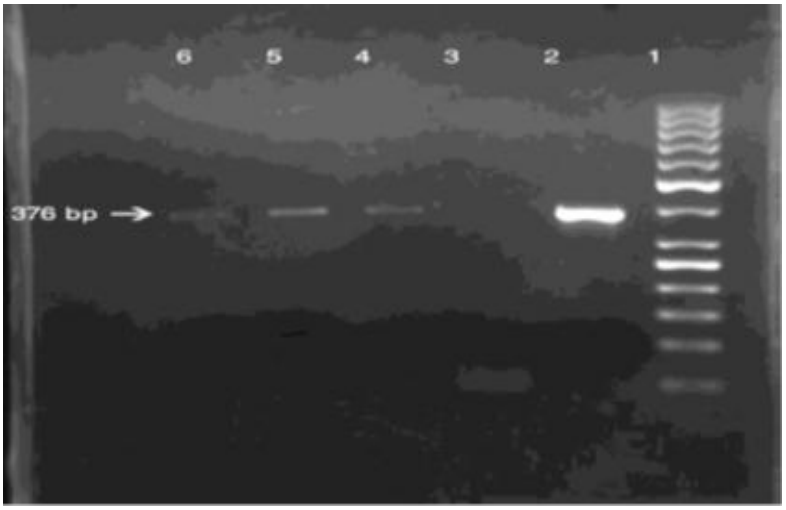

Fig. 1. Amplification of EV71-PV1 gene from clinical samples
1, 50 bp DNA ladder; 2, positive control; 3, negative control; 4-6, clinical samples

Table 2. Comparison between the frequency of non-polio enteroviruses, human parechoviruses, and enterovirus 71 infections with respect to gender

Gender	Positive Test Results			
	HEV	HPeV	EV71	Total
Male	84	103	56	243
Female	34	51	38	123
Total	118	154	94	366

DISCUSSION

Most enteroviral infections are subclinical and self-limited, but neurological illness, such as aseptic meningitis, encephalitis, and other manifestations, including paralysis, Guillain-Barré syndrome, transverse myelitis, cerebellar ataxia, and peripheral neuritis, also occur [11, 12].

Many studies have shown that CSF PCR is more sensitive and rapid for diagnosis of enteroviral meningitis, as compared to virus isolation in cell cultures. Thus, a definite diagnosis of enteroviral meningitis can be established by direct detection of virus in CSF specimens [13, 14].

There are some published data on the frequency of HEVs in aseptic meningitis in our country [5-8, 15, 16]. Enterovirus detection rate was 10.16% in patients less than one year old in 2009-2010 [6]. In a study in the south of Iran (Shiraz city in Fars province), enteroviruses were detected in 46.15% of children with viral meningitis followed by other viruses (46.2%) [6]. In another study from Ahvaz city in Khuzestan province, enteroviruses were detected in 52.63% of samples [16].

HPeVs used to be classified as non-polio enterovirus genus, but recent investigations on molecular properties have led to the distinction of these viruses from other enteroviruses, which resulted in reclassification of them as members of the genus parechovirus in 1999 [17-20].

During this study, a relative high frequency of HPeV was detected in children >1 year old in comparison with HEV and EV71. However, this difference was not significant. It has been shown that, like enterovirus infections, children are infected with HPeVs early in life, mostly with asymptomatic or mild illness [17-20]. In this study, the age distribution of HPeV infection was not consistent with findings of other studies, in which the prevalence of HPeV was more in >1-year-old age group, so HPeV should be considered as an important cause of aseptic meningitis, especially in >1-year-old children [17-19].

According to various studies from all over the world, HEVs are one of the major causes of aseptic meningitis, but it appears that parechoviruses are surpassing them because of the development of molecular detection methods [11, 12, 20]. EV71 with 3 genotypes and 11 subgenotypes is most recently described as a non-polio enterovirus genus [20, 21]. EV71 has been known as a major cause of epidemics of hand, foot and mouth disease (HFMD) associated with severe neurological disorders in young children, especially in southeast Asian countries [7, 18, 21-26].

According to the epidemiological data, males have more exposure to HEVs infection than females [19-23]. In the present study, an obvious increased risk of EV71 infection was also seen in young boys (<2 years old), which is similar to results from other countries [21-24]. These findings are in agreement with recent studies supporting the introduction of routine HPeV and EV71 screening of CSF specimens [1-7, 21-26]. The introduction of such screening would significantly increase better understanding of disease burden of these viruses, especially in aseptic meningitis. Also, it could lead to reduce hospitalization and use of antibiotic to prevent other complications in neonates and young children.

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Improvements in personal hygiene and public sanitation may reduce transmission of non-polio enteroviruses within the community. Also, Use of molecular diagnostic methods for these viruses can prevent antibiotic consumption and emergence of drug-resistant micro-organisms.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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