


RESEARCH ARTICLE

Detection of Enterovirus, Herpes Simplex, Varicella Zoster, Epstein-Barr and Cytomegalovirus in cerebrospinal fluid in meningitis patients in Iran

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Abstract

Background: Despite medical advances, central nervous system (CNS) diseases put a pressure on the health care system. A number of risk factors, especially infectious agents can accelerate the progression of meningitis. As viruses probably account for most cases of meningitis, the diagnosis of them can reduce antibiotic prescriptions. Among various types of infectious diseases, the relationship between two important virus families, including Picornaviridae and Herpesviridae, and meningitis has attracted attention.

Methods: In this study, one hundred and two samples were collected from patients who experienced symptoms, such as the loss of consciousness, seizures, muscle weakness, fever, headache, rash, and severe dementia, between November 2018 and September 2019. After RNA and DNA extraction, the prevalence of Enterovirus (EV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Herpes simplex virus type 1 (HSV-1), Herpes simplex virus type 2 (HSV-2), and Varicella zoster virus (VZV) was evaluated using PCR, multiplex PCR, and nested PCR.

Results: Results indicated that there were two VZV DNA-positive specimens, while six and five samples were infected with HSV-1 and EBV, respectively.

Conclusion: We reported that the prevalence of EBV, HSV-1, and VZV in patients, suffering from meningitis cannot be ignored; however, further investigation is needed.

KEYWORDS

CSF, Cytomegalovirus, Enterovirus, Epstein-Barr, Herpes Simplex, Varicella Zoster

1 | INTRODUCTION

Despite medical advances, central nervous system (CNS) diseases have detrimental effects on the health care system. A number of risk factors, especially infectious agents can cause brain parenchymal inflammation, and different types of neurologic symptoms called

meningitis.¹⁻⁴ Meningitis is one of the life-threatening diseases, resulting in functional brain failure. Previous studies have reported that infectious agents can play an important role in meningitis progression, affecting more than 1.2 million cases every year.⁵⁻⁷ Among different types of infections, much attention has been given to the role of virus families, especially *Herpesviridae*. This virus family may be responsible

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for blood-brain barrier (BBB) destruction and brain tissue necrosis. At least five members of *Herpesviridae*, including CMV, EBV, HSV-1, HSV-2, and VZV, which spread around the world, can cause tumorigenic transformation, and gene overexpression.⁸ The most common clinical symptoms of patients, suffering from these viral health problems, are blisters, fever, meningitis, changes in an individual's behavior, cognitive disability, aphasia, seizures, and neuropsychological deficits.^{9,10} Immunocompromised people, such as AIDS patients and organ-transplant recipients, usually have these symptoms as these viruses are opportunistic pathogens.^{11,12} Moreover, *Enterovirus* (EV) is another virus that can be considered one of the leading causes of aseptic meningitis in children.¹³⁻¹⁵ This virus, accounting for 60%–80% of all aseptic meningitis cases, belongs to *Picornaviridae*.¹⁶ EV infections cause a wide variety of symptoms, such as pleocytosis (an increase in the number of lymphocytes in cerebrospinal fluid).¹⁷ As viruses probably account for most cases of meningitis, the diagnosis of them can reduce antibiotic prescriptions.¹⁸ In the light of what is mentioned above, we attempted to investigate the prevalence of these viral diseases in CSF of patients, suffering from meningitis.

2 | MATERIAL AND METHODS

2.1 | Samples preparation

The present study is financially supported by “Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran” (IR.SBMU.RETECH.REC.1397.697, Grant No 15259). In this cross-sectional study, we collected one hundred and two samples from patients (between 1 month-old and 55 years old), suffering from the loss of consciousness, seizures, muscle weakness, fever, headache, rash, and sudden severe dementia from Emam Hossin, Loghman, and Mofid Hospitals between 2018 November and 2019 September. In this study, lumbar puncture was performed by three experts to analyze the characteristics of cerebrospinal fluid, including WBC, glucose, protein, and microbiological properties. (Table 1) All samples were stored at -20°C .

2.2 | Nucleic acid extraction

The RNA and DNA of filtered CSF were extracted by High Pure Viral Nucleic Acid Kit (Roche Diagnostics), in which 200 μl of filtered CSF sample was eluted by 50 μl of elution, according to manufacturer's protocol.

2.3 | The synthesis of cDNA

In this study, we used a Bio fact cDNA kit. After combining 25 μl of eluted purified viral nucleic acids with 24 μl reverse transcriptase and 1 μl Random hexamer, the incubation was carried out for 40 min at 50°C and 10 min at 95°C in Bio Intellectica PCR. The cDNA was then diluted twice in sterile water.

TABLE 1 Demographic data and laboratory findings in VZV-infected patients and total of children with suspected meningitis

Variables mean (range) or No (%)	VZV positive	HSV-1 positive	EBV positive
Male	2	2	3
Female	0	4	2
Fever	38°C	38.3°C	37.9°C
Headache	2	1	1
Nausea or vomiting	2	0	1
Seizure	0	2	2
Rash	1	1	0
CSF-WBC count (0–21,200) / mm^3	1567	1675	1932
CSF-RBC count (0–85) / mm^3	5.4	5.2	5.4
CSF-protein (5–670) mg/dl	107.5	152.1	135
CSF-glucose (10–80) mg/dl	48.8	53.3	49.1
Serum-CRP (1–149) mg/dl	44	65	52

2.4 | GAPDH gene PCR

β -globin gene was employed to evaluate the quality of DNA extraction. 12.5 μl master mix, 1 μl forward primer (10 pmol), 1 μl reverse primer (10 pmol), 1 μl DNA, and 8.5 μl sterile water in final 25 μl were mixed. The mixture was incubated in the following PCR schedule: 5 min at 95°C as a 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. Final PCR production was about 100 base pairs and every positive sample was evaluated to detect HSV-1, HSV-2, CMV, VZV, EBV, and EV.

2.5 | Nested PCR for EV

In this study, we evaluated EV prevalence with nested PCR. In the first step, 12.5 μl master mix PCR, 1 μl forward primer, 1 μl reverse primer (Table 2), 5 μl DNA, and 5.5 μl sterile water were mixed. The first PCR schedule was 3 min initial denaturation at 94°C , 40 cycles of denaturation at 94°C for 30 s, annealing at 44°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min. Then, in the second step, 12.5 μl master mix, 1 μl forward primer, 1 μl reverse primer, 8.5 μl sterile water, and 2 μl DNA of the first PCR products were mixed. The PCR schedule was as follow: 3 min initial denaturation at 94°C , 40 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The second PCR product was about 360 μl , and run into 2% agarose gel electrophoresis.

2.6 | Multiplex PCR and PCR

Multiplex PCR was employed to investigate the prevalence of VZV, HSV-1, and HSV-2 in CSF samples. 25 μl master mix was combined with 18 μl sterile distilled water, 4 μl DNA template, 0.5 μl forward and 0.5 μl reverses primers of HSV-1, HSV-2, VZV (1.5 μl forward

primer and 1.5 μ l reverse primer), in final 50 μ l. The multiplex PCR schedule was: initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 58°C for 40 s, and 72°C for 1 min and 15 s, with a final extension at 72°C for 10 min.

Furthermore, 2 PCR protocols were utilized to detect EBV and CMV. 12.5 μ l master mix, 6.5 μ l sterile distilled water, 4 μ l DNA

templates, 1 μ l forward and 1 μ l reverse primers (Table 2) were mixed and incubated in an initial denaturation step at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 55°C for 60 s, and 72°C for 1 min and 15 s, with a final extension at 72°C for 10 min. (Figure 1.)

2.7 | Molecular cloning

In this step, the DNA sequences were amplified and the glycoprotein gB, glycoprotein gG, and glycoprotein gB were encoded to detect CMV, HSV-1,2, VZV respectively. Indeed, after subcloning these genes in *Escherichia coli* (strain BL21), we used them as positive controls.

2.8 | Statistical analysis

The results, obtained from the present study, were analyzed to find the correlation between the prevalence of VZV, HSV-1, EBV and patient's sex and age by IBM SPSS statistics software version 22, and Chi-square test. Moreover, ANOVA test was employed to confirm the relation between age and VZV, HSV-1, EBV infections. Experimental data were expressed by mean \pm standard deviation of three independent assays. p -value less than ($p < 0.05$) was used for the differences.

TABLE 2 The primers of multiplex PCR, PCR, and nested PCR

PCR-HSV1-F	GACTCTCCACCGCCATCAG
PCR- HSV1-R	TGTCTCGGGGACTGGTCT
PCR- HSV2-F	TATGCTATCCCCGGTTGGA
PCR- HSV2-R	CGTGCCATCCGAATAAACGTG
PCR-VZV-F	TTGTGTCGGTCTCTCCAAGC
PCR-VZV-R	TACGTCTCAACCTCACGCC
PCR-CMV-F	TGGCTTTCTTGAACGTGCG
PCR-CMV-R	CCTTGACGCTGGTTGGTTG
EBNA F	TGAATACCACCAAGAAGGTG
EBNA R	AGTTCCTTCGTGGTAGTC
GAPDH F	ATGTTCCATGGGTGTGAA
GAPDH R	GGTGCTAAGCAGTTGGTGGT
224-F (nested PCR, first step)	GCIATGYTIGGIACIAYRT
222 (nested PCR, first step)	CICIGGIGGIAYRWACAT
AN89 (nested PCR, second step)	CCAGCACTGACAGCAGYNGARAYNGG
AN88-F (nested PCR, second step)	TACTGGACCACCTGGNGNAYRWACAT

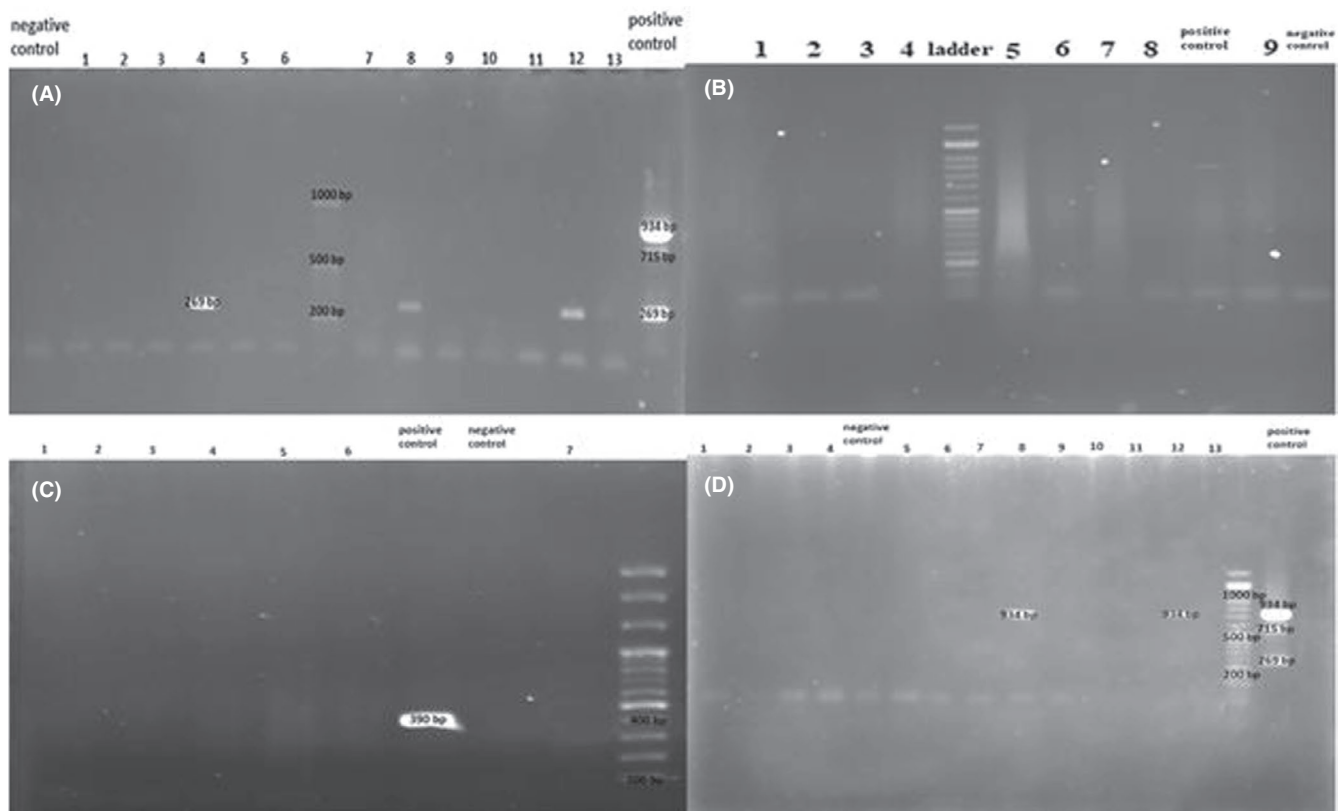


FIGURE 1 (A) The final PCR production for HSV-1 was 269 bp. Glycoprotein gG was considered as a positive control (B) The PCR production for CMV was 716 bp, and we used Glycoprotein gB as a positive control (C) EBV PCR production was 390 bp (D) VZV final PCR production was 934 bp, and Glycoprotein gB was selected as a positive control

3 | RESULTS

One hundred and two patients (53 male and 49 female) with symptoms, including fever, nausea, headache, rash, were selected to evaluate EV, CMV, EBV, HSV-1, HSV-2, and VZV prevalence with nested PCR, PCR and multiplex PCR. Clinical data of these patients, hospitalized in Emam Hossin, Loghman, and Mofid Hospitals, showed bacterial infections in almost 30% of the samples. Most patients were children under 18; one month and less ($n = 15$), 1–12 months ($n = 37$), 1–7 years ($n = 10$), 7–18 years ($n = 17$), and more than 18 years old ($n = 23$). More than 35% and 20% of cases had nausea or vomiting, and seizure, respectively. Headache was reported in 32.4% of patients; however, body temperature for 66.7% of patients was 38°C, and only 5.9% had rash and dizziness. The average WBC count from the CSF was 5520/ μ l (ranging from 0 to 21,200), and the average RBC count from the CSF was 2.1/ μ l (ranging from 0 to 85.0). Moreover, protein and glucose levels of CSF were 91 mg/dl (ranging from 5 to 878) and 47 mg/dl (ranging from 3 to 116), respectively. In most patients, serum showed high CRP levels (median 36 mg/dl; ranging from 1 to 149).

Our results indicated that 6, 5, and 2 samples (7 male and 6 female) were infected with HSV-1, EBV, and VZV, respectively. The mean age in these patients was 11 months and fever was seen in all of them. In patients, suffering from VZV, headache and nausea or vomiting were reported, and one of them had a rash. (Table 1).

4 | DISCUSSION

Central nervous system (CNS) diseases can be considered one of the leading causes of death all around the world. Statistics have shown that meningitis is responsible for 0.6% of all deaths, and survivors usually suffer from various types of disabilities.¹⁹ There are a number of tight relationships between different risk factors and central nervous system disorders, but the role of viral infections has drawn much attention.^{13,20–22} As symptoms of this disease are nonspecific, molecular analysis is essential to identify causative agents.^{23,24} In this study, the prevalence of *Herpesviridae* and *Picornaviridae* was investigated in CSF of patients, suffering from meningitis.

In this study, we detected 6, 5, and 2 samples infected with HSV-1, EBV, and VZV, respectively, but we did not find CMV and HSV-2 DNA. This study confirmed the results of previous researches, reporting that the prevalence of these viruses is dependent on multiple factors, especially the place of study and patient's age. For example, in Finland from 1999 to 2003, 26% and 8% of patients were infected with EV and VZV, respectively; however, 17% of total samples and 25% of females showed HSV-2 infections.²⁵ Another study in 2017 which was conducted in Turkey reported that the prevalence of HSV1, HSV2, CMV, EBV, VZV, HHV6, and EV was 1.80% (24/1333), 0.08% (1/1333), 3.28% (19/580), 4.35% (22/506), 0.46% (1/216), 1.05% (5/478),

and 3.37% (6/178), respectively.²⁶ HSV-1 DNA in 351 patients (70.8%), HSV-2 DNA in 83 samples (16.7%) and undefined HSV DNA type in 62 patients (12.5%) were identified by PCR assay.²⁷ Highlighting the importance of PCR assay, Smith and et al reported 2 patients with HSV-1 meningoencephalitis.²⁸ After analyzing 21 patients with acute bacterial meningitis in Sweden, none of the CSF samples showed the DNA of HSV-1, HSV-2, VZV, EBV, and HHV-6.²⁹ In patients, who did not have any blister or rash, *herpesviruses* (HHVs) were detected in 12 (6.9%) CSF samples (6.3% in tick-borne encephalitis cases, 7.8% in people suffering from enteroviral meningitis and 1 (0.9%) in control group). Furthermore, total protein in HHV DNA-positive samples was higher than HHV-negative ones.³⁰ Multiplex PCR showed EV, VZV and HSV-1 infections in 26, 4 and 2 cases out of 140 enrolled patients, respectively, but mumps and HSV-2 DNA were not detected.³¹ In Ukraine among 107 patients with some clinical features, including fever, stiff neck, and focal neurological signs, HSV-1 and 2 in (12.1%), VZV in (1.8%), CMV in (13%), EBV in (20.5%), HSV-6 in (4.7%), HSV-7 in (12.1%), and Co-infection (≥ 2 HVs) in 35.5% were detected.³² In another study, 21 HSV-1, and 74 HSV-2 positive samples were found (most patients with HSV-1 had encephalitis and a number of HSV-2 infected patients had meningitis).³³ In 2011, Paticheep S and et al found EV in 1.67% of their samples,³⁴ but Bastos MS and et al detected EVs in 16 out of 49 CSF specimens (16/49; 32.6%). In another study, the results of EV-PCR showed that one hundred thirty-seven patients (49.6%) were EV positive.³⁵

Along with the geographic distribution and the year of study, more factors can contribute to the progression of meningitis, including age, sex, the method of detection, and sample size. The limitation of this study was the low number of samples. Moreover, in this study, the majority of samples were taken from patients under 18 years of age. Method is another issue that should be considered carefully, and our methods were PCR, nested PCR and multiplex PCR.^{36–38} In the light of what was mentioned above, the HSV-1, EBV, and VZV prevalence is high in Iran and can play a pivotal role in the progression of meningitis, requiring immediate detection and treatment.

5 | LIMITATION

The limitation of this study was the low number of samples.

6 | ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present study is financially supported by "Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran" (IR.SBMU.RETECH.REC.1397.697, Grant No 15259).

7 | CONSENT FOR PUBLICATION

Not applicable.

ACKNOWLEDGEMENT

Not applicable.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTIONS

E.F., I.A and S.H.T designed the study and performed the molecular experiments. H.G performed the statistical analyses. All authors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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