Epidemiology of herpes simplex and varicella zoster virus in cerebrospinal fluid of patients suffering from meningitis in Iran

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Abstract

From the early 18th century that "meningitis" outbreak was firstly recorded in Geneva, it is one of the alarming health problems worldwide. Different infectious risk factors may contribute to the progression of meningitis. Herpes simplex virus (HSV) and Varicella-zoster virus (VZV) are just some noticeable risk factors among many involved in the progression of this disease. In this study, 415 meningitis suspected patients were recruited with some symptoms, such as fever, headache, nausea or vomiting, seizure, rash, dizziness from four different hospitals of Iran and molecular examinations of samples were performed by using specific primers of $HSV^1/_2$ and VZV via real-time PCR. Out of 415 included patient 41 (9.8 %) were VZV and six (1.4 %) cases were $HSV ^1/_2$ positive. Fever was the most frequent symptom by 315 (76 %) of patients with median temperature of 38 °C in all included patients. The median WBS counts of CSF in VZV positive, $HSV^1/_2$ positive, and all included cases were $1567 × 10^6$ /L, $1257 × 10^6$ /L, and $766 × 10^6$ /L (range 0-21200), respectively. In conclusion, as the rate of VZV infection was high among children patients and it was associated with the absence of vaccination program for chickenpox in Iran, we suggested that VZV is one of the plausible hallmarks in meningitis.
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Background

Central nervous system (CNS) infections still remain a clinical challenge because of their potential morbidity and mortality. Different risk factors are supposed to contribute to the deterioration in mental function of individuals suffering from meningitis, especially infectious ones; therefore, urgent care and medical attention to diagnose, and consequently provide emergency treatment, is essential [1]. The symptoms of these infectious agents are different, and depended on the location of the infection. Hence the different names, including meningitis, encephalitis and localized brain abscesses can be considered. Meningitis, the inflammation of the protective membranes of the CNS, has a dramatically important position on the spectrum of CNS infections. Aseptic and bacterial (purulent) meningitis can be differentiated based on the leucocyte content of the cerebrospinal fluid (CSF) and other characteristics of CSF, such as glucose and protein content [2]. Bacterial meningitis is more severe than aseptic meningitis; which is usually caused by viral agents. The use of conjugate vaccines has reduced the incidence of meningitis by bacterial agents, but viral meningitis is one of the most common types of meningitis [3]. Although there are significant differences in the age of patients and their geographical distribution, many studies have shown that non-polio Enteroviruses, herpes simplex virus (HSV) and varicella zoster virus (VZV) are the main aetiological agents of viral meningitis [4–7]. In a recent report by the US Centers for Disease Control, it was stated that other viral risk factors, such as mumps orthorubulavirus, measles morbillivirus, influenza virus subtypes A and B, arboviruses (such as West Nile virus), and lymphocytic choriomeningitis mammarenavirus can result in meningitis [1].

Herpesviridae is a large family of DNA viruses and their double-stranded DNA genome is surrounded by an icosahedral capsid. HSV and VZV, which are, respectively, known by their taxonomical names human alphaherpesviruses 1 and 3, are neurotropic herpesviruses that establish latent infection in dorsal root ganglia, located in the peripheral nervous system for the entire life of the patient [2]. The infection may cause acute, sub-acute and chronic disorders of the CNS in adults and children. Although serious problems in most individuals occur rarely, immunocompromised hosts may be at risk as the *Herpesviridae* can be considered as opportunistic agents [3,4]. The most common trigger of Mollaret's meningitis, the benign recurrent lymphocytic form of meningitis, is HSV-2, although HSV-1 is probably responsible for some viral meningitis [5,6]. Moreover, individuals with neurological complications of VZV infection have a high rate of chemokines and inflammatory responses in their CSF, which attract immune cells and cause inflammation of CNS tissues, leading to meningitis [7].

In this study, we attempted to investigate the prevalence of HSV and VZV in the CSF of the children under 18 who were suspected of having meningitis by using a real-time PCR assay, which is a fast and accurate way to detect viral infections.

Methods

Sample

This study has been submitted, and approved by the Ethics committee of the School of Medicine Shahid Beheshti University of Medical Sciences; IR.SBMU. MSP.REC.1397.384 (Grant no 11477). The samples were collected from hospitalized children under 18 years old at Mofid, Taleghani and Bighiatallah Hospitals of Tehran, Iran and *Logman Hakim*, Iran, between February 2016 and Jan 2019. A total of 415 CSF samples were obtained from individuals with suspected meningitis who had three or more symptoms of meningitis (headache, fever, seizure, nausea/vomiting and neck rigidity). Samples were evaluated by smear staining and culture and differential tests in the microbiology laboratory of each hospital. Individuals with malignancies, a focal neurological dysfunction, alteration in behaviour or consciousness, or photophobia were excluded from this study. The last two are common symptoms of patients diagnosed with encephalopathy and are outside the scope of our study. The samples were obtained by lumbar puncture and after analysis for white blood cell count, red blood cell count, glucose and protein, and microbiological examinations, they were stored at −20°C until transfer to the laboratory and further molecular examination. Demographic data, including gender, age, disease and surgery history, meningeal symptoms and signs, and laboratory test results on blood and CSF, were gathered from clinical records.

DNA extraction

DNA was extracted from CSF samples using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol, in which 500−1000 μL of filtered CSF samples were eluted to 50 μL. The qualification of DNA was evaluated by Nano-Drop (Thermo Scientific NanoDrop 2000 Spectrophotometer; ThermoFisher, Waltham, MA, USA) and the β-globin gene was used as an internal control of the PCR. The β-globin sample was generated in a mixture of 12.5 μL master mix, 1 μL forward and reverse primer (10 pmol), 1 μL DNA and 8.5 μL sterile water in a final 25-μL reaction. The primer sequences are shown in Table 1. The PCR schedule for the β-globin gene was: 5 min at 95°C as the first denaturation, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and 72°C for 7 min. Purified DNA samples were stored at −20°C.

Real-time PCR

The uniplex real-time PCR was designed to quantify the DNA of VZV, HSV-1 and HSV-2 based on the principle of SYBR Green technology using a Rotor-Gene 6000 real-time PCR system (Corbett Life Sciences, Sydney, Australia), and two sets of primers whose sequences are shown in Table 1. The products were subjected to 40 cycles of amplification in a total volume of 20 μL of reaction mixture, containing 2 μL of extracted DNA, 10 μL of 1 × real-time PCR Master Mix (Intron, Gyeonggi-do, Korea), 1 μL forward primer, 1 μL reverse primer and 6 μL of sterile water.

The cycling conditions consisted of initial denaturation at 94°C for 10 min and 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The analytical melting curve was programmed from 60°C to 90°C with a 0.1°C/s ramp rate.

Statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences, version 21.0 (SPSS Inc., Chicago, IL,

TABLE 1. Sequences of primers used in the study

Gene	Primer sequence	Ref.
HSV-1, HSV-2 (*gpB*) F	CCACCGTCAGCACCTTCAT	[30]
HSV-1, HSV-2 (*gpB*) R	CGCTGGACCTCCGTGTAGTC	[30]
VZV (*DNApol*) F	GCGCTCTAACGTTCGAGAAAGT	[31]
VZV (*DNApol*) R	CGCATAGCCAACCAGTCTTTT	[31]
IC (β-globin) F	GAAGAGCCAAGGACAGGTAC	[32]
IC (β-globin) R	CAACTTCATCCACGTTCACC	[32]

HSV, herpes simplex virus; IC, internal control; VZV, varicella zoster virus.

USA). Pearson chi-square test was used to detect the relationship between categorical variables and analysis of variance was used to analyse the relationship between age and VZV. A p value of <0.05 was considered statistically significant.

Results

Participants

In this study 415 patients (56.6 % male and 43.4 % female) were collected, and clinical data from Bighiatallah, *Logman Hakim*, Mofid and Taleghani hospitals. Our study defined 4 age groups for patients including patients with the age of one month and less (n = 52), 1-12 months (n = 179), 1-7 years (n = 125), and 7-18 years (n = 59). Most patients were between 1-12 months old (43%).

Clinical symptoms and data

Fever was the most probable symptom, seen in 76% of all individuals with median temperature of 38°C. Nausea or vomiting was reported in 43% and <21% had seizures. Headache was observed in only 16% of individuals and about 7% showed rash and dizziness. The clinical data of other symptoms, such as neck stiffness, was missing for some patients. The median white blood cell counts in CSF samples were 766×10^6/L (range $0-21\ 200 \times 10^6$) and median red blood cell counts were 1.4×10^6/L (range $0-85.0 \times 10^6$). Also, the median protein content in CSF was 87 mg/dL (range 5−878) and the median glucose concentration in CSF was 54 mg/dL (range 3−116). Furthermore, serum C-reactive protein levels were high (median 36 mg/dL; range 1−149).

Real-time PCR assay

In melting curve analysis, VZV was distinguishable by an average melting temperature of 78.6°C and HSV by 84.5 °C in positive samples.

Viral detection

Overall, VZV infection was identified in 9.8 % of all patients (n = 41). Out of 415 included patients, six (1.4 %) was HSV $^1/_2$ positive. Out of 41 VZV-infected patients, 26 were men and 15 were female with the median age of 21 months old. Fever was the most frequent clinical symptom among VZV-infected patients (median = 38 °C). Although nausea or vomiting was seen in 25 VZV-infected patients, one of them represent rash and three patients reported headache. The median white blood cell counts of CSF were 1567×10^6/L (range $0-21\ 200 \times 10^6$) and median red blood cell counts were 5.4×10^6/L (range $0-85.0 \times 10^6$). The median protein content in CSF was 107.5 mg/dL (range 5−670) and the median glucose concentration in CSF was 48.8 mg/dL (range 10−80). Furthermore, the median serum C-reactive protein level was 44 mg/dL (range 1−149).

Furthermore, in the presence of positive control, and despite repeated tests, no HSV infections were reported among participants (Table 2).

Discussion

In this prospective study, we evaluated the prevalence of herpesvirus infections (HSV and VZV) in the CNS of hospitalized children (<18 years old) who were suspected of having meningitis using real-time PCR. This molecular diagnostic method was able to detect pathogen genomes in small amounts of CSF samples.

By reduction of bacterial aetiologies and improvements in screening of viral agents, the role of viruses in CNS infections becomes clearer, and aseptic meningitis becomes more common than bacterial meningitis. Although it causes less severe disease, the mortality rate of herpesvirus-related CNS diseases in immunocompromised patients without proper antiviral therapy can be high [8−11]. Flaviviruses are regarded as neurotropic

TABLE 2. Demographic data and laboratory findings in varicella zoster virus-infected patients and total of children with suspected meningitis

Variables mean (range) or n (%)	VZV positive (n = 41)	VZV negative (n = 374)	Total (n = 415)
Demographics			
Age (months)	21 (0.6−90)	32 (0.1−168)	33 (0.10−180)
Male/Female	26/15	198/176	233/182
Fever	38 (37−40)	38.4 (36−40)	38 (36−40)
Headache	3 (0.7%)	62 (15 %)	68 (16.2%)
Nausea or vomiting	25 (6%)	128 (39.6%)	178 (43%)
Seizure	10 (2.5%)	77 (18.6%)	90 (21.6%)
Rash	1 (0.3%)	26 (6.2%)	24 (5.7%)
Dizziness	5 (1.5%)	20 (4.9%)	32 (7.7%)
Laboratory			
CSF WBC count	1567 (0−21 200)	677 (0−21 200)	766 (0−21 200)
CSF RBC count	5.4 (0−85)	1.6 (0−85)	1.4 (0−85)
CSF protein	107.5 (5−670)	100.5 (5−878)	87 (5−878)
CSF glucose	48.8 (10−80)	54 (3−107)	54 (3−116)
Serum CRP	44 (1−149)	39 (1−149)	36 (1−149)

CRP, C-reactive protein; CSF, cerebrospinal fluid; RBC, red blood cell; VZV, varicella zoster virus; WBC, white blood cell.
The reference ranges of laboratory tests for WBC, RBC, protein, glucose in CSF are $0-10$/mm^3, $0-10$/mm^3, 20−40 mg/dL, 45−80 mg/dL, respectively.

agents that are transmitted through mosquitoes bites; they infect the CNS asymptomatically and should be considered in differential diagnosis during outbreaks in endemic areas [12].

Identification of a proven aetiology in CNS-infected individuals contributes to better management of disease and hospital costs by reducing the length of hospitalization and the unnecessary use of antibiotics. Despite the obvious reasons for using molecular diagnostic methods, there is still no suitable platform for commercializing and using them in medical laboratories.

In our study, out of 415 included patient 41 (9.8 %) were VZV and six (1.4 %) cases were HSV $^1/_2$ positive. The VZV infection incidence was similar to the estimated data in Choi et al., who reported 11.9% of VZV meningitis in an adult Korean population [13]. In a prospective observational cohort study in Denmark, VZV (13%) was most common in viral meningitis followed by enterovirus and HSV-2 (nearly 10% each) [14]. In Turkey, a country near to Iran, in a population of children and adults, herpesviruses were found to be more frequent than enteroviruses [15]. However, evidence collected by Xie's group from hospitalized Chinese children showed the primary pathogens of viral meningitis to be human enteroviruses (37.7%), followed by HSV-1 (14%) and VZV (11.5%) [16]. In Iran in 2012, Sasan et al. detected enterovirus in 13 of 102 samples from individuals diagnosed with aseptic meningitis by RT-PCR (12.7%); and in 20 patients considered to have mumps due to parotitis and negative RT-PCR (19.6%) [17]. Azadfar et al. detected four HSV-1 samples (8.8%) by PCR in children in the southeast of Caspian Sea area [18]. In 2016, Shahroodi et al. reported enterovirus to be the common cause (>30%) of meningitis in neonatal and young patients in Mashhad, Iran [19]. In general, most studies in different parts of the world have identified herpesviruses and enteroviruses as the most common cause of viral meningitis [20–22]. Unfortunately, due to conditions in the laboratory, we were unable to extract and detect the sensitive RNA of the enterovirus in CSF samples, which is an important limitation of our research. Our results may be related to the epidemiology of the region, the lack of a defined vaccination programme for VZV in Iran, the use of different age groups, PCR detection limits, methodologies and limitations of the study.

The hospital data showed 14 VZV-positive samples that were diagnosed as bacterial meningitis. They show how diagnosing acute viral meningitis in children with pleocytosis is important, because the main concern in children with CSF pleocytosis is bacterial meningitis [23]. Although several cut-off points have been established to help the clinician in differentiating the aetiologies [24–26], the important barrier in using such prediction models is that in clinical practice many other aetiologies might need to be considered and the population they were tested in will not be the same as target groups. For example, there is no available model to differentiate meningitis in infants from other conditions, so the clinical view is dominant when considering

whether to start empiric antibiotic and adjunctive therapy [27]. Ericsdotter et al. reported the reactivation of HSV-1 in pneumococcal meningitis in a 67-year-old woman [28]. So there may be a possible co-infection of viral and bacterial aetiologies in individuals with a weakened immune system.

Between all included patients just 6 (1.4 %) cases were HSV $^1/_2$ positive. It may be that in our study most patients were under 1 year old and initial infection with HSV is less likely to occur for this age group, except for HSV transmission during delivery or through postnatal contact with an individual who is actively shedding virus, which may result in CNS infection [29]. The fact that the prevalence of HSV-2 in Asia is less could be another reason for our study results.

Conclusion

Real-time PCR was found to be a good method for the diagnosis of herpesvirus-related meningitis. It is an efficient, specific and quick tool for detecting the aetiology of viral meningitis and should be commercialized for use in routine diagnosis. As the rate of VZV infection was high among hospitalized children and it was associated with the absence of a vaccination programme for chickenpox in Iran, we suggest that VZV is one of the plausible hallmarks in meningitis. Generally, misdiagnosing viral meningitis as bacterial meningitis results in elevated hospital costs and prescription of antibiotics that may cause side effects. Molecular examination of CSF would help to solve this problem in hospitals.

Ethics approval and consent

This study has been submitted, and approved by the Ethics committee of the School of Medicine Shahid Beheshti University of Medical Sciences; IR.SBMU. MSP.REC.1397.384 (Grant no. 11477). Written consent for publication was obtained for all participants, and as all participants were under 18 years of age, consent was collected from the parents.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Availability of data and materials

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

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