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Original Article

Challenges in Interpreting Cerebrospinal Fluid Viral Polymerase Chain Reaction Results: Understanding the Results Related to HHV-6, HHV-7, and Enterovirus

Beyin Omurilik Sıvısında Viral Polimeraz Zincir Reaksiyonu Sonuçlarının Yorumlanmasındaki Zorluklar: HHV-6, HHV-7 ve Enterovirüs ile İlgili Sonuçların Anlaşılması

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ABSTRACT

Objective: We have aimed to evaluate our experience in interpreting polymerase chain reaction (PCR) test results of cerebrospinal fluid (CSF) samples for human herpesvirus (HHV)-6, HHV-7, and enterovirus in children with suspected viral meningoencephalitis.

Method: Children aged 1 month to 5 years underwent PCR analyses. Samples were collected via lumbar puncture and assessed using real-time PCR for the identification of enterovirus, HHV-6, and HHV-7.

Results: Most (79.8%) of 109 CSF samples analyzed did not show the presence of any viral particles. Among the positive samples, 8.3% were positive only for HHV-6, 6.4% for HHV-7, and 1.9% for enterovirus. Two samples showed positivity for both HHV-6 and HHV-7; one sample for HHV-7 and enterovirus; and another sample for HHV-6, HHV-7, and enterovirus. Among the PCR-positive patients, fever (77%) and seizures (59%) were the most prevalent presenting symptoms. A statistically significantly higher incidence of seizures was observed in patients with HHV-7 positivity compared to those in whom no virus was detected (p=0.003). At discharge, three patients received alternative diagnoses.

Conclusion: The most frequently detected virus was HHV-6, followed by HHV-7. Enterovirus was detected at a lower frequency than expected, most probably due to the rapid clearance of enterovirus from the CSF and coronavirus disease 2019 mitigation. Considering the possible latency or chromosomal integration (for HHV-6), clinical presentations, CSF findings, and patient-specific additional diagnostic work-up were influential on the decision-making process for diagnosis. In the absence of advanced molecular techniques, it is crucial to recognize that HHV-6 and HHV-7 may be bystanders, and other potential pathogens and diagnoses should be considered.

Keywords: HHV-6, HHV-7, enterovirus, meningoencephalitis

ÖZ

Amaç: Viral meningoensefalit şüphesi olan çocuklarda insan herpes virüsü (HHV)-6, HHV-7 ve enterovirüs açısından beyin omurilik sıvısı polimeraz zincir reaksiyonu (PCR) sonuçlarını yorumlama deneyimimizi değerlendirmeyi amaçladık.

Yöntem: Bir ay ile beş yaş arasındaki çocuklar analiz edildi. Numuneler lomber ponksiyon yoluyla elde edildi ve enterovirüs, HHV-6 ve HHV-7 tespiti için gerçek zamanlı PCR kullanılarak değerlendirildi.

Bulgular: Analiz edilen 109 beyin omurilik sıvısı numunesinin %79,8'inde herhangi bir viral partikül bulunmadı. Pozitif numuneler arasında %8,3'ü sadece HHV-6, %6,4'ü HHV-7 ve %1,9'u enterovirüs pozitifti. İki numunede hem HHV-6 hem HHV-7, bir numunede HHV-7 ve enterovirüs, bir numunede ise HHV-6, HHV-7 ve enterovirüs pozitifliği

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saptandı. PCR pozitif hastalar arasında en sık görülen semptomlar ateş (%77) ve nöbet (%59) idi. HHV-7 pozitif olan hastalarda, hiçbir virüs saptanmayanlara göre istatistiksel olarak daha yüksek nöbet insidansı gözlendi (p=0,003). Üç hastaya meningoensefalit dışında tanılar konuldu.

Sonuç: En sık tespit edilen virüs HHV-6, ardından HHV-7 oldu. Enterovirüs, muhtemelen beyin omurilik sıvısından hızlı bir şekilde temizlenmesi ve koronavirüs hastalığı 2019 önlemleri nedeniyle beklenenden daha düşük sıklıkta tespit edildi. HHV-6 için olası latens veya kromozomal entegrasyon göz önünde bulundurulduğunda, klinik bulgular, beyin omurilik sıvısı bulguları ve hastaya özgü ek tanısal incelemeler tanı sürecinde etkili olmuştur. İleri moleküler tekniklerin bulunmadığı durumlarda, HHV-6 ve HHV-7'nin yalnızca "bystander" virüsler olabileceği ve diğer potansiyel patojenler ve tanıların göz önünde bulundurulması gerektiği unutulmamalıdır.

Anahtar kelimeler: HHV-6, HHV-7, enterovirüs, meningoensefalit

INTRODUCTION

In recent years, the landscape of central nervous system (CNS) infections in children has undergone a significant shift, primarily attributed to the widespread administration of conjugated vaccines. As a result, viruses have emerged as the leading cause of CNS infections in this population[1]. Among viral pathogens, enteroviruses have been identified as responsible for over 75% of the cases with viral meningitis caused by a specific pathogen[2,3]. However, other T-lymphotropic viruses, such as human herpesvirus (HHV)-6 and HHV-7, which exhibit high seroprevalence rates in childhood, are believed to contribute to CNS infections less frequently. Seroprevalence studies have revealed that HHV-6 exhibits a seroprevalence rate of 80% in children older than 2 years, while HHV-7 antibody prevalence rate reaches 75% in children aged between 3 and 6 years[4-7]. Infections caused by enteroviruses, HHV-6, and HHV-7 can present with a range of clinical signs, including fever, rash, and seizures[8-10]. When these viruses infect the CNS, the clinical manifestations can vary widely, spanning from asymptomatic infections to severe cases of encephalitis[10,11]. The analysis of cerebrospinal fluid (CSF) samples using polymerase chain reaction (PCR) tests has indeed facilitated the rapid diagnosis of viral nucleic acids, including HHV-6. However, interpreting the results of these tests to determine whether the infection is acute, latent, or due to chromosomal integration can be challenging[12,13]. The bystander effect refers to the presence of HHV-6 and/or HHV-7 in CSF that does not directly contribute to the emergence of infection. Clinically, the bystander effect complicates diagnosis and treatment, as it becomes challenging to determine whether the detected pathogen is causing an active infection or is merely a result of latency or chromosomal integration. Misinterpreted results may lead to incorrect diagnoses. Therefore, clinical symptoms, laboratory findings, and the patient's immune status must be carefully evaluated to ensure accurate interpretation of test results. Studies have suggested that up to 80% of HHV-6 DNA isolated on multiplex panels of CSF may be clinically irrelevant.[14-16] Similarly, HHV-7 has been considered to cause CNS infections in immunocompromised patients or co-infections with other viruses. However, CNS infections due to HHV-7 in immunocompetent children is considered as rarely seen entities.[17,18] As HHV-7 establishes lifelong latency; it is often difficult to interpret the clinical relevance of HHV-7 detection in the CNS.[17] As a result, the actual frequency of HHV-6 and HHV-7-related diseases remains uncertain.

The use of molecular tests has facilitated the rapid diagnosis of viral meningitis. Nevertheless, challenges have arisen in interpreting molecular test results about both prevalent and rarely seen viral CNS pathogens. In this study, we aimed to review our experience with in-house PCR to detect HHV-6, HHV-7, and enteroviruses in children younger than 5 years old with suspected viral meningoencephalitis.

MATERIALS and METHODS

Study Design and Population

This prospective study was conducted at a tertiary care children's hospital, a referral center for pediatric patients, between December 28, 2019, and March 31, 2022.

A total of 110 children aged 1 month to 5 years with suspected CNS infection were included in the study. The diagnostic inclusion criteria for viral meningoencephalitis were: nausea, vomiting, neck stiffness or bulging fontanelle, seizures, irritability, and altered mental status, which are commonly observed in the clinical course of such infections.[19] The patients with bacterial growth in their conventional CSF cultures were excluded from the study.

CSF samples obtained through lumbar puncture were analyzed using real-time PCR test to detect enteroviruses, HHV-6, and HHV-7. The following variables were recorded: age, gender, presenting symptoms, CSF white blood cell counts, protein, and glucose levels, PCR test results, and discharge diagnosis of the patients. CSF samples with >5 white blood cell (WBC)/μL were

considered pleocytic. Protein values under 45 mg/dL and glucose above 50 mg/dL were considered normal[19].

Detection of the Viral Nucleic Acid

Viral nucleic acid extraction from 200 µL of each CSF sample was performed using the spin column extraction method, resulting in an average yield of approximately 4-6 µg nucleic acid per sample.

The detection of enterovirus, HHV-6, and HHV-7 was performed using kits (Procomcure Biotech PhoenixDX®, Thalgua, Austria) containing primers targeting the DNA and RNA sequences of these viruses. Real-time PCR tests were performed, and virus detection method was verified using signals from the Fluorescein amidite (FAM) channels to detect HHV-6 and HHV-7 DNA, while Cy5, and Rhodamine X (ROX) probes were used to detect the PCR positive control, and passive reference (if required) for HHV-6 and HHV-7, respectively. The three-step process included 1 cycle at 94 °C for 5 minutes, followed by 45 cycles at 94 °C for 15 seconds and at 55 °C for 70 seconds. A comparison of the signals obtained with positive controls allowed for the identification of PCR-positive and negative samples. The analytical sensitivity of the kit is 95%, and 40 copies can be detected at each reaction.

Enterovirus RNA was detected with the signals from the FAM channel (for enterovirus enterovirus RNA), from the Hexachlorofluorescein VIC channel (for human extraction control), and from the ROX channel (for passive reference, if requested). The four-step process included 2 cycles at 50 °C for 5 and at 95 °C for 5 minutes, followed by 45 cycles at 95 °C for 15 and at 62 °C for 1 minute.

Statistical Analysis

Statistical analysis for this study was performed with IBM SPSS Statistics 22 (Chicago, IL, USA). Continuous variables are presented as medians, and ranges varying between minimum and maximum values. Categorical variables are presented as frequencies and percentages, and compared using Pearson's χ^2 and Fisher's exact tests (Table 1). A p-value of <0.05 was considered to be statistically significant.

Ethics approval for the study was obtained from the Institutional Review Board of University of Health Sciences Turkey, Dr. Behçet Uz Children's Hospital (approval number: 2019/364, dated: 19.12.2019).

Informed consent for this study was obtained from the parents of the patients.

RESULTS

A total of 110 CSF samples were obtained from 110 patients. Unfortunately one of these samples that did not contain sufficient volume of CSF sample for testing was excluded from the statistical analysis. As a result, a total of 109 patients were included in the study, with a median age of 8.5 months (range: 1-60 months). Study population consisted of 44 (40%) female, and 66 male (60%) patients. With the exception of Patient 7 with DiGeorge syndrome, none of the patients had a previously known immunodeficiency.

CSF samples of 109 patients were analyzed, and 79.8% (n=87) did not show the presence of any viral particles. CSF samples were reverse transcription-PCR positive only for HHV-7 in 9 (8.3%) for HHV-6 in 9 (8.3%), for HHV-7 in 7 (6.4%), enterovirus in 2 (1.9%), for both HHV-6 and HHV-7 in 2, HHV-7 and enterovirus in 1, and HHV-6, HHV-7, and enterovirus in 1 patient. No bacterial growth was observed in the conventional CSF cultures.

Table 1 illustrates the demographic data, clinical features, laboratory findings, and discharge diagnoses of 22 patients with at least one positive viral particle in their CSF samples. Among the PCR-positive patients, fever (77%, 17/22) and seizures (59%, 13/22) were the most prevalent presenting symptoms. Notably, 27% (6/22) of patients exhibited significantly higher WBC counts in their CSF samples indicative of meningoencephalitis, with the exception of 2 patients with bloody CSF samples. Furthermore, 41% (9/22) of patients demonstrated elevated protein levels, and 14% (3/22) displayed decreased glucose levels. At discharge, three patients received alternative diagnoses. No antiviral treatment was used in any of the patients for the treatment of HHV-6, HHV-7, and enterovirus detected in the CSF.

Our study could not reveal any statistically significant difference in terms of the incidence rates of altered mental status, fever, and seizure between patients with and without at least one positive HHV-6 or HHV-7 identified in their CSF samples. Similar symptom frequencies were noted in patients with only HHV-6 positivity and those without detectable viral particles. Nonetheless, a statistically higher incidence of seizures was observed in patients with HHV-7 positivity compared to those without (p=0.003).

No antiviral treatment was used in any of the patients for the treatment of infections caused by HHV-6, HHV-7, and enterovirus detected in the CSF samples. However, all patients who tested positive for influenza A/B virus via the respiratory viral panel received oseltamivir treatment.

Table 1. Demographics, clinical characteristics, laboratory results and discharge diagnoses of the patients in whom at least one viral particle was identified in CSF samples

Patient number	Age (month)	Gender (M/F)	Presenting symptoms	WBC/ mm^3	Protein (mg/dL)	Glucose (mg/dL)	Positive for HHV-6	Positive for HHV-7	Positive for enterovirus	Discharge diagnoses
1	60	M	Seizure	0	23.6	83	Yes	No	No	Influenza A + *Bordetella pertussis*
2	19	F	Fever, seizure	0	25.1	60	No	Yes	No	Influenza B
3	2	M	Fever	0	48	47	Yes	No	No	Influenza A
4	11	F	Fever, seizure	0	27.5	63	Yes	Yes	No	Meningitis
5	2	M	Fever	0	76.4	52	No	No	Yes	Meningitis + myocarditis
6	24	F	Seizure	50	20.9	46	No	Yes	No	Meningitis
7	5	M	Fever, seizure	0	37.5	61	Yes	Yes	No	Meningitis,
8	3	M	Fever	30	46.6	60	No	Yes	Yes	Meningitis + Kawasaki syndrome
9	60	F	Fever, altered mental status	BloodyLP	31.6	79	No	Yes	No	Encephalitis
10	19	F	Fever, seizure	0	43.8	70	No	Yes	No	Meningitis
11	1	F	Fever, seizure	0	132.5	50	Yes	Yes	Yes	Meningitis
12	7	F	Fever, seizure	10	23.6	62	No	Yes	No	Meningitis
13	13	M	Fever, seizure	>500	77.9	46	No	Yes	No	Meningitis
14	60	M	Seizure, altered mental status	0	317	74	Yes	No	No	Encephalitis
15	21	M	Fever, seizure	0	4	93	No	Yes	No	Meningitis
16	1	F	Seizure	0	26.9	57	Yes	No	No	Meningitis
17	2	M	Fever	0	41.3	51	Yes	No	No	Meningitis
18	10	F	Fever	>500	55.3	53	Yes	No	No	Meningitis
19	4	M	Fever	10	14.6	68	Yes	No	No	Meningitis
20	10	M	Fever, petechial rash	0	45.4	52	Yes	No	No	Meningitis
21	18	M	Seizure	0	28.5	55	No	No	Yes	Meningitis
22	2	M	Fever	Bloody LP	153.8	54	Yes	No	No	Meningitis

CSF: Cerebrospinal fluid, HHV-6: Human herpes virus-6, HHV-7: Human herpes virus-7, WBC: White blood cell, LP: Lumbar puncture

DISCUSSION

In this prospective study conducted over 15 months in children under 5 years of age with suspected CNS infection, most frequently HHV-6 followed by HHV-7 were identified in CSF samples. However, enterovirus was detected at a lower frequency than expected. Concomitantly, three viruses were also identified. HHV-6 and enterovirus were not specifically associated with any particular symptoms, but in patients with HHV-7 infection, seizures were significantly more frequent. Clinical presentations, CSF findings, and patient-specific additional diagnostic work-up were influential in the diagnostic decision-making process.

Identifying HHV-6 DNA in CSF samples could indicate primary infection, latency, reactivation, or chromosomally integrated HHV-6 (ciHHV-6) complicates interpretation of the results obtained[14]. Immunohistochemical analysis of nasopharyngeal swabs of our solely HHV-6-positive patients revealed concomitant influenza A virus and *Bordetella pertussis* positivities in Patient 1, and influenza A positivity in Patient 3 possibly associated with symptoms which made us to consider the plausibility of latency or chromosomal integration. Moreover, Sugaya et al.[20] reported that certain instances of influenza-related encephalopathy might result from a co-infection with influenza virus and HHV-6, HHV-7, or both. Additionally, an alternate scenario was the reactivation of latent HHV-6 or HHV-7 virus in the brain by influenza virus, leading to encephalopathy or febrile convulsions[20]. Although based on their symptoms, and results of immunohistochemical examination of their CSF samples, Patients 14 and 18 likely had HHV-6 as the causative agent, normal CSF biochemistry and the absence of cells in direct CSF examination in Patients 16, 17, and 20 led us to consider the presence of a causative culpritt microorganism as a lower possibility. However, CSF abnormalities are rarely reported in patients with HHV-6 infection[21,22]. Pleocytosis may be absent or minimal during primary HHV-6 infection of the central nervous system[23]. Since HHV-6 meningitis was observed at postnatal 1 and 2 months as in Patients 16, and 17, the possibility of congenital infection was entertained. Finally, if lumbar puncture specimen is contaminated with blood as in Patient 22, the source of HHV-6 positivity could not be definitively assessed.

Limited data exists on HHV-7 infections involving CNS. A systematic review indicated that the prevailing symptoms commonly include headache and fever, with documented cases of rash and seizures[24]. Furthermore, analysis of spinal tap specimens typically reveals an elevated cell count with lymphocytic predominance and normal to slightly elevated protein levels, aligning with patterns observed in other viral CNS infections[24]. In our study, several cases (Patients 6, 12, and 13) were suspected of having an acute HHV-7 infection based on presenting symptoms and/or analysis of CSF specimens. Despite the presence of symptomatic meningitis, analysis of CSF samples may not reveal evidence of an alternative causative agent as in Patients 10 and 15. As in Patient 9, despite the potential contamination of spinal tap specimen with peripheral blood the altered mental status was considered indicative of HHV-7 encephalitis.

The simultaneous detection of both HHV-7 and influenza B virus in Patient 2 implies the possibility of coinfection or reactivation of HHV-7 induced by influenza B.

Co-detection of multiple viruses in CSF samples sometimes makes the determination of the causative agent more challenging. HHV-6 and HHV-7 might represent bystanders; however, it is established that enterovirus induces acute infection, does not persist latently, and undergoes rapid elimination by the immune system akin to other RNA viruses.[25,26] Hence, as in our study, in instances where HHV-6 and/or HHV-7 are concurrently detected with enterovirus, the causative agent is deemed to be the enterovirus, considering the patient's clinical characteristics. Nevertheless, the possibility of coinfection could not be ruled out. Notably, Patient 8, testing positive for HHV-7 and enterovirus, received the diagnosis of Kawasaki disease during the hospitalization. It has been reported in the literature that both HHV-6 and HHV-7 may reactivate in Kawasaki patients and aggravate symptoms of Kawasaki disease[27]. In addition, Kawasaki disease triggered by enterovirus or Kawasaki disease concurrent with enteroviral infection can also be seen[28].

In our study, the detection rate of enterovirus, recognized as the most prevalent cause of aseptic meningitis in childhood, was lower than anticipated. It has been reported that enterovirus is swiftly eliminated from the CSF, resulting in decreased viral load and lower rates of positive detection in the CSF samples among patients with symptoms lasting over 2 days[29]. We did not consider duration of symptoms as one of the study parameters. Consequently, it is plausible that enterovirus may not have been detected at the expected frequency. On the other hand, since the study was carried out during the coronavirus disease 2019 (COVID-19) pandemic

period, it was hypothesized that there may have been a decline in the frequency of enterovirus, like some other viruses, due to the effect of the mitigation strategies. Sun et al.[30] investigated the impact of non-pharmaceutical interventions on enterovirus infections in children in Hangzhou, China, and found a significant decrease in enterovirus-positive cases during the pre-COVID-19 and COVID-19 pandemic, with a gradual increase observed after the relaxation of nonpharmaceutical interventions in 2023. Their findings support our hypothesis.

Study Limitations

Our study has certain limitations. Firstly, no serologic confirmation was conducted on peripheral blood samples. Although detection of maternal antibody in young infants was observed, immunoglobulin detection in older children could provide insight into acute infection. Additionally, quantitative real-time PCR tests in serum or plasma samples were not performed. It has been noted that individuals with ciHHV-6 exhibit a persistent presence of HHV-6 DNA in their serum or plasma, indicating that identification of viral DNA alone is inadequate for diagnosing active infection. Moreover, it has been proposed that whole blood HHV-6 DNA viral loads exceeding >5.5 log 10 copies of HHV-6 DNA per mL strongly indicate the presence of ciHHV-6[31]. Due to concerns of cost-effectiveness, access to these tests is limited in many centers across Turkey. The second limitation of our study was its small patient sample size.

CONCLUSION

Our study holds importance in assessing CSF HHV-6, HHV-7, and enterovirus positivity without utilizing advanced molecular or serological tests. When evaluating the clinical significance of HHV-6 and HHV-7 positivity, it is necessary to assess the symptoms, immune status, and CSF characteristics of the patients in combination. In the absence of advanced molecular techniques, it is crucial to recognize that HHV-6 and HHV-7 may be bystanders, and other potential pathogens and diagnoses should not be discounted.

Ethics

Ethics Committee Approval: Ethics approval for the study was obtained from the Institutional Review Board of University of Health Sciences Turkey, Dr. Behçet Uz Children's Hospital (approval number: 2019/364, dated: 19.12.2019).

Informed Consent: Informed consent for this study was obtained from the parents of the patients.

Footnotes

Author Contributions

Concept: İ.D., N.B., Design: E.B., İ.D., N.B., Data Collection or Processing: E.B., E.K., Ş.Ş., A.A.K., K.Ö.O., H.A., F.Y.A., D.Z., S.K., S.T., Analysis or Interpretation: E.B., İ.D., E.K., F.Y.A., D.Z., S.K., Literature Search: E.B., İ.D., E.K., Ş.Ş., A.A.K., K.Ö.O., H.A., S.T., Writing: E.B., İ.D., N.B.

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