FREQUENCY OF ENTEROVIRUS IN PATIENTS WITH SUSPECTED BACTERIAL MENINGITIS IN TURKEY

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ABSTRACT

Objective: *Enteroviruses are responsible for the majority of cases of aseptic meningitis. Among these, the human parechoviruses (HPeV) have recently been recognised as important contributors. Discrimination between bacterial and viral meningitis is important for proper treatment and prognosis; however, this distinction is not always possible based on clinical presentation because viral meningitis can mimic bacterial meningitis. The aim of this study was to investigate the frequency of enterovirus and HPeV infection in cerebrospinal fluid (CSF) samples collected from children diagnosed with bacterial meningitis but negative for bacteria in Turkey between 2006 and 2009.*

Material and Method: *CSF samples were collected from children with suspected bacterial meningitis from 37 clinical centres in Turkey. Among 1,460 CSF samples available, 1,184 were negative for bacteria and were included in the study. Enteroviral and HPeV RNA were detected in CSF samples by rRT-PCR, and specific genotypes were identified by direct sequencing of the VP1 region.*

Results: *Enteroviruses were detected in 13 (1%) of the 1,184 CSF specimens analysed and included echovirus 14 (n=1), echovirus 9 (n=1), coxsackievirus B4 (n=1), and unknown serotype (n=10). No HPeVs were detected.*

Conclusion: *Neither clinical nor CSF laboratory criteria routinely used to diagnose bacterial meningitis can definitively rule out viral aetiology, so viral infections should be considered during meningitis surveillance and in patient care.*

Keywords: *Enteroviral meningitis, bacterial meningitis, enterovirus, human parechovirus, Turkey.* ***Nobel Med 2016; 12(1): 49-54***

TÜRKİYE'DE BAKTERİYEL MENENJİTTEN ŞÜPHELENİLEN HASTALARDA ENTEROVİRÜS SIKLIĞI

ÖZET

Amaç: Enterovirüsler aseptik menenjit olgularının çoğundan sorumludur. İnsan parechovirusleri (HPeV) son zamanlarda çocuklarda menenjite neden olan önemli viral patojenler olarak tanımlanmaktadır. Bakteriyel ve viral menenjit ayırımı tedavi ve prognoz açısından önemlidir; ancak sadece klinik tabloya dayanarak bu ayırımı yapmak her zaman mümkün olmayabilir ve viral menenjit bakteriyel menenjiti taklit edebilir. Bu çalışmanın amacı, Türkiye'de 2006-2009 yılları arasındaki ulusal bakteriyel menenjit sürveyansı sırasında bakteriyel menenjit tanısıyla toplanan ancak bakteri açısından negatif beyin omurilik sıvısı (BOS) örneklerinde gerçek zamanlı, ters transkripsiyon-polimeraz zincir reaksiyonu (rRT-PCR) ile enterovirüs ve HPeV enfeksiyon sıklığını araştırmaktı.

Materyal ve Metot: BOS örnekleri Türkiye'deki 37 klinik merkezden bakteriyel menenjitten şüphelenilen çocuklardan toplandı. Mevcut 1.460 BOS örneği arasında 1.184'ü bakteri açısından negatifti ve çalışmaya alındı. Enteroviral ve HPeV RNA BOS örneklerinde rRT-PCR ile araştırıldı ve spesifik genotipler VP1 bölgesinin direkt sekanslanması ile tanındı.

Bulgular: Enterovirüs analiz edilen 1.184 BOS örneğinin 13 (%1)'ünde saptandı ve ekovirüs 14 (n=1), ekovirüs 9 (n=1), koksakivirüs B4 (n=1), ve tanımlanmayan serotipleri (n=10) içerdi. HPeV saptanmadı.

Sonuç: Bakteriyel menenjit tanısı için rutin olarak kullanılan klinik ve BOS laboratuar kriterleri kesin olarak viral etyolojiyi dışlayamaz, bu yüzden menenjit sürveyansı sırasında hastaların tanısı ve tedavisinde viral enfeksiyonlar dikkate alınmalıdır.

Anahtar kelimeler: Enteroviral menenjit, bakteriyel menenjit, enterovirüs, human parechovirus, Türkiye.
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INTRODUCTION

Acute viral infections of the central nervous system are prevalent worldwide and can occur in a sporadic, endemic, or epidemic manner. These infections, usually consisting of meningitis, encephalitis, acute flaccid paralysis (such as poliomyelitis), mononeuritis, polyneuritis, and Reye's syndrome, cause high morbidity rates and serious sequelae, especially in children.[1] Non-polio enteroviruses are responsible for 85% of cases of viral meningitis. Enteroviruses (EVs) are members of the Picornaviridae family and have positive uni-helixed RNA genomes and include the human parechoviruses (HPeVs), previously known as echovirus 22 and 23.[2,3] The clinical disease characteristics of parechovirus infection are similar to those caused by echoviruses, including fever, respiratory tract infections, exanthems, viral meningitis, encephalitis, myocarditis, and serious neonatal infections.[4] Human enteroviruses and parechoviruses are omnipresent and transmitted from person to person via the faecal-oral route. Enterovirus and parechovirus infection occur throughout the year in temperate climates, especially in summer and fall.[2] Enteroviruses infect all age groups, but are several-fold more virulent in new-borns and infants; similarly, HPeVs cause infection mainly in young children.[3,5]

Infection with EVs and HPeVs has been reported worldwide. While the causative agents of aseptic meningitis have been investigated in small-scale or single-centre regional serological studies in Turkey, the epidemiology of aseptic meningitis due to EVs and HPeVs has not been investigated countrywide.[6-10]

Distinguishing between bacterial and viral meningitis is important for proper treatment and prognosis. Though the World Health Organization (WHO) and others have defined standards for diagnosing the two causes of infection based on multiple large-scale studies, this distinction remains difficult because viral meningitis can clinically mimic bacterial meningitis.[2,11-15]

Rapid and specific detection of EVs and HPeVs would help differentiate viral from bacterial meningitis. The aim of this study was to investigate the frequency of EV and HPeV in patients suspected of bacterial meningitis, whose cerebrospinal fluid (CSF) was negative for bacteria during a national bacterial meningitis survey between 2006 and 2009.

MATERIAL AND METHOD

This study was carried out using CSF samples collected for the polymerase chain reaction (PCR)-based national survey of bacterial meningitis funded by the Turkish Scientific and Technical and Research Institute (TUBITAK) in collaboration with the Turkish Ministry of Health and the U.S. Centers for Disease Control and Prevention (CDC) between May 2006 and January 2009. A total of 37 hospitals located in 23 cities in 7 geographic regions of the country participated. These centers were chosen because they were reference centers for admission

of children diagnosed with meningitis in their city or region. Ethical approval for the study was obtained from the Ethics Committee of Marmara University Faculty of Medicine, study number MAR-YÇ-2009-0171. Written informed consent was obtained from the parents of each enrolled child.

Case Identification

Children under 17 years of age (excluding new-born infants) who were admitted to emergency departments and underwent lumbar puncture with signs and symptoms of meningitis, including fever, vomiting, headache, seizure, meningeal irritation, impaired consciousness, were eligible for enrolment in the study. CSF samples for PCR studies were stored at -20^{0}C until transport to Marmara University Hospital Pediatric Infectious Diseases Research laboratory under cold-chain conditions. CSF samples negative for bacteria, either by culture or by PCR, were included in the study. CSF samples were stored at -80^{0}C until being sent to the CDC.

Epidemiological Data

Age, sex, vaccination status, fever, nuchal rigidity, level of consciousness, meningeal symptoms (fontanelle bulge, Kernig and Brudzinski signs), focal neurologic signs, cranial nerve injury, cyanosis or skin eruption, CSF findings (cytology, protein, glucose, Gram staining, latex agglutination), and results of CSF culture were recorded. Patients with chronic illnesses, ventriculoperitoneal shunt, or immune deficiency were excluded.

Molecular Identification

For EV and HPeV testing, 200 µL of each CSF sample were sent to the CDC under cold-chain conditions.

RNA Extraction

RNA was extracted from each specimen using the QIAamp viral RNA minikit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted in 50-µL nuclease-free water.

Real-Time RT-PCR (rRT-PCR)

To ensure comparability, the same RNA preparation was used for both PCR tests and the two assays were performed simultaneously. rRT-PCR assays for EV and HPeV were performed as described previously, using primers and Taqman® probes targeting sites in the 5'-non-translated region that are fully conserved among all enteroviruses; all serotypes can be amplified and detected.[16-18] rRT-PCR assays were performed using 5-µL RNA and the SuperScript III® Platinum® One-Step qRT-PCR System (Invitrogen, Carlsbad, CA) in a Stratagene MX3000P (Agilent Technologies, La Jolla, CA), using a CT value of 45 as the cut-off for positivity.

Tablo 1: Clinical findings according to enterovirus results

Clinical symptom	EV-negative (%)	EV-positive (%)	*p*
Fever	76.8	69.2	0.514
Nuchal rigidity	47.1	38.5	0.370
Altered consciousness	44.9	42.9	0.612
Bulging fontanelle	23.4	25	0.987
Kernig sign	22.9	23.1	0.601
Brudzinski sign	18.1	23.1	0.431
Cranial nerve injury	15	0	0.783
Focal sign	9.7	7.7	0.635
Skin eruption	6.5	15.4	0.210
Cyanosis	1.5	0	0.265

EV: Enterovirus

VP1 RT-Semi-Nested PCR (RT-snPCR) and Sequencing

The RT-snPCR typing assays for EV and HPeV were performed as described previously using 5 L of RNA.[19,20] The reaction products were separated and visualised on 1.2% agarose gels containing 0.5 g/ mL ethidium bromide and were purified from the gel using a QIAquick gel extraction kit (QIAGEN). Slight variations in the sizes of the PCR products (350 to 400 bp) were observed due to VP1 gene length differences among serotypes, as described previously.[19,20] The resulting DNA templates were sequenced using a BigDye Terminator v1.1 ready reaction cycle sequencing kit on an ABI Prism 3100 automated sequencer (both from Applied Biosystems, Foster City, CA).

Sequence Analysis

Amplicon sequences were compared to the VP1 sequences of EV reference strains, including at least one representative of each recognised serotype, by script-driven sequential pairwise comparison using the Gap program (Wisconsin Sequence Analysis Package, version 10.2; Accelrys, Inc., San Diego, CA).[20] In cases with indeterminate results (highest score less than 75% or second-highest score greater than 70%), the deduced amino acid sequences were compared by a similar method.[17,20]

Statistical Analysis

The SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to assess all demographic and

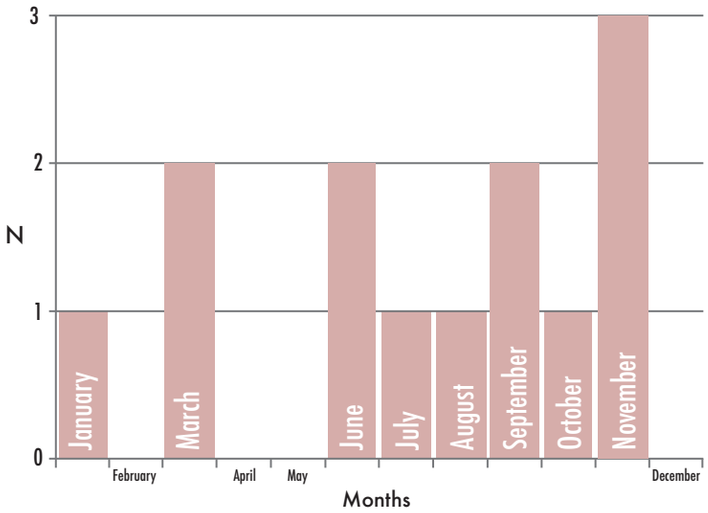

Figure: Monthly distribution of cases of enteroviral meningitis.

Table 2: CSF findings according to enterovirus infection

CSF Findings	EV-negative	EV-positive	*p*
Leukocyte number (/mm^3)	30 (0-8318)	35 (0-730)	0.489
Lymphocyte number (/mm^3)	0 (0-500)	35 (0-730)	<0.001
PNL number (/mm^3)	30 (0-8318)	0	<0.001
CSF protein (mg/dL)	28 (0-253)	63 (23-141)	0.421
CSF/blood glucose	0.63 (0.06-1.03)	0.64 (0.43-0.72)	0.54

CSF: Cerebrospinal fluid, **EV**: enterovirus, **PNL:** polymorphonuclear leukocytes. Values are medians and ranges.

laboratory data. The Mann-Whitney U test was used to compare CSF findings. Clinical data were compared using chi-squared and Fisher's exact chi-squared test.

RESULTS

Between May 2006 and January 2009, 1,460 CSF specimens were collected from children with suspected meningitis. Among these, bacterial DNA was detected in 246(17%) and bacteria were cultured from 29(2%) samples. Of the bacteria-negative samples, 1,184 were included in the present study. Of these, 754(63%) came from male patients, and 430(37%) from females. Patient ages ranged from 1 to 201 months (median, 31 months).

Enteroviruses were detected in 13(1%) of the 1,184 CSF specimens analysed. One of these was echovirus 14, one was echovirus 9, one was coxsackievirus B4, and the remaining 10 were of unknown serotype. EV-positive patients' ages ranged from 1 to 126 months (median, 52 months). While the clinical symptoms of EV-positive patients did not differ from those negative for the virus, some CSF findings did (Table 1,2). CSF lymphocyte count in EV-positive children was significantly higher than in EV-negative patients, and polymorphonuclear leukocytes (PML) were not detected in the CSF of EV-positive patients (p<0.001). The monthly incidence of enteroviral meningitis is shown in Figure. All CSF samples were negative for HPeVs.

DISCUSSION

Eighty-three percent of CSF samples included in the national bacterial meningitis survey were negative for bacteria by both culture and PCR methods. This high rate likely results from widespread use of antibiotics before diagnosis (40% in our study) and loss of sample quality during storage and transportation. In addition, the WHO criteria for the diagnosis of bacterial meningitis are not specific enough to rule out viral meningitis.[11] Therefore, we hypothesised that viral meningitis may contribute to the low rate of detection of bacteria and bacterial DNA in CSF samples. Enteroviral meningitis mimicking bacterial meningitis has previously been reported by others.[12-15] Since EVs are reported to be responsible for 85% of viral meningitis, we investigated only EVs and HPeVs.[2] We detected enteroviruses in only 1% of the bacteria-free samples. This low rate may result from our inclusion of all patients with suspected bacterial meningitis rather than only those whose symptoms were consistent with viral meningitis.

Normal CSF leukocyte counts are often seen in cases with enteroviral meningitis.[21] A predominance of mononuclear cells is usually expected in cases with viral meningitis, although a polymorphonuclear leukocyte predominance may represent the early stage of enteroviral meningitis.[22] Furthermore, we did not observe polymorphonuclear leukocytes, with predominance of lymphocytes in the CSF of enterovirus-positive patients.

Most surveys of enteroviral infections have been conducted in developed countries.[5] In the USA from 1970 to 2005, the most common serotypes were coxsackieviruses A9, B5, and B1, and echoviruses 6, 9, 13, 18, and 30.[23]

Single-centre regional studies on the causative agents of aseptic meningitis in Turkey have reported that the most common enteroviruses are coxsackievirus B, echoviruses 4, 5, 6, 9, 11, 13, 14, 18, 25, and 30, and coxsackievirus A9.6-10 Most (n=10) of the 13 enteroviruses in our study could not be typed, while echovirus 14, echovirus 9, and coxsackievirus B4 were identified in three samples. Enteroviral aseptic meningitis surveys in Iran and Greece found that the most common serotypes in these neighbouring countries were echoviruses 4, 6, 9, 14, and 25, and coxsackieviruses A6, A15, A24 and B1, similar to our results.[24-30]

Enteroviral infections are more common in summer and autumn, similar to our findings, where 77% of cases (10/13) occurred in these seasons.[2]

EV infections affect all age groups, although the rate of infection in infants under 1-year-old is often higher than that in older children and adults.[2,5] In this study, however, 77% of enteroviral aseptic meningitis cases were in patients over that age (median age, 52 months), which may result from our focus on cases suspected of bacterial meningitis.

The methods of detecting EVs used in our study, real-time RT-PCR, VP1 RT-snPCR, and molecular typing based on sequencing, allow rapid EV molecular phylogenetic analysis. VP1 RT-snPCR is 100-fold more sensitive than cell culture methods.[20] Rapid detection and recognition of EVs are important in controlling the early stages of an epidemic.[31]

HPeV is an important causal agent of central nervous system infections, sepsis-like illness, fever, and viremia in young children in Canada, the United Kingdom, the Netherlands, the USA, and Italy. The age distribution of infected patients and types of HPeV may vary geographically.[3,32-37] We did not identify HPeVs in any of the 1,184 CSF samples examined here, possibly due to selection of our sample based on bacterial meningitis symptoms or to geographic differences.

CONCLUSION

In conclusion, this study demonstrates that enterovirus infection can mimic bacterial meningitis and should be considered in patients whose CSF is negative for bacteria.

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* The authors declare that there are no conflicts of interest.

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