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ORIGINAL ARTICLE

Prevalence of *herpes simplex viruses* types 1 and 2 infections among suspected children of encephalitis in Kermanshah, Iran

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ABSTRACT

Background and Objectives: Various infectious and non-infectious factors can cause encephalitis in the central nervous system (CNS), the most important of which are viruses. *Herpes viruses* are one of the most important causes of encephalitis worldwide. PCR detected the virus on the cerebrospinal fluid (CSF) sample. The aim of this study was to set up an in-house PCR to identify *herpes simplex virus* type 1 (*HSV-1*) and *herpes simplex virus* type 2 (*HSV-2*) and determine the prevalence of these viruses in suspected children of encephalitis.

Materials and Methods: This cross-sectional study was conducted on 160 suspected children with encephalitis cases referred to Dr. Kermanshahi Children Hospital, Kermanshah, Iran, from April to March 2021. CSF samples were extracted using a viral extraction kit, and a PCR was performed. The level of glucose and total protein of the samples was measured.

Results: The total prevalence of *HSV* was 16.25%. 17 samples were positive for *HSV-1* (10.6%), and 9 samples for *HSV-2* (5.6%). There was a significant correlation between glucose, total protein, and *HSV* PCR positive, but no significant correlation between age and *HSV* PCR positive results.

Conclusion: Rapid diagnosis of a virus may reduce the hospitalization rate and the use of unnecessary therapies and crease mortality, morbidity, and disability in children. Results in this study show that the distribution of *HSV* types in children with encephalitis predominantly was type 1 compared with type 2.

Keywords: Encephalitis; *Herpes simplex virus*; Cerebrospinal fluid

INTRODUCTION

CNS can be affected by several diseases and disorders due to various factors. One of the major diseases of the CNS is encephalitis. These diseases can be caused by multiple factors, including trauma, noninfectious cause, and infectious diseases such as viruses, bacteria, and fungi (1). Encephalitis is acute inflammation in the brain tissue, and the most common type is viral encephalitis (2, 3). The virus can enter the host's nervous system through the blood or other routes, such as the motor nerves, and disrupt its function. The common signs of the disease are unbalanced walking, coma, and death. Diagnosis of the disease is generally based on signs, symptoms, and laboratory diagnosis by CSF analysis (4). The main viruses causing viral encephalitis are *enteroviruses* and *herpes viruses* (5).

The *herpesviridae* includes important human pathogen viruses that have DNA genomes. The most important viruses of this family causing CNS disorders are *HSV-1* and *HSV-2* (6). After entering the virus into the host's body, these viruses can enter the host's nervous system and become latent in the senso-

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ry nerve nodes (7, 8). They have a worldwide distribution and can be transmitted through various ways such as close contact, respiratory secretions, sex, etc. Humans are their only host, infecting many people worldwide annually (9). Latent viruses are activated under certain conditions such as stress, weak immune system, fever, and environmental factors and cause active disease in the host. The most important disease caused by this virus is encephalitis (10). Encephalitis or acute inflammation in the brain may occur after infection by *HSV* or its reactivation. Lesions of this disease are generally restricted to the temporal lobe, and lymphocytosis in CSF usually leads to focal neurological seizures (11, 12). It is prevalent in all age groups and all seasons of the year (13).

Early diagnosis of encephalitis caused by the *herpes virus* is very important because the progress of the disease is fast. To diagnose this disease in CNS, a CSF sample is usually collected from the patient and transported to the laboratory to measure the amount of glucose and total protein. The clinician diagnoses the disease by considering both clinical and laboratory data. At this point, developing a test with high specificity is essential. One of the most reliable and commonly used laboratory methods is PCR assay, which is widely used in biological sciences and can determine the cause of the disease, which can be very helpful in the beginning and process of the patient's treatment (14). The aim of this study was to set up an in-house PCR to identify *HSV-1* and *HSV-2* and determine the prevalence of these viruses in suspected children of encephalitis.

MATERIALS AND METHODS

Study population. This cross-sectional study was conducted on 160 suspected children with encephalitis cases referred to Dr. Kermanshahi Children Hospital in Kermanshah city from April to March 2021. This study was reviewed by the Ethics Committee and approved by the ethics code number of IR.MODARES.REC.1398.120 and the informed consent form were filled out by the patient's families. Inclusion criteria for the study included suspicion of encephalitis in patients' signs, the negative bacterial culture of samples, and the non-using of antiviral drugs before sample collection. Exclusion criteria for the study included the age of patients being more than 15 years and delay in sending the sample to the laboratory.

Sample collection. For the investigation of DNA of *HSV,* CSF samples were collected from patients in sterile tubes. Fresh samples were extracted on the same day and stored in a freezer at -70°C.

Laboratory evaluation of patient samples. The glucose and total protein samples were measured, cultured in a bacterial culture medium, and prepared for virus detection by PCR.

Primer design for *HSV-1* and *HSV-2*. Reference sequences of *HSV-1* (NC_001806.2) and *HSV-2* (NC_001798.2) were downloaded from the NCBI website. After analyzing the genome of the viruses and selecting the conserved regions of the viral gene, primers were designed using primer design of Oligo 7 software, and the sequences of the primers were checked on the NCBI website for specificity in humans and bacteria (Table 1).

Viral DNA extraction from patient samples. Since the CSF sample is unstable and poor in terms of nutrients compared to other samples, such as serum and plasma, samples were extracted daily. Genome extraction was done by INVITEK Universal DNA/RNA purification kit (Lot:5F7EBEBF/Exp:2023.03-Germany/IVD). The extracted genomes were transferred to a freezer at -70°C. Beta-globin internal control primers were used to ensure the quality of the extracted DNA and check the presence of inhibitors (Table 1).

Polymerase chain reaction (PCR) and electrophoresis. The PCR reaction was optimized using a Tm gradient and selecting a 60°C for primer annealing Tm as an optimal temperature. To calculate the limit of detection (LOD) primers, after extraction of *HSV-1* and *HSV-2* DNA from the confirmed samples by cell culture, DNA concentration was determined using a nanodrop, and the number of copies of DNA template was calculated by the following formula:

$$\text{Number of copies of DNA template per µl} = \frac{\text{DNA concentration (ng/µl)} \times \text{Avogadro`s number}}{\text{Length of template (bp)} \times \text{Conversion factor to ng} \times \text{Average weight of a base pair (Da)}}$$

Then, using distilled water, a serial dilution of DNA was prepared from 10^1 to 10^7 and used as a template for PCR to determine the sensitivity of the study

Table 1. Details of designed primers for *HSV-1*, *HSV-2* and beta-globin

Virus name	Primers	Region	SEQUENCE (5'-3')	Size (bp)
HSV-1	Forward	UL19	CCGTTGTTCCCATTATCCCATTCC	225
	Reverse		CTTCCTCCTCCTCCAGTCCGATAC	
HSV-2	Forward	UL18	CGTCGGAAAATCAAACCCAATGC	345
	Reverse		CTGGAGCGTCTTATCGTGGAAAC	
Beta-globin	Forward	-	GAAGAGCCAAGGACAGGTAC	268
	Reverse		CAACTTCATCCACGTTCACC	

primers. The last dilution with a visible band on an agarose gel was considered the LOD of primers. Finally, the LOD of primes was determined to be 10^6.

For beta-globin gene amplification, PCR reaction components including 12.5 microliters of master mix (Amplicon, Denmark), 7.5 microliters of deionized distilled water, 1 microliter of each of forward and reverse primers (10 pmol), and 3 microliters of purified DNA, in a final volume of 25 microliters were prepared and placed in a thermocycler. PCR temperature program with 35 thermal cycles, including initial annealing at 95°C for 3 minutes, annealing at 94°C for 30 seconds, primers binding at 56°C for 30 seconds, and amplification was applied at 72°C for 1 minute, and final amplification at 72°C for 5 minutes.

To perform the PCR test of *HSV-1* and *HSV-2* on samples, microtubes in a final volume of 25 microliters, including 12.5 microliters of master mix (Amplicon, Denmark), 6.5 microliters of deionized distilled water, 0.5 microliters of each of the forward and reverse primers (10 pmol), and 5 microliters of purified DNA were prepared and placed in a thermocycler. The PCR temperature program was carried out by the conditions mentioned above, in 40 thermal cycles and the primers annealing Tm at 60°C.

The PCR electrophoresis was carried out with a 2% agarose gel using a 1× TAE buffer. The 5 microliter of samples and 2 microliters of 100 bp ladder were loaded on an agarose gel. Then, electrophoresis was performed for 40 minutes. The results were analyzed based on the size of the bands compared to the ladder.

RESULTS

The optimization of extraction on *HSV1*-positive and *HSV2*-positive controls was done and demonstrated in Fig. 1 and the results of PCR by beta-globin primers on two extracted CSF samples are shown in Fig. 2, respectively. The statistical analysis was performed using SPSS, version 21. Pearson chi-square test was used to evaluate the relationship between variables such as age, glucose, and protein. Of 160 samples; 26 were positive for *HSV* (17 for *HSV-1* and 9 for *HSV-2*). The total prevalence of *HSV* was 16.25% (10.6% in *HSV-1* and 5.6% in *HSV-2*). The patient in this study was 73 boys (45.62%) and 87 girls (54.37%).

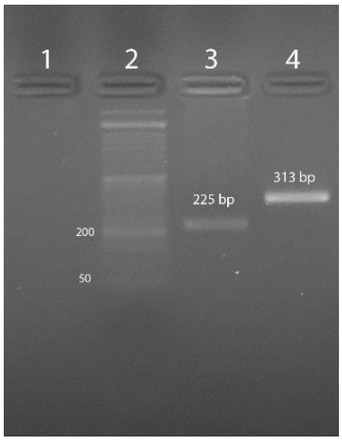

Fig. 1. Result of PCR by *HSV-1* and *HSV-2* primers.
1: Negative control. 2: 50 bp ladder. 3: *HSV-1*positive control. 4: *HSV-2* positive control.

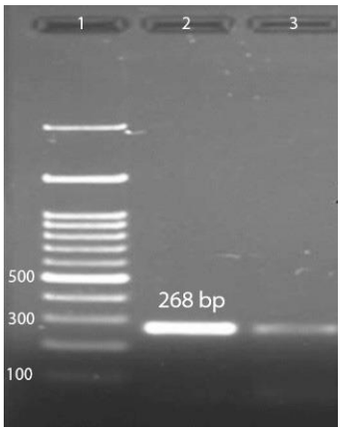

Fig. 2. Results of PCR by beta-globin primers on two extracted CSF samples. 1:100 bp ladder. 2: beta-globin. 3: beta-globin

All patients were aged between <1 and 14 years (Table 2). There was a significant correlation between glucose (P Value=0.003), total protein (P Value=0.000), and *HSV*-positive samples, but there was no significant correlation between age (P Value=0.958) and *HSV*-positive samples. All samples were negative for bacterial culture (Table 3).

Table 2. PCR test results of 160 samples based on age

Age groups (Years)	<1	1-5	5-10	>10
HSV-1&2 Negative	67	42	22	3
HSV-1 Positive	5	9	3	-
HSV-2 Positive	2	3	3	1
Total	74	54	28	4

DISCUSSION

HSV can cause various human diseases and affect different body parts, such as the skin, mouth, genital tract, eyes, etc. One of the most important diseases caused by this virus is encephalitis, referred to as herpes simplex encephalitis (HSE). The progression of the disease is rapid. If HSE is untreated, it causes about 70% mortality (12). HSE has an annual incidence rate of about 1 case per 250,000 to 500,000 individuals (15), which is caused respectfully by *HSV-1* and *HSV-2* (16). Rates of virus encephalitis, generally higher among children than adults, range from 3 to 30 or more per 100,000 persons of all ages annually, depending upon the location of the epidemiological study (17). Since patient recovery from encephalitis

Table 3. Demographics and laboratory evaluation of *HSV*-positive samples

Gender	Age (years) (P Value=0.958)	Glucose* (mg/dl) (P Value=0.003)	Total protein# (mg/dl) (P Value=0.000)	WBC (Cell/µl)	PCR results	Bacterial culture
F	1	60	45	0	HSV-1	Negative
F	3	58	40	200	HSV-1	Negative
M	1	57	40	170	HSV-1	Negative
F	6	46	28	30	HSV-1	Negative
M	1	43	32	35	HSV-1	Negative
F	1	48	10	70	HSV-1	Negative
M	7	49	35	400	HSV-1	Negative
M	2	66	53	1200	HSV-1	Negative
M	8	50	39	200	HSV-1	Negative
F	3	48	42	85	HSV-1	Negative
M	4	45	29	50	HSV-1	Negative
M	<1	50	38	10	HSV-1	Negative
F	<1	54	33	10	HSV-1	Negative
M	<1	53	40	140	HSV-1	Negative
F	<1	40	26	0	HSV-1	Negative
M	<1	73	50	0	HSV-1	Negative
M	1	57	24	10	HSV-1	Negative
M	2	49	32	150	HSV-2	Negative
M	2	48	29	100	HSV-2	Negative
F	7	43	21	90	HSV-2	Negative
F	7	54	37	68	HSV-2	Negative
F	<1	46	38	0	HSV-2	Negative
F	4	53	30	150	HSV-2	Negative
F	3	60	38	100	HSV-2	Negative
M	7	59	47	900	HSV-2	Negative
M	<1	42	41	25	HSV-2	Negative

* Reference value: 40-60 mg/dl - # Reference value: 15-45 mg/dl

can be long, slow, and difficult, many individuals will never fully recover; the on-time diagnosis and treatment of encephalitis is essential. In HSE, the PCR method is a gold standard diagnostic test for the rapid detection of *HSV* DNA genome in CSF samples of patients. Sample analysis should be done as soon as possible, increasing the chance of virus identification. An important issue that should be paid attention to is taking the sample in a sterile tube and performing genomic extraction as soon as possible (18).

Cross-sectional studies from various regions of the world have shown that *HSV* is the main viral agent of encephalitis (19). Studies have shown that the prevalence in patients with suspected HSE in Syria, and Egypt is 30% (20), and 1%, respectively (21). In the current study, for detecting *HSV* in the CSF samples from 160 HSE-suspected patients, a PCR test was performed by a designed primer for *HSVs*, and 26 patients (16.25%) were positive for the virus genome. This result may be due to the sensitivity and specificity of the designed primer sets or the difference in the sensitivity of the kits and extraction methods. A study in Hamedan reported the prevalence of HSE as 15% (22). In another study in Shiraz, the prevalence of HSE was reported 11.11% (23). In a study in northern Iran, 33% of patients were positive for *HSV* (19). In different studies in various regions, and clinical reference textbooks, the prevalence of *HSV-1* in CNS disease is usually higher than *HSV-2*. In a study conducted in the East of the Caspian Sea, Iran, out of 45 CSF samples of children suspected of having meningitis, 4 cases were positive, all of which were *HSV-1*, the predominant type of this virus in that area (24). In another study, On CSF samples of suspected meningitis patients, the PCR test for *HSV-1* was positive along with VZV and EBV, and *HSV-1* was considered the main cause of this disease (25). The results of this study are consistent with the results of other mentioned studies. As said before, the high or low rate of *HSV* infection in some research may be due to the quality of sampling and laboratory diagnosis methods. The current cross-sectional study is a new report of herpes simplex encephalitis in children in western Iran, which can provide scientific information on the prevalence of HSE.

CONCLUSION

Identification of *HSV-1* and *HSV-2* was performed by detecting the virus in the CSF sample with a PCR test. Screening and timely diagnosis of suspected HSE patients are very important, for treating patients should be done on time to decrease mortality, morbidity, and disability in children. On the other hand, rapid diagnosis of the virus, may reduce the hospitalization rate and the use of unnecessary therapies. Results in this study show that the distribution of *HSV* types in children with encephalitis predominantly was type 1 compared with type 2. For future studies, we need to set up multiplex PCR (M-PCR) tests for simultaneous detection of *HSV-1* and *HSV-2* due to the obvious advantage of M-PCR, including higher throughput, cost-effectiveness, and time saving, for rapid management of the disease.

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