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Enterovirus Detection in Patients Suspected Aseptic Meningitis by RT-PCR in Kermanshah, Iran

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Abstract

Background: Aseptic meningitis is frequently caused by viral agents, particularly human enterovirus. Several methods of Reverse Transcription PCR (RT-PCR) have recently been introduced and modified for better diagnosis of enteroviral infection in meningitis. This study aimed to determine enteroviruses in patients with suspicion of aseptic meningitis using RT-PCR in the West of Iran.

Methods: In this study, 120 CSF samples were collected from patients hospitalized with the suspicion of aseptic meningitis in Imam Reza Hospital of Kermanshah, Iran. RT-PCR was used to diagnose enteroviruses. The cDNA recovered from RT-PCR was purified using a DNA purification kit and sequenced to confirm viral genome. Sequence data were analyzed for homology using the Gen Bank database.

Results: The samples were collected from 63 (52.5%) men and 57 (47.5%) women with an average age of 31.5 ± 29.4 years. Of the samples tested, 4 cases (3.33%) yielded positive results for enterovirus. The results of sequence data analysis confirmed all positive cases as Enterovirus type 71. The biochemical (protein and glucose) and cytological analyses of positive CSF samples showed no significant changes.

Conclusion: According to the results, Enterovirus type 71is one of the common causes of enteroviral meningitis in patients in Kermanshah. The RT-PCR technique can rapidly diagnose Enterovirus type 71 and help better treatment of the patients and prevent the unnecessary use of antibiotics. So, molecular methods can reduce the cost of patients' treatments and prevent drug resistance among bacteria. It can also provide a better picture of enteroviral infection in our region.

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Introduction

Aseptic meningitis (AM) is a severe, potentially fatal infection of the central nervous system (CNS) which is characterized by meningeal inflammation, but it is not associated with identifiable bacterial pathogens in the cerebrospinal fluid (CSF) (1). Most patients show some symptoms and signs such as fever, headache, stiff neck, lethargy, anorexia with or without vomiting, diarrhea, sore throat and rash (2). AM is frequently caused by viral agents particularly human enterovirus (EV) (3). EVs are members of the genus Enterovirus in the family *Picornaviridae* (4). EVs are small, non-enveloped with a single strand positive-sense RNA and their capsid is made up four structural proteins (VP1 to VP4) (5). EVs are classified into four species, EV-A, EV-B, EV- C, and EV- D (6, 7). More than 90 EV serotypes are currently recognized by the International Committee on Taxonomy of Virus (ICTV), for example, EV-A (25 serotypes), EV-B (63 serotypes), EV-C (23 serotypes), and EV–D (5 serotypes) (7). Human is the only reservoir for human enteroviruses which are transmitted from person to person through the fecal-oral route and respiratory droplets (1). Enterovirus type 71 (EV71) causes several diseases, including: herpangina; myocarditis; hand, foot and mouth disease (HFMD); polio-like acute flaccid paralysis (AFP) and AM (8). EV71 was first isolated from the stool sample of an infant in 1969 in California (9). In the pediatric population, EVs are the pathogens associated most commonly with acute meningitis worldwide and can also cause sporadic cases, outbreaks and epidemics (1, 3). The risk of EV infection is mostly associated with poor hygiene, overcrowding, and inadequate vaccination (10). Previous studies have shown that most of the infections may associate with a single serotype in a particular region (2). Therefore, the human EV causing meningitis, need to be identified in each area for better management of the disease. The cell culture is a gold standard for EV identification but this method is time-consuming and high cost, therefore, diagnostic tests based on the molecular techniques such as RT-PCR have been widely used to identify EV in recent years. Since RT-PCR can provide rapid results, about 5 to 24 hours after receiving the samples, therefore, it is an effective alternative method for viral culture (11, 12).

This study aimed to determine the prevalence of enteroviruses among patients suspected AM using RT-PCR in the West of Iran. In the pediatric population, enteroviruses (EV) are the most frequent causes of benign aseptic meningitis, which require neither treatment, nor extensive investigations.

Materials and Methods

Patients

In this study, 120 CSF samples were collected from AM patients hospitalized in Imam Reza Hospital of Kermanshah, Iran, during 2012-2013. All samples were negative by Gram stain and bacterial culture for common bacterial pathogens.

RNA Extraction

One hundred micro-litters (µL) of CSF samples were used for RNA extraction by Cinna Pure RNA viral kit (SinaClon Co., Iran) according to manufacturer's instructions. Extracted RNA was kept at -70°C until PCR testing.

RT-PCR

Enteroviruses RNA was detected by 2-steps RT-PCR Kit (Vivantis, Malaysia). Briefly, 5 µl of the extracted CSF RNA

with Primer Mixture (1 µl of oligo (dT)18, 1 µl of dNTP mix (10 mM) and top up to 10µl of Water) were incubated at 65°C for 5 minutes and chilled on ice for 2 min followed by spinning down the mixture. The cDNA synthesis mix was prepared in the following order, 2µl of 10X Buffer M-MuLV, 100 units of M-MuLV Reverrse transcriptase and top up to 10µl of Water. 10 µl of the prepared cDNA Mix was added into each RNA-primer mixture and incubated at 37°C for 60 min. The reaction was terminated by incubation of the tubes at 85°C for 5 min and chilling the tubes on ice followed by brief centrifugation of the tubes. Finally, 2µl of the mixture was used as template for PCR reactions. PCR reaction for viruses was prepared in a final volume of 25 µl containing 12.5 µl of Master Mix Ampliqon III (Ampliqon, Denmark), 1 µl of each primers 224 and 222 (12) (Table 1), 5.5 µl of H_2O and 5 µl of cDNA. PCR program was set as follows: 1 cycle at 95ºC for 6 min, followed by 40 cycles at 95ºC for 30 sec, 42ºC for 30 sec and 60ºC for 45 sec in the BioRad Thermocycler C1000 (USA). A final extension was performed at 72ºC for 2 min and 2 µl of PCR product was added into 23 µl PCR reaction containing 12.5 µl of Master Mix Ampliqon III (Ampliqon, Denmark), 1 µl of each primers AN88 and AN89 (12) (Table 1), and 8.5 µl of H_2O. PCR program was set as follows: 95°C for 6 min prior to 40 cycles of amplification at 95°C for 30 sec, at 50°C for 20 sec, and at 72°C for 15 sec. Primers were synthesized by SinaClon company (Tehran, Iran). PCR products were detected by 1.5 % agarose gel electrophoresis, and gels were visualized using Gel Doc (BioRad, USA) after staining with safe stain (SinaClon Co., Iran). The approved RNA of enteroviruses, used as a positive control, was kindly provided by the Virology Department of Tehran University of Medical Sciences (TUMS). The characteristics of primers developed in this study, are presented in Table 1.

Table 1. The characteristics of primers.

Primer Name	Sequences 5'-3'	Number of Bases
224	GCIATGYTIGGIACICAYRT	20
222	CICCIGGIGGIAYRWACAT	19
AN88	TACTGGACCACCTGGNGGNAYRWACAT	27
AN89	CCAGCACTGACAGCAGYNGARAYNGG	26

Sequence and data analysis

PCR products for enteroviruses were purified using a DNA purification kit (SinaClon Co., Iran) and sequenced. DNA samples were sequenced using ABI 3730XL DNA analyzer. Sequence data were analyzed for homology using the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/).

Statistical Methods

In this study, all collected data were analyzed using SPSS version 21 (SPSS Inc, IL, USA), and correlation between data groups was assessed using chi-square and t-test.

Results

The CSF samples were taken from 47 children (mean age= 1.86 years; age range: 1 day to 11 years) and 73 adults (mean age= 49.76 years) with AM who were hospitalized in Imam Reza Hospital of Kermanshah, Iran. The samples were

63 (52.5%) men and 57 (47.5%) women with an average age of 25.8 ± 29.4 years. Of the samples tested, 4 (3.33%) cases were positive for EV (Figure 1), which all of them were confirmed as EV71 by sequencing (Table 2 and Figure 2). The biochemical (protein and glucose) and cytological analysis of positive CSF samples are presented in Table 2.

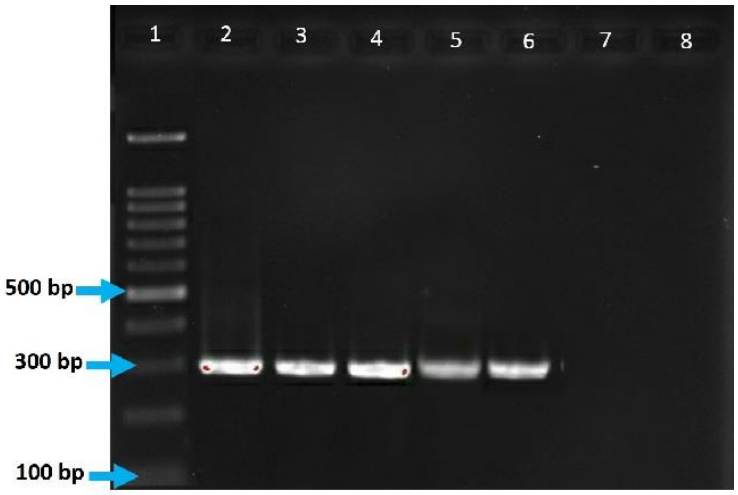

Figure 1. PCR of CSF cases; Line1: Ladder 100 bp, Line 2: Positive Control, Line 3-6: Positive Samples, Line7: Negative Sample, and Line 8: Negative Control.

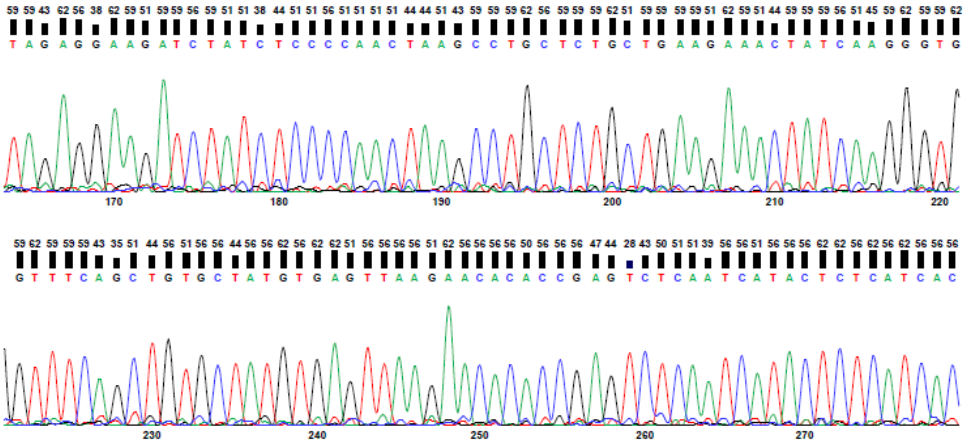

Figure 2. A part of the sequence results of Enterovirus type 71.

Table 2. The characteristics of positive CSF cases.

Virus	Sex	Age	Protein mg/dl	Glucose mg/dl	WBC	RBC	Season
Enterovirus type 71	Male	7 months	18	72	42	20	Winter
	Female	27 years	20	87	0	130	Fall
	Female	5 days	30	28	52	20	Winter
	Male	40 years	27	91	0	0	Fall

Four cases of viral meningitis were two males and two females aged 5 days to 40 years with an average age of 16.7± 20.0 years. There was no significant relationship between two genders (P>0.05). There was also no significant difference in CSF protein or glucose levels between positive and negative samples (Table 3). All four positive cases occurred in winter and fall. More detailed characteristics of the positive and negative cases are presented in Table 3.

Table 3. The comparison of average characteristics in positive and negative cases.

PCR Results	Age (year)	Protein mg/dl	Glucose mg/dl	WBC Count	RBC count	Number of Samples
Negative	25.8±29.4	28±12	63±27	7±21	424±1190	116
Positive	1±16.7±20.0	23±5	69±28	23±27	42±59	4
P-value	0.324	0.562	0.353	0.310	0.952	-

Discussion

Rapid diagnosis of viral meningitis is a crucial phase for patient management. The clinical features of AM caused by EV are similar to bacterial meningitis, and consequently, unnecessary diagnostic tests and antibiotics may be used for patients with viral meningitis (13). The detection of EV sporadic cases is essential in order to understand the epidemiology of infection (13, 14), surveillance for the emergence of new virus types or possible changes in virulence of the virus in circulation (14). As a result, rapid and accurate diagnosis of EV meningitis can result in the better patient management and reduction of health care costs (7).

More than 90% of viral meningitis are caused by enterovirus and it is the leading cause of AM / encephalitis in children, but is much less common in adults (15, 16). Since 1997, the several outbreaks of EV71 have been reported among children in some parts of Asia-Pacific countries(17). The first outbreak was detected in Kuwait and then in Taiwan (1998), West Australia (1999) and Singapore (1, 15, 18, 19). In a study in Vietnam, the enterovirus was detected by RT-PCR in the CSF samples with the rates of 8% and 10% for adults and children, respectively (20). In the present study, positive samples were EV71 indicating the importance of this virus in developing AM in Kermanshah, Iran. Several studies have shown that Enteroviruses is a common cause of AM. For example, Kupila et al. in Finland (2006), reported that Enteroviruses were the major causative agents (26%) of AM (21). The prevalence of enterovirus infections in different countries has been reported with varying rates, from 4.9% to 91% with the average of 29% (2, 3, 10, 12, 22). This variation can be due to several reasons such as age, number of patients, sampling method, sample storage and transport, type of disease, molecular methods and seasonal prevalence. Accordingly, several studies in Iran, have reported various rates of EV meningitis. For example, in a study in Tehran (2013) on CSF samples of 118 children under 13 years, the rate of EV was 10.16%, which is higher than that reported in the results of this study (23). Also, Roohandeh et al. (performed a study in Tehran and detected EV71 in 14% of the patients and demonstrated the seasonal peaks of this virus during autumn and winter (24). In a study by Hosseininasab et al. (2011) in Shiraz, the prevalence of AM by enterovirus infection in children was estimated 43.3% (25). In another research in Ahvaz (2002), the prevalence of EV infection in AM was reported 59.6% (2). The former results indicate that some factors such as outbreak and the specific age groups

(children) may cause this difference among the results of studies in Iran. Studies in Korea and Turkey (2003) have reported the rates of 7.5% and 4.9% for EV meningitis, which are similar to the results of the present study (26, 27). However, some studies on CSF samples have reported relatively higher rates for EV meningitis. For examples, studies in India and France reported the rates of 11 to 14.7% for this infection. In these studies, the patients were totally children, which may explain the reasons of higher rates in these studies (15, 28).

EV71 is transmitted from person to person by fecal-oral route and direct contact with nose and throat secretions or sometimes by vertical transmission of the infection (perinatal infection) (22). EV71 has caused several epidemics in different parts of the World as well as outbreaks in Asiaian countries (8). Also it has been reported with high rates among children in South-East Asia (29), which is consistent with the results of this study because two of the patients included in this study were children, one aged less than 1 year and another was a newborn baby. In most studies, the EV outbreaks has been reported in summer and autumn (1, 3, 7, 30). However, some research has reported outbreaks of enterovirus in cold seasons (31). In this study, all positive cases occurred in winter and fall, which is statistically significant (P=0.01). Since Kermanshah is a mountainous region with relatively warm and dry summer and cold winter, the results of this study may be reasonable.

However, there were some limitations in this study. First, the long-term storage of samples in freezer with multiple freeze-thaw cycles may damage the enterovirus RNA. Second, the presence of PCR inhibitors in the CSF samples may affect the results. Third, instead of children, CSF samples of patients with a wide range of age were used in this study, so a lower rate of enterovirus infection can be expected.

The results of this study and previous studies have shown that PCR could be routinely performed to evaluate specimens from patients with meningitis syndrome whose initial CSF examination does not allow a differentiation between bacterial and viral meningitis.

Conclusion

According to the results, EV71 is one of the common causes of enterovirus meningitis in patients in Kermanshah. Using RT-PCR technique in diagnostic laboratories, enteroviral meningitis can be rapidly diagnosed. This can improve the management of patients and prevent the unnecessary use of antibiotics. Therefore, using molecular methods may reduce the cost of patients' treatments and prevent drug resistance among bacteria.

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