

Detection of HSV-1, HSV-2 and VZV Isolated from Cerebrospinal Fluid Samples of Children Suspected to Encephalitis

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Abstract

Background: Encephalitis is an uncommon, serious brain infection that can cause fatality among children. The cause of most encephalitis remained unknown, yet viruses are the most well-known infectious agents that cause encephalitis. This study aimed to determine varicella-zoster virus (VZV) and herpes simplex virus type 1, 2 (HSV1/2) among individuals who are under 5 years of age in Iran.

Materials and Methods: In this study, 149 cerebrospinal fluid samples of suspected patients of encephalitis were analyzed with some symptoms, such as seizure, fever, nausea loss of consciousness, and dizziness from Mofid Children's Hospital in Tehran, Iran. Then, the molecular evaluation of samples was performed using multiplex Polymerase Chain Reaction (PCR) for detecting HSV1/2 and VZV.

Results: The mean age of the patients was 1.8 years. Also, 63.4% of children were male and 36.6% were female. Out of 149 tested samples, 11 (7.3%) showed the viral DNA for one of the herpes viruses (7.3%). Nine samples were HSV1 positive (6.0%) and two samples were VZV positive (1.3%). Fever and vomiting were the most frequent symptoms. The mean \pm standard deviation (SD) WBC and counts in cerebrospinal fluid (CSF)-positive samples, and all included samples were 298.8 ± 552.7 cells/ μ L and 131.1 ± 474.6 cells/ μ L, respectively.

Conclusions: Although viral encephalitis is considered a threat to children's health, with accurate diagnosis and appropriate antiviral drugs, death and neurological complications in children can be prevented.

Keywords: Cerebrospinal fluid, children, encephalitis, herpes simplex virus, multiplex PCR, varicella-zoster virus

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INTRODUCTION

Most of the time, encephalitis is characterized by fever, headache, lack of consciousness, seizures, and altered mental status.^[1] This condition can affect anyone but more often occurs in children than in adults, with 3.9 times more common in infants than in 20–44-year-old adults.^[2] According to a study of children with suspected encephalitis in the United States, 40% of these children needed hospitalization in the pediatric intensive care unit. It has been found that the mortality rate or severe outcome caused by encephalitis in

children under 1 year of age can be 5 times greater than in older children.^[3] Encephalitis can be caused by a mistake in the immune system and an attack on brain cells, or by the presence of infectious agents such as viruses, bacteria, and fungi.^[4,5] With the eradication of polio, rubella, measles, and mumps as the most important causes of childhood encephalitis, today herpes simplex viruses (HSV), followed by varicella-zoster virus (VZV), are the most common factors assumed to cause encephalitis in infants and children.^[6]

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Members of the family herpesviridae (includes eight human viruses) has a genome, which is surrounded by an envelope.^[7]

HSV-1 is more frequent in childhood Herpes simplex encephalitis (HSE). HSV-2 is mostly present in neonatal HSE because it is associated with infants born to mothers with genital herpes.^[7] Because appropriate administration of antivirals such as acyclovir or valacyclovir can significantly prevent mortality and morbidity, rapid and accurate diagnosis is crucial.^[8,9]

Blauw *et al.* performed a 10-year retrospective cohort study to investigate the characterization of encephalitis among children in the Netherlands. They found viral encephalitis in 33.1% and immune-mediated encephalitis in 10.7% of patients. HSV was the most frequent viral pathogen in viral encephalitis, and it mostly affected children under 5 years of age. They published a high mortality rate (about 20.8%), and higher mortality was seen in viral or bacterial encephalitis than in immune-mediated encephalitis.^[10] However, vaccination against VZV seems to play a vital role because VZV-induced encephalitis is less common in countries with VZV vaccination, and vice versa. In Eastern European countries where vaccination is less common, VZV is more common in children with encephalitis.^[11]

There are not many studies on Herpes virus-mediated encephalitis in Iran. However, Sawadkahi and Ahmadpour-Kacho in Northern Iran on 50 children with encephalitis HSV was illustrated by PCR in 34% of cerebrospinal fluid (CSF) samples. All these children were discharged from the hospital after acyclovir treatment.^[12] Therefore, due to the available antivirals for herpes viruses such as acyclovir and valacyclovir, using fast and reliable diagnostic techniques as well as timely administration of effective antivirals can significantly reduce the mortality and complications of encephalitis in children.

We assessed the laboratory findings in patients admitted due to encephalitis to Mofid Children's Hospital, Tehran, Iran. Then, all CSF samples were examined for HSV-1, 2, and VZV presence by multiplex PCR.

MATERIALS AND METHODS

Encephalitis is an inflammation of the brain parenchyma and can affect anyone but more often occurs in children than in adults. Thus, we monitored HSV-1, 2, and VZV prevalence the children ≤5 years in Iran. A total of 149 CSF specimens were collected from children 5 years of age with clinical suspicion of encephalitis by lumbar puncture with at least one symptom of ataxia, headache, nausea, lethargy, seizures, and fever in Mofid Children's Hospital, Tehran, Iran, between September 2019 and September 2020. Cell blood counts (CBC) and characteristics of CSF, including red blood cell count (RBC), white blood cell count (WBC), glucose, and protein concentration were obtained. For further molecular examination, samples were transferred to the laboratory. The present study has been approved and supported by the Ethics Committee of the

School of Medicine, Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1399.158, grant No. 22317).

Nucleic acid extraction

To evaluate the nucleic acid of CSF specimens, High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) was utilized. Accordingly, 20 µg of proteinase K was added to each CSF sample. Afterward, the solutions were incubated at 70°C for 10 min. Then, the viral DNA genome was extracted by adding kit solutions according to manufacturer instructions.

Then, spectrophotometry evaluated the qualification of DNA.

GAPDH gene PCR

We controlled the quality of extracted DNA by the *GAPDH* gene as an internal control of the PCR. The mixture of 12.5 µL master mix, 1 µL of forward and reverse primers (10 pmol), 2 µL of DNA, and 8.5 µL of sterile water in a final 25 µL reaction was prepared [Table 1].^[13] Then, it was incubated in the following PCR schedule: 30 cycles of denaturation at 95°C for 30 s, hybridization at 55°C for 30 s, and elongation at 72°C for 30 s and 72°C for 10 min in the final elongation step. All DNA-positive samples were selected for multiplex PCR.

Multiplex PCR

The multiplex PCR was utilized to evaluate the presence of HSV-1, 2, and VZV. In a total volume of 50µL containing 25 µL of a master mix, 18 µL of sterile distilled water, 4 µL of DNA template, and 1.5 µL of forward and reverse primers, which we used in our previous study are listed in Table 1.^[13] Sterile water was used as a negative control and plasmids containing positive viral genes was used as a positive one.

The multiplex PCR schedule was: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 40 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. The glycoprotein G gene for HSV-1 and HSV-2 and the glycoprotein B gene for detecting VZV were encoded,

Table 1: List of primers used for multiplex PCR

Assay	Sequence (5' → 3')	Gene	Product size (bp)
GAPDH	F: ATGTTTCGTCATG GGTGTGAA	GAPDH	110
	R: GGTGCTAAGCA GTTGGTGGT		
HSV-1	F: GACTCTCCCACC GCCATCAG	Glycoprotein G	269
	R: TGTCTTCGGGCG ACTGGT		
HSV-2	F: TATGCCTATCCCC GGTTGGA	Glycoprotein G	715
	R: CGTGCCATCCGA ATAAACGTG		
VZV	F: TTGTGTCCGGTCT CTCCAAGC	Glycoprotein B	934
	R: TACGTCTTCAAC CTCACGCC		

F=Forward primer, R=Reverse primer, bp=base pair

and subcloned into *Escherichia coli* (strain BL21). All PCR products were loaded onto a 1% agarose gel. As a negative control, a 25 μ L reaction without any DNA was run.

Statistical Package for the Social Sciences, version 21.0 (SPSS Inc., Chicago, IL, USA) was employed to analyze the statistics. The relationship between categorical variables was evaluated by the Pearson Chi-square test. A *P* value of < 0.05 was considered significant.

RESULTS

From 149 CSF samples of children with clinical suspicion of encephalitis 63.4% of them were male and 36.6% were female. In this study, we defined three age groups for children including patients <1 month (21.7%), 1–12 months (22.3%), and between 1 and 5 years (56%)(with a mean age of 1.8 years. Most patients were in the group between 1 and 5 years of age. There was no statistical difference between age and gender in positive samples compared to negative samples [Table 2].

Clinical data and laboratory procedures

Among the symptoms of encephalitis, fever, and vomiting were the most common symptoms in patients (22.1%) followed by seizures (7.3%). Dizziness, headache, and lack of consciousness were less reported.

Leukocytosis was defined with CSF WBC cells >30 cells/ μ L for patients aged 1 month to 1 year, and >20 cells/ μ L for patients aged >1 year, high CSF proteins if >45 mg/dL and high CSF glucose if >80 mg/dL.

In our study, the mean \pm SD of WBC and RBC counts in CSF samples were 131.1 ± 474.6 cells/ μ L (range 1–4480) and 2418.9 ± 7376.4 cells/ μ L (range 0–79000), respectively. In 40 samples (26.8%) leukocytosis was seen. The mean \pm SD for the content of protein in CSF was 214.8 ± 570 mg/dL (range 2–3900) and the mean \pm SD for the concentration of glucose was 54.3 ± 37.1 mg/dL (range 8–417). In 33 samples (22.1%), the content of glucose was reduced. Also, an increased amount of protein was observed in 57 samples (38.2%).

Viral detection by multiplex PCR

Among the 149 samples collected from children with suspected encephalitis, using multiplex PCR, it was found that 11 samples illustrated one of the herpes viruses (7.3%). Nine samples were HSV1 positive (6.0%) and two samples were VZV positive (1.3%). These samples were seven men (63.6%) and four females (36.3%) (mean age 1.25 ± 1.47 years). Most positive samples were in the age range of 1 to 5 years (54.5%), followed by the age range of 1–12 months (36.3%) and the age range of less than 1 month (9.09%). The most common symptoms seen in infected people were fever and vomiting (5/11, 45.5%). The mean \pm SD WBC counts in CSF of positive samples was 298.8 ± 552.7 . In seven of the positive samples (63.6%), the amount of WBC in CSF was higher than normal. Overall, the count of WBC was significantly higher in positive samples than in negatives [Table 2]. CSF

Table 2: Demographic data and clinical findings of patients in positive and negative viral samples

Variables	Positive samples <i>n</i> =11	Negative samples <i>n</i> =139	<i>P</i>
Age (mean \pm SD)	1.25 \pm 1.47	1.91 \pm 1.89	0.700
Gender (female/male)	4/7	50/88	0.938
Fever and vomiting	5 (45.5%)	26 (18.8%)	0.097
CSF analysis			
CSF WBC count (mean \pm SD)	298.8 \pm 552.7	115.8 \pm 465.2	0.014
CSF RBC count (mean \pm SD)	8581.9 \pm 22382.4	1892.6 \pm 3938.6	0.165
CSF protein (mg/dL)	213.3 \pm 351.5	213.5 \pm 581.6	0.116
CSF glucose (mg/dL)	42.2 \pm 19.6	55.4 \pm 37.8	0.258
Hematology analysis			
WBC counts (mean \pm SD)	10.25 \pm 5.7	10.7 \pm 6.1	0.171
RBC counts (mean \pm SD)	3.9 \pm 0.4	3.8 \pm 0.6	0.017
Hemoglobin (g/dL)	10.8 \pm 2.2	10.9 \pm 2.0	0.008
HCT (%)	32.9 \pm 5.4	35.7 \pm 31.0	0.004
MCV (fL)	84.0 \pm 8.2	85.6 \pm 8.1	0.267
MCH (pg/cell)	27.4 \pm 3.6	28.3 \pm 3.4	0.300
MCHC (%)	31.2 \pm 2.0	31.7 \pm 2.4	0.019
Platelet (10^3 /mm ³)	339.2 \pm 175.5	365.4 \pm 180.1	0.108
Neutrophil (%)	49.0 \pm 23.3	54.7 \pm 20.4	0.134
Lymphocyte (%)	44.3 \pm 22.3	37.3 \pm 19.3	0.012
BUN (mg/dL)	10.7 \pm 8.9	10.9 \pm 8.7	0.462
Creatinine (mg/dL)	0.60 \pm 0.20	0.60 \pm 0.1	0.067
Na (meq/L)	136.8 \pm 2.1	135.7 \pm 11.1	0.518
K (mmol/L)	4.0 \pm 0.4	4.2 \pm 0.7	0.810

Data are presented as *n*/*N* (%), median or mean (\pm SD). Significant differences in bold. CSF=cerebrospinal fluid, RBC=red blood cell, WBC=white blood cell, HCT=hematocrit, MCV=mean corpuscular volume, MCH=mean corpuscular hemoglobin, MCHC=Mean corpuscular hemoglobin concentration, BUN=blood urea nitrogen, Na+= sodium, K=potassium

glucose level decreased in 54.5% (6/11) of the samples and CSF protein content increased in 63.6% (7/11) of the positive samples [Table 2]. According to the hematology analysis of patients, which is shown in Table 2, significant statistical differences were seen in RBC counts, hemoglobin content, Hematocrit Test (Hct), mean corpuscular hemoglobin concentration (MCHC), and lymphocytes in positive viral samples compared to negative samples.

DISCUSSION

Encephalitis is an uncommon but important cause of neurological complications and childhood mortality that can be caused by numerous factors, including neurotropic viruses but with the advent of emerging viruses in the list of causes of viral encephalitis, the diagnosis can be challenged such as outbreaks of Japanese encephalitis virus (JEV) in northern India and even SARS-CoV-2, which has recently been reported by Cheraghali *et al.* as a causative agent of encephalitis.^[14-17] However, nowadays, with the formation of anti-vaccine groups

of people in different countries, we are facing disruptions in the vaccination of children, such as measles, which can have serious consequences, including increasing the incidence of encephalitis in this group.^[18] However, currently, according to studies around the world and also in Iran, the most important causes of viral encephalitis are HSV.^[19,20] Similarly, the most common infectious agent found in this study was HSV-1 (6%) followed by VZV (1.3%). HSV-1 causes encephalitis in children and adults, and HSV-2 is the most frequent cause of encephalitis in infants who were born to mothers with HSV-2 genitalia.^[21] Therefore, it is probable that because most of the children we studied were between 1 and 5 years (56%), we could not find a positive sample of HSV-2. However, according to the study of Arabsalmani and her colleagues, it is related to the low prevalence of HSV-2 in pregnant women in Iran (0.26%).^[22]

In this study, there was a predominance of males (63.6%), which was consistent with most studies that can be related to the underlying genetic makeup of males. Neonatal HSE is often associated with fever, seizures, temperature instability, vesicles, sepsis-like illness, and lethargy and in older children with fever, lethargy, seizures, and confusion, likewise, our study reported fever was the frequent symptom.^[23]

Most people with HSE eventually have abnormal CSF. Viral encephalitis during CSF analysis is often defined by an increase in the amount of WBCs, elevated content of protein, and a normal or decreased amount of glucose.^[23] In our study, WBCs surged in 63.6% of positive samples, and an elevation in protein and a decrease in glucose were observed in 63.6% and 54.5% of infected samples, respectively.

One of the patients did not show any viral infection in the first sampling; however, in the second CSF sample, which was taken after 12 days, the presence of HSV-1 was confirmed. This may be related to early sampling or laboratory errors in the initial sampling. Also, in the analysis of the second CSF sample, the amount of WBC had increased significantly and the glucose content had also decreased.

In another patient, the initial PCR test showed the presence of HSV-1; however, in his second sample, taken 5 days later, no HSV-1 was found and the VZV was positive instead. During this time, he was hospitalized in the infectious ward of the hospital. It is possible that the presence of the VZV virus could be related to nosocomial infection and because children are not vaccinated against chickenpox in Iran, the VZV can easily infect children hospitalized as a highly contagious agent. In this regard, the seroprevalence of the VZV among nurses in a hospital in China was analyzed. The overall seroprevalence of VZV was 88.4%, which increased considerably with age.^[24] Similarly, in another study conducted in 2018 in northern Iran on nursing students, the seroprevalence was 72.3%, and 26.7% of them were seronegative and susceptible to VZV infection. Therefore, hospital health workers who are seronegative can easily transmit VZV to hospitalized patients.^[25] This issue makes the need for VZV vaccination of children in Iran.

After human papillomavirus, HSV is the most common sexually transmitted disease (STD) worldwide and the majority of the Iranian population is young people in the age's sexual activity and pregnancy, the identification and control of HSV infection in pregnant women can be a crucial factor in preventing the incidence of HSE in infants. However, it shows the importance of developing an effective HSV vaccine for young people.

According to a cross-sectional study conducted in Iran on 50 children with suspected HSE, although 34% of children tested positive for PCR, all were treated with acyclovir and discharged from the hospital.^[12] Therefore, early detection and timely administration of antivirals can significantly prevent HSE mortality and morbidity in children. Because reports of herpes virus resistance to certain antivirals, such as acyclovir, have prompted researchers to research new drugs, such as helicase-primase inhibitors (HPIs), that have shown high potential for inhibiting viral replication.^[26-28]

CONCLUSION

Because various studies around the world have identified other viral agents such as Epstein–Barr virus (EBV), Cytomegalovirus (CMV), and arboviruses such as West Nile virus and dengue virus as causes of viral encephalitis, it is necessary to conduct more comprehensive studies with larger sample sizes to determine the cause of viral encephalitis, finding the proper antiviral and the convenient diagnostic technique.^[29]

Acknowledgments

The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran, under the Ethics code of IR.SBMU.MSP.REC.1399.158, and Grant Number 22317). All subjects gave their written informed consent.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran, under the Ethics code of IR.SBMU.MSP.REC.1399.158, and Grant Number 22317). All subjects gave their written informed consent.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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