

Investigating the etiologic agents of aseptic meningitis outbreak in Iranian children

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ABSTRACT

Introduction: This study aimed to determine the viral agent (s) and their genome burden associated with an aseptic meningitis outbreak that occurred in Yasuj, Iran. **Materials and Methods:** During April to August 2015, 104 CSF samples from 104 patients under 14 years old admitted to the hospital of Yasuj, Iran, with aseptic meningitis associated clinical signs were collected. 200 µl CSF specimens was prepared for DNA and RNA viral genome extraction each and then subjected to diagnostic Taq-man real time PCR assays for the present of Enteroviruses, HSV, VZV, mumps, measles and rubella in the samples. **Results:** The majority of them had experienced clinical meningitis sign. Primary laboratory differentiated tests were in favor of viral meningitis. Among a total of 104 patients diagnosed with clinically aseptic meningitis, enterovirus as the most significant viral agent was detected in 53 subjects. However, mumps, HSV and VZV, as the endemic causes of viral meningitis, were detected in 6, 6 and 2 of the affected patients. It was revealed that two HSV and one VZV affected patients were co-infected with enteroviruses. All affected children with relatively variable viral load recovered without any sequels. **Conclusion:** The present study revealed enterovirus as the main predominant cause of pediatric aseptic meningitis that broke out in Yasuj-Iran. Also, the co-circulation of mumps, HSV and VZV, as the endemic cause during the same aseptic meningitis outbreak, was demonstrated in some cases.

Keywords: Aseptic meningitis, Iran, outbreak, quantitative PCR

Introduction

Aseptic meningitis is characterized by acute onset of clinical manifestation of the central nervous system (CNS) infection, cerebrospinal fluid (CSF) pleocytosis and no identifiable bacterial agent in gram stain or culture.^[1] Affected patients may exhibit different clinical features such as fever, photophobia, stiff neck, seizure, headache, anorexia, sore throat and rash.^[2-3] The most frequent cause of aseptic meningitis is viruses. This inflammatory disorder, as the most common CNS infection worldwide, can

occur at any age, but younger children especially those under 1 year are most at risk.^[3,4]

Nowadays, in MMR post-vaccination era, the frequency pattern of viral meningitis has been altered and enteroviruses (EVs) have supplanted mumps, as the most common cause of viral meningitis in children.^[5] It accounts for 80 to 90% of known endemic cases; however, it can appear with occasional outbreaks.^[4,6] Older infants and children could be susceptible to other forms of viral meningitis.^[7,8]

Enteroviruses are members of picornaviridae with many species. EV-A to EV-D can potentially infect humans. EV-B is the most

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abundant species with 58 human types accounting for vast EVs meningitis.^[9] This viral infection, as the name implies, is transmitted through fecal-oral route with summer-fall seasonality, except that a few documented cases were reported in winter.^[4]

Since the development of sensitive molecular techniques for the diagnosis of enterovirus infection, detection and molecular typing of enterovirus have been facilitated during endemic and epidemic cases during past years.

The aim of this study was to determine the etiology of viral meningitis outbreak in Yasuj-Iran, using quantitative PCR assay.

Materials and Methods

We surveyed 104 children younger than 14 years of age for viral etiological agent associated with an aseptic meningitis outbreak in Yasuj, Iran. From April to August 2015, patients were admitted to pediatric emergency of Yasuj hospital with acute onset of clinical symptoms and signs of meningitis. Their demographic (age and sex) and clinical vaccination status, underlying diseases including primary and secondary immunodeficiency, malignancy, severe malnutrition and clinical presentations were recorded.

Lumbar puncture was performed in all suspected patients upon obtaining their parents or legal guardians' consent up to 6 hours after hospitalization. Glucose and protein concentrations and leukocyte count were analyzed for each CSF specimen. Leukocytes higher than 5 per microliter of CSF were considered as pleocytosis.^[10] Gram stain and bacterial culture were performed by standard methods for all CSF specimens in Yasuj hospital.

Approximately, 500 µl of each CSF sample was stored with proper cold chain storage and transported to the virology department of Prof. Alborzi Clinical Microbiology Research Center, Nemazee Hospital, Shiraz and kept at -80°C until further process. All patients received conservative treatment and were discharged based on the attending physician's decision. Outpatient follow-up of all patients was done for 3 months by a pediatric infectious diseases specialist (A.G.M). It is noteworthy that the Ethics Committee of Yasuj University of Medical Sciences approved the study.

Genome extraction

Two 200 µl of each CSF specimen were subjected to DNA and RNA extraction, respectively. Viral RNA extraction was done by Invisorb® spin virus RNA mini kit (stratec-Germany) and Viral DNA extraction using Invisorb® spin virus DNA mini kit (stratec-Germany), according to the manufacturer's instructions.

Taq-man real-time PCR assay

Taq-man real-time PCR assay was carried out to detect possible meninges-tropism viruses. Totally, for each CSF specimen, five tests were done. Five standard assay kits were purchased

from Primerdesign Company (UK) for detecting HSV, VZV, EVs, mumps and measles viruses. Lyophilized standard stocks in all 6 kits were rehydrated, according to the manufacturer's instructions. Serial dilutions from 1×10^5 – 10 copies/reaction were prepared, as mentioned in the kits guideline. $2 \times$ one-step master mixes (Applied Biosystem company, USA) was used to perform RNA virus quantitative PCR assay and gene expression master mix was applied to the assessment of DNA viral agents. 5µ of each extracted DNA and RNA was used as template in separate real-time PCR assays.

The RT-PCR conditions for detecting RNA viruses were as follows: 50°C for 30 min and 95°C for 10 min, followed by 50 cycles of 95°C for 10 s and 60°C for 1 min; this program for detecting DNA viruses was as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The reactions were performed in a 7500 real-time PCR system (Applied Biosystems, United States).

Statistical analysis

Data were analyzed using SPSS, version 19.0, statistical software. Comparisons were made using *t*-test and the Chi-squared test.

Results

In the current 2015 outbreak during less than 6 months, 104 children less than 14 years of age (1 month to 14 y/o), consisting of 56 (54%) male and 48 (46%) female, were diagnosed clinically. 31%, 34% and 35% of patients were in the age range of ≤ 1 , 1.1-5, and 5.1-14 years old, respectively.

The diagnosis of this disease was based on symptoms and signs indicative of meningeal irritation in patients. Fever, headache, vomit and rash were the main clinical manifestations with an incidence of 100%, 85%, 56% and 18%, respectively. Other clinical signs included neck stiffness (100%), Brudzinski sign (90.4%), and Kernig sign (84.5%).

According to the patients' records, bacterial gram staining and culture were negative. The CSF glucose levels in all patients were normal. Protein concentrations ranged 30-90 mg/dl. CSF pleocytosis (more than 5 leukocytes) was defined as another laboratory hallmark of viral meningitis. Other laboratory findings are shown in Table 1.

In the current survey, all partially treated patients received up to 14-day treatment, as standard duration of antibiotic therapy, while all initially confirmed cases of viral meningitis have been discharged from the hospital without receiving any antibiotic. The patients were followed up for 2 and 4 weeks afterwards.

Among 104 affected children, EVs were detected in 53 (51%) patients, as the etiologic cause of the aseptic meningitis associated with outbreak. Mumps and HSV were found each in 6 (5.7%) affected children; also, 2 patients with HSV were co-infected with EVs.

VZV was another meningeal tropism virus found in CSF specimens of two affected patients, one of whom were co-infected with EVs. It is notable that rubella and measles were not detected in any CSF sample. Totally, in 37 remaining patients, no specific viral agent was identified [Figure 1].

As shown in Figure 2, enterovirus, as the prominent cause of aseptic meningitis, had a significantly higher distribution than others. The EV load in CSF specimens ranged from 37 to 68644 copies/ml. HSV viral load was between 144-926 copies/ml, while mumps viral load was lower and ranged from 54 to 175 copies/ml.

Although a high variability was seen in the EVs load, it is notable that in 2 EV/HSV and 1 EV/VZV co-infected patients, the high

| Table 1: Laboratory findings of aseptic meningitis affected patient-serum and CSF parameter | |
|---|------------------------|
| A) CRP rate | |
| CRP | Percentage of patients |
| CRP- | 62% |
| CRP+ | 18% |
| CRP++ | 14% |
| CRP+++ | 6% |

| B) ESR rate and C) WBC count of CSF | |
|-------------------------------------|------------------------|
| ESR | |
| | Percentage of patients |
| ESR <20 | 69% |
| ESR 20-50 | 23% |
| ESR >50 | 8% |

| WBC count of CSF | |
|------------------|------------------------|
| | Percentage of patients |
| 5-50 | 17% |
| 50-100 | 33% |
| 100-1000 | 38% |
| >1000 | 12% |

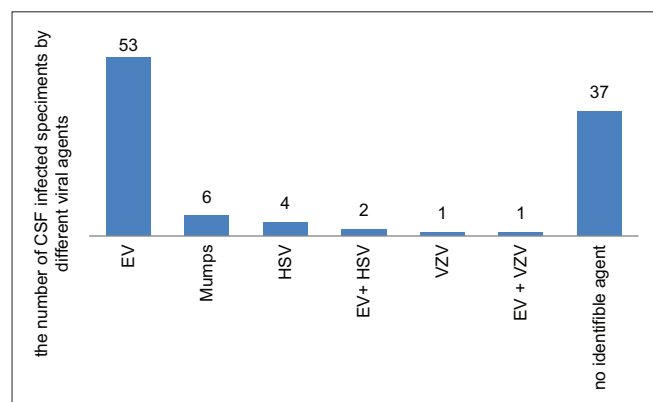


Figure 1: Notification of viral meningitis agents associated with outbreak in Iran and the number of Enterovirus (EV), mumps, Herpes Simplex Virus (HSV) and Varicella Zoster Virus (VZV) infected and EV/HSV and EV/VZV co-infected patients. No rubella and/or measles-infected CSF specimen was identified. Among 104 clinically and laboratory diagnosed aseptic meningitis, in 37 cases no specific viral agent was detected

EV load was associated with high HSV or VZV load and vice versa. None of the patients had underlying disorders. No fatalities occurred among the patients during 3 the month follow-up and all patients recovered without any sequels.

Discussion

The study reports the viral etiology of an outbreak of aseptic meningitis in children younger than 14 years old in Yasuj, 2015. Enterovirus, mumps, HSV, VZV and measles, as the most important causative viral agents in endemic cases in our region,^[11] were considered and assessed.

Given that PCR is up to 1000-fold more sensitive than cell culture in the detection of HSV, VZV and enterovirus in the suspected CSF specimens,^[12] Taq-man real PCR was used as the initial approach to the diagnosis of causative agents of aseptic meningitis.

The current study is the first documented aseptic meningitis associated with recent outbreak in Iran caused by EVs with 51% incidence. Some studies conducted in different geographical regions of Iran showed that EVs were the most important cause of endemic aseptic meningitis with an incidence of 20 to 59%.^[11,13]

More than 50 subtypes of EVs were associated with aseptic meningitis. Coxsackie B5, echo viruses 6, 9 and 30 were the most common etiological agents of epidemic enteroviral meningitis, while Coxsackie A9, B3 and B4 were predominant causes of endemic enteroviral meningitis. It is worth noting that all of them belong to enteroviruses B species.^[14,15]

So far, several outbreaks of meningitis have been reported worldwide with typed or un-typed EVs. Some enteroviral meningitis outbreaks have been reported in Asia, especially Southeast Asia, during the past decade. For example, in 2012, Xio *et al.* reported an outbreak of aseptic meningitis associated with EVs in Guangdong province-China (68%),^[16] followed by another

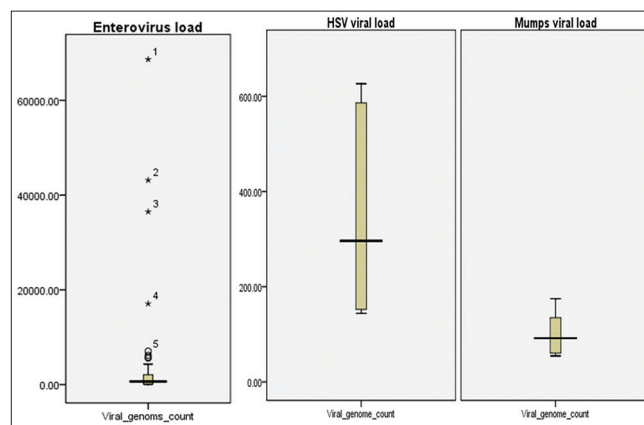


Figure 2: Box and whisker plot represents viral loads (copies/ml) in patients infected by: EVs, Mumps, Herpes Simplex Virus (HSV) and Varicella Zoster Virus

outbreak reported by Zhang *et al.*, in 2003 in Jiangsu-China.^[17] EVs caused a viral meningitis outbreak in Korea in 2008^[18] and in Taiwan,^[19,20] and Jordan^[21] in the past decade.

In other parts of the world, such as Brazil,^[6,22] China^[23] France^[24] Canada,^[15] Poland, USA,^[25,26] and South Africa,^[27] EVs were the main cause of viral meningitis associated outbreak.

Frequent mutation in circulating enterovirus B and inter- and/or intra-typic recombination and their adaptation to environmental challenge mainly account for the emergence of new outbreaks in different countries.^[9]

Mumps, as one of the most important endemic causes of aseptic meningitis even in post-vaccination era, affected 4 vaccinated and 2 unvaccinated children. Some popular studies revealed the adverse effects of Leningrad-Zagreb and Leningrad-3 strains contained within mumps vaccine in development of aseptic meningitis, in contrast to Jeryl-Lynn and RIT 4358 strains.^[28] There are no informative data about S-12 mumps vaccine, the current vaccine strain used in Iran. In addition to the likelihood of meningitis induction by mumps vaccine, vaccination failure is another probable cause of aseptic meningitis.^[11] In the present study, since the 2 children were 3.5 and 6 years old respectively, vaccination failure seems to be more reasonable.

In post-MMR vaccination era, mumps vaccine rather than measles and rubella may cause vaccine associated aseptic meningitis. In the current study, no measles and/or rubella CSF infected specimen was detected; however, 6% of the CSF samples were infected with mumps; this is consistent with other studies in endemic aseptic meningitis events in Iran.^[11]

HSV was considerably detected in six CSF specimens, two of which co-infected with enterovirus. Meningeal sign and decreased level of consciousness were observed in all 6 HSV-affected patients. Determination of HSV and VZV viral loads seems not to be necessary in the prognosis of the disease, but it may be helpful in monitoring the effectiveness of antiviral therapy.^[29]

It is notable that negative viral quantitative PCR results may interfere with the sampling time and limitation in viral genome detection.^[11] Since residence in low-income areas, and poor hygienic and sanitation are the predisposing factors for neurotrophic infections,^[30] Yasuj in Iran is predisposing these infections. It is suggested that environmental and personal hygiene, especially among those in close contact with children such as nursery workers, parents and sibling, should be carefully considered and promoted so that such infections are diminished.

In this study as well as other documented reports, it was revealed that rapid detection of viral agents in aseptic meningitis cases, using PCR assays, can shorten the length of antibiotic therapy, decrease hospital stay, and save health costs.^[11]

Conclusion

This study, as the preliminary report on the aseptic meningitis outbreak which happened in Iran in 2015, revealed that enterovirus was the main causative agent associated with the outbreak. Other viruses such as HSV, Mumps and VZV which are the endemic causes of viral meningitis were detected in some CSF samples, as well. Taq-man real-time PCR provides a quick and accurate diagnosis of viral agents associated with aseptic meningitis as well as viral load as complementary information.

Findings from this study recommended that healthcare providers, after mumps and rubella vaccination, provide the warnings about symptoms of meningitis for the parents, as well as healthcare providers would be sensitive about the symptoms of meningitis, that the patient can be quickly diagnosed and be treated.

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Conflicts of interest

There are no conflicts of interest.

Ethical approval

Ethical approval was given by Ethics Committee of Yasuj University of Medical Sciences and Shiraz University of Medical Sciences.

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