Parechovirus and enteroviruses among young infants with sepsis in Iran

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ABSTRACT

Background and Objectives: Human parechoviruses (HPeV) and Human enteroviruses (EV) frequently cause a sepsis-like illness in young infants (younger than three months). Therefore, this study was conducted to determine the frequency of HPeV and EV among the young infants with clinical signs and symptoms of sepsis in Ahvaz city, Iran.

Materials and Methods: The blood specimens were collected from 100 (younger than 90 days hospitalized infants) including 54 (56.25%) males and 46 (43.75%) females with clinical signs and symptoms of sepsis-like disease. The RNA was extracted and tested for detection of VP1 region of HPeV and 5 UTR (Untranslated Region) of EV by RT-PCR. The sequences of positive HPeV were further analyzed to determine HPeV genotyping.

Results: 5/100 (5%) of patients including 2/46 (2%) females and 3/54 (3%) males tested positive for HPeV (P=0.85). The analysis of 5 positive VP1 region of HPeV revealed the genotype 1. The analysis of sequencing and phylogenetic tree revealed that the isolated HPeVs were genotype 1. While 38/100 (38%) specimens including 16 (16%) females and 22 (22%) males were tested positive for EV (P=0.68).

Conclusion: The frequency of HPeV genotype 1 was 5% among the young infants with sepsis. While frequency of EV was 38% among the young infants with sepsis. This study showed HPeV genotype 1 and EV are dominant in this region.

Keywords: Sepsis; Parechovirus; Enterovirus; Reverse transcriptase polymerase chain reaction; Genotype

INTRODUCTION

Sepsis describes as a complicated disease with greatly diverse appearance. Annually, over 5 mil-...
may prevent unnecessarily prolonged antibacterial treatment (4). Both parechovirus and enteroviruses are involved in neonates and infant sepsis.

Human parechoviruses (HPeVs) are non-enveloped, single stranded RNA, positive-sense and belong to the family Picornaviridae (5). The HPeV genome is approximately 7350 nucleotides (nt) and encode three structural proteins (VP0, VP3 and VP1) seven non-structural proteins (2A, 2B, 2C, and 3A-3D) and two untranslated regions; 5 UTR and 3 UTR (6).

Based on the sequences of VP1 region and phylogenetic analysis, HPeVs have been classified in the genus Parechovirus, which is divided into four species: Parechovirus A (formerly Human parechovirus; human host) is classified into 19 genotypes, HPeV-1 to -19 (7), Parechovirus B (previously called Ljungan virus; rodent host) comprises 1 to 4 genotypes, parechovirus C (formerly Sebokele virus 1; rodent host) (7), and parechovirus D (ferret parechovirus) (8). The prevalence of HPeVs -A genotypic varies globally. HPeVs -A1 genotype is dominant in Europe and USA followed by PeV-A3 and PeV-A4, while PeV-A2 and PeV A7-A9 genotypes are rarely reported (7). In Asia, similar to Europe and the USA, PeV-A1, A3, and A4 genotypes are the most prevalent and a higher diversity of genotypes has been reported in India and Pakistan (9, 10) while PeV- A4, and A8 were in China (11). In the African continent, PeV-A1, A2, and A3 are the most prevalent but nearly all HPeVs genotypes have been detected, indicating a much wider circulation of genotypes in this continent (12). The prevalence of HPeVs have been reported in young children with aseptic meningitis, sepsis-like illness and gastroenteritis in Iran (13-15).

PeV-A1 has been detected in acute gastroenteritis, upper respiratory tract symptoms, fever, rash, paralysis and encephalitis in children age 6 months to 5 years (16-19).

Enteroviruses (EVs) are single positive RNA and belong to Picornaviridae (20). Based on the sequences of the VP1 region, the EVs have been classified into polioviruses (PV, three serotypes), coxsackie A viruses (CAV, twenty three serotypes), coxsackie B viruses (CBV, six serotypes) and echoviruses (enteric cytopathic human orphan (ECHO) virus) (twenty eight serotypes) (20, 21). The enteroviruses were grouped into four species (EV-A to D) among them six different species A (CAV2,CAV4, CAV6, CAV10, CAV16 and EV71) and four serotypes species B (E6, E9, E30 and CAV9) have been diagnosed in young children with sepsis- like illness (22-24).

Within EV species B (the echoviruses, CBV1-6 and CAV9) have been detected in neonatal with sepsis-like illness (5-8). Sepsis symptoms are non-specific with abdominal distention, apnea, fever, irritability and fatigue, seizures, rash, aseptic meningitis, paralysis, myocarditis and feeding problems (25). Both EVs and HPeVs can be transmitted via fecal-oral and respiratory routes (25-27).

The sequencing of VP1 region generally are used for determination of the EV and HPeV genotyping by Reverse Transcription Polymerase Chain Reaction PCR (RT-PCR), which are faster and more sensitive than viral cell culture (28, 29).

Therefore, this study was carried out to determine the frequency of parechoviruses and EVs in the hospitalized young neonates with sepsis in Ahvaz city, Iran.

**MATERIALS AND METHODS**

The young infants with clinical signs and symptoms of sepsis including rapid respiration, confusion, hyperthermia (≥38°C), hypothermia, chills, poor feeding, tachycardia, hypotension, abdominal distension, nausea, vomiting, and diarrhea who were admitted to the intensive care unit, Aboozar Children Medical Center, Ahvaz city, Iran, during September 2015- September 2016. Patients with apparent malformations, Apgar score less than seven, prematurity, on antibiotics treatment were excluded from this study.

The blood culture was tested for bacteria, 1 mL of blood of the each specimen placed in 2 culture bottles containing broth to grow aerobic and anaerobic microorganisms. All assays were carried out accordance with the manufacturers’ instructions. Finally, 100 (younger than 90 days) specimens negative for blood culture including 54 (56.25%) males and 46 (43.75%) females were proceeded for molecular diagnosis of enteroviruses and parechoviruses using RT-PCR.

**RNA extraction.** RNA was extracted from sera samples using High Pure RNA Isolation kit (Roche/ Germany) according to manufacturer’s protocol. The cDNA was prepared (Thermo scientific/ USA) based on the supplier’s instruction. The nested PCR and semi-nested PCR were carried out for detection of HPeV and enterovirus genomes respectively.
Nested-PCR for parechovirus detection. The positive parechovirus and double-distilled, filtered water were used as a positive and negative control respectively. For the first round PCR the outer primers, forward: AN353 (2126–2154) 5’- GAAATAGTTTGGAAATNACNATHCCNTA- 3’ and reverse: AN355 (3119–3086) 5’- AACTATAATGCCATARTGYTT- TARAANC- 3’ were used to amplify VP3/2A regions of HPeV genome (30). First round PCR was performed with 25 µl volume, containing 5 ng of cDNA as template, 2.5 µl 10× PCR buffer, 0.75 µl MgCl₂ (50 mM), 0.5 µl (10 µM) forward/reverse primer, 1 µl dNTP (10 mM),0.2 Taq DNA Polymerase (Cinnagen Inc, Iran) (5 u/µl), D/W up to 25 µl. The reaction mixture was subjected to thermal cycler (TC-512, Techne, Staffordshire, UK) with the following program: 1 cycle with initial denaturation at 95°C for 5 minutes, followed by 35 cycles, 94°C for 30 s, 42°C for 40 s, 72°C for 60 s, and final extension at 72°C for 3 minutes. For the second round PCR, the inner primers, forward: AN369 (2531–2559) 5’ - ACCAAGGTGTA- CAACATTTTYGGNGNCG -3’ and inner primers, reverse AN367 (2829–2798), 5’ GAATAAAATGG- TACTGANARG TCATYTGYTC -3’ were used to amplify the VP1 region of HPeV genome. 1 µl of PCR product of the first round of amplification was used as the template for the second round. The amount of PCR components was the same as described for the first round. The thermal cycler was programmed as follows: initial denaturation at 95°C for 5 minutes, 35 cycles consisting of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and final extension at 72°C for 3 minutes. All reactions were performed in the presence of negative and positive controls. The PCR product of 293 bp showed the positive reaction (30).

Semi-nested PCR for EV detection. The Sabin vaccine was used as positive control. The PCR reaction mixture comprising 2.5 µl PCR buffer, 0.5 µl dNTP, 0.75 µl of EV-F1 primer 5’-CAAGCACCCTTTGTTCCCCCGG and EV-R ATGTCCACCAAGAGCAGCCA primer were used to amplify the 5’ UTR region of EV genome (31), 0.75 µl MgCl₂, 0.12 µl Taq, 4 ng template and D/W up to 25 µl. The reaction mixture was subjected to thermocycler (TC-512, Techne, UK) for 35 cycles of amplification (94°C for 45 s, 54°C for 45 s, 72°C for 45 s and 72°C for 10 min). Then 1 µl the product was used as the template for the second round which consisting 2.5 µl 10× PCR buffer, 0.5 µl dNTP, 0.75 µl of each EV-F2: TCCCTCCCG- CCCCTGAATGCG and EV-R: ATGTCCACCAAGCAGCCA primers, 0.75 µl MgCl₂, 1 µl template, 0.12 Taq and D/W up to 25 µl was subjected for 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and 72°C for 5 min to amplify the 5’ UTR region of EV genome. The final product of 155 bp on the 2% gel electrophoresed indicated the positive test (31).

Sequencing. Two amplicons of VPI regions of HPeVs were selected and were sequenced (Bioneer company, South Korea). The sequences were blasted using available databases https://blast.ncbi.nlm.nih.gov/Blast.cgi. The positive samples were sent to Gen- eBank to obtain accession number.

Phylogenetic tree. A phylogenetic tree was constructed by Neighbor joining method using the partial nucleotide sequences of VPI region of HPeVs isolated. Neighbor joining method with MEGA software version 6 was performed under Kimura two-parameter model, with Bootstrap 1000 replication (32).

Ethic status. This project was approved by the ethic committee of Ahvaz Jundishapur University of Medical Sciences Ahvaz, Iran. The consent was obtained from each young neonate parent.

Statistical analysis. Statistical analysis was carried out for mean and standard division of for neutrophils, lymphocytes platelet counts and level of C-reactive protein. The frequency of parechovirus and enterovirus among the gender, age groups, and season were analyzed by the chi-square. Data were analyzed by SPSS Statistics ver. 22 P values of <0.05 was considered to be statistically significant.

RESULTS

The patient ages were between 20-90 days with mean age of 54 ± 22 with clinical criteria; fever > 38°C, poor feeding (76%), irritability (97%), tachypnea (78%), tachycardia (91%), rash (67%), vomiting (27%), lethargy (47%), diarrhea (41%), poor perfusion (54%) and apnea (12%). The mean WBC (mm³) (9990-22000), lymphocytes count (mm³) 11550 ± 1200, neutrophil count (mm³) (4766-5233), Platelet (x10³/mm³) (315-419) and the mean level of ± SD CRP C-reactive protein level was 10.2 ± 1.8 (mg/L). Out of 100 samples, five cases including 2/46 (2%)
females and 3/54 (3%) males tested positive for HPeV (P=0.85). The analysis of 2 positive VP1 region of HPeV revealed the genotype 1 and were recorded in the GenBank.

**Sequencing.** The sequences of two isolated HPeVs were deposited in GenBank under accession numbers, MN845069 and MN845070. Both isolated HPeVs showed 97% nucleotide identity with HPeV genotype 1 (MK792785.1) isolated from Tehran, Iran.

**Analysis of phylogenetic tree.** The phylogenetic tree was constructed by Neighbor joining method for partial VP1 region of two isolated HPeV and compared with different HPeV genotypes retrieved from GeneBanks. The Neighbor joining was built under Kimura two-parameter model, with reproducibility of 1,000 of bootstrap and scale bar = 0.05 (Fig. 1).

While 38/100 (38%) specimens including 16 (16%) females and 22 (22%) males tested positive for EV (P=0.68), 47 (47%) of cases were negative for parechovirus and enterovirus. Table 1 shows the distribution of parechovirus and enterovirus among the gender, age groups and different seasons.

**DISCUSSION**

Both Parechovirus and Enteroviruses are the most agents which cause high morbidity and mortality in neonates and young children worldwide. In the present study 5% of cases including 2/46 (2%) females and 3/54 (3%) males tested positive for HPeV (P=0.85). The results of sequencing of VP1 region of the two isolated HPEV1 (MN845069 and MN845070) showed 97% nucleotide identity with HPeV -1 isolated from Tehran, Iran (MK792785.1) and were cluster with HPEV1 isolated from MK792785.1 (Tehran, Iran) and Belarus (MK167999.1, MK168003.1, MK167997.1, MK167996.1, MK167994.1, MG570229.1). Rahimi et al. (2013) have detected HPeV 1 infections in 64/148 (43.24 %) of young children with aseptic meningitis in Tehran (14). There are several reports indicated that the prevalence of HPeV3 is much more associated with sepsis disease than HPeV1 (33, 34). In our survey the frequency of HPeV 1 was 3% in winter and 2% in the spring. While Rahimi et al. have detected HPeV1 throughout the seasons in Tehran, Iran (14). The frequency of HPeV1 in Tehran (43.24%) was higher compared to our study (5%) (P<0.00001).

In the present study 38/100 (38%) cases including 16 (16%) females and 22 (22%) males tested positive for EV (P=0.68). Rahimi et al. have detected Human enterovirus (HEV) in 31/148 (20.94%) of young children with aseptic meningitis in Tehran (13). The frequency of HEV in Tehran (20.94%) was lower compared to our study (38%) (P=0.005). The frequency

**Fig. 1.** A phylogenetic tree was constructed with Neighbor joining method for partial VP1 region of two isolated HPeV from two patients with sepsis in Ahvaz city. The two isolated HPeV-1 with accession numbers (MN845069, MN845070) were compared with the different HPeVs genotypes retrieved from GenBank. The results of phylogenetic revealed that the Iranian isolates with black circle color are cluster with HPeV genotype 1 isolated from MK792785.1 (Tehran, Iran), and Belarus (MK167999.1, MK168003.1, MK167997.1, MK167996.1, MK167994.1, MG570229.1). The accuracy was assessed by 1000 bootstrap replicates. Scale bar=0.05
Table 1. Shows the profile of patients with positive EV and HPeV

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>EV positive</th>
<th>P value</th>
<th>HPeV positive</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>38 (38%)</td>
<td>5 (5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N=100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>16 (16%)</td>
<td>0.43</td>
<td>2 (2%)</td>
<td>0.85</td>
</tr>
<tr>
<td>Male</td>
<td>22 (22%)</td>
<td></td>
<td>3 (3%)</td>
<td></td>
</tr>
<tr>
<td>Age (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-7</td>
<td>12/24 (12%)</td>
<td>1/24 (1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-30</td>
<td>14/47 (14%)</td>
<td>0.48</td>
<td>2/47 (2%)</td>
<td>0.54</td>
</tr>
<tr>
<td>31-90</td>
<td>12/29 (12%)</td>
<td></td>
<td>2/29 (2%)</td>
<td></td>
</tr>
<tr>
<td>Season</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>11/25 (44%)</td>
<td>3/25 (3%)</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>12/30 (40%)</td>
<td></td>
<td>0.5</td>
<td>2/30 (2%)</td>
</tr>
<tr>
<td>Summer</td>
<td>7/23 (30.43%)</td>
<td></td>
<td>0.23 (0%)</td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>8/22 (36.63%)</td>
<td></td>
<td>0.22 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 shows the frequency of EVs and HPeV among the males and females were not significant (p=0.43) and (p=0.85) respectively. The distribution of EVs and HPeV among the different age groups was not significant (p=0.48) and (p=0.54) respectively. The frequency of EVs and HPeV in different season were not significant (p=0.5) and (p=0.26) respectively.

of enterovirus have been reported in neonates with aseptic meningitis in Mashhad city 37% (35) and Tehran 32% (36) and 55.8% in Ahvaz city (37).

Harvala H et al. (2011) have detected enterovirus in 46% young infants (<3 months) during 5-year period in Edinburgh (22). Maguire et al. detected enteroviruses in the 29% of young neonates with sepsis in England and Wales (23). Khetsuriani et al. have detected EV in 44% of young neonates with sepsis in the USA (24).

Verboom-Macielek et al. have reported EVs in 57% of neonates with sepsis syndrome in the Netherlands (19). Jordan, et al. from Spain has reported 10/72 (13.88%) of neonates with febrile showed positive for EVs (38). Ji-Hyun et al. (2015), have described the detection of enterovirus among the young children with a sepsis-like illness was 7.5% in Jinju, South Korea (39).

In our study the detection of EVs have been observed among the young neonates with sepsis in winter (44%), spring (40%) summer (30.43%) and autumn (36.63%).

The peak EVs detection was observed in the winter (44%), while the lowest detection was in summer (30.43%) (p=0.5). Ji-Hyun et al. (2015) have described the peak EVs detections was observed in spring season in Jinju, South Korea (39).

At present, there is no specific treatment for enterovirus. To date, an effective specific antiviral drug to preclude HPeV is not available. The intravenous immunoglobulins (IVIGs) is used to treat dilated cardiomyopathy caused by HPeV-1 (8).

In the present survey, low detection of 5% HPeV were found among young infant with sepsis. The remaining 57/100 (57%) of the patients were tested negative for HPeV 1 and EVs. The role of other viruses such as human respiratory syncytial virus RSV, human Enterovirus EV, human adenovirus (HAdV), human cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), varicella zoster virus (VZV), and influenza have not been investigated in patients with sepsis, but it requires comprehensive investigation (40). About 17% of sepsis can be caused by Candida species, with 2% to 3% caused by Aspergillus and others (41). Fungal sepsis can kill at a rate of 40% to 60% (42). The role of fungal sepsis was not evaluated in this study, although needs further investigation.

We could not follow the time course of EV and HPeV 1 detection in patients with sepsis. That was the limitation of this study and requires further investigation.

In conclusion, The results of this survey indicate that the frequency of HPeV1 and EV was 5% and 38% among the patients with sepsis was respectively. HPeV1 and EV are dominant in this region. The screening of HPeV RNA and EV should be implemented in young infants to reduce the use of antibiotics and shorten the duration of hospitalization.

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