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# Assessment of a multiplex RT-PCR for Simultaneous, Rapid Screening of Common Viral Infections of Central Nervous System: A Prospective Study for Enteroviruses and Herpesviruses

Original Study

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**Abstract**

**Introduction.** Acute meningitis is a common neurological disorder that affects both children and adults and has a high mortality rate. This study aimed to create a multiplex reverse transcriptase PCR system for screening clinical samples for the presence of the two viruses currently considered to be the most common causes of acute meningitis in Asia.

**Materials and Methods.** A single-tube RT multiplex PCR assay was developed and tested for sensitivity and specificity using primers that have been commonly used to screen for herpes simplex viruses 1 and 2 (HSV-1/2) and enterovirus (EV) in clinical samples. The procedure was then used to screen 303 clinical samples for the target viruses, which included 101 feces samples, 101 throat swabs, and 101 cerebrospinal fluid (CSF) samples obtained from 101 hospitalized Iranian children with suspected viral meningitis/meningoencephalitis, and the findings were compared to those of an RT monoplex PCR method.

**Results.** The RT-PCR approach demonstrated high precision, with no non-target virus amplification. The results of using this assay to screen clinical samples revealed that RT monoplex PCR had the same sensitivity as RT multiplex PCR for the three different types of specimens.

**Conclusions.** This newly developed multiplex RT-PCR method is a simple, fast diagnostic tool that can be used to screen clinical samples for viruses that cause acute meningitis/meningoencephalitis in children.

**Keywords**

multiplex PCR • central nervous system disease • meningitis • children • enteroviruses • herpes simplex virus-1/2

## Introduction

Infections of the central nervous system (CNS) are linked to debilitating consequences such as cognitive deficiencies, vision and hearing deficiency, motor and sensory deficits, and epilepsy in more than half of those that survive [1, 2]. To minimize the associated morbidity and mortality, CNS infections must be diagnosed and treated quickly. Unfortunately, medical signs and symptoms are also not specific to the causative pathogen, making the diagnosis of suspected CNS infections problematic [3]. Aseptic meningitis (ASM), one of the most common CNS infections, accounting for the vast majority of total viral CNS infections, is diagnosed when there is an acute onset of meningeal symptoms and cerebrospinal fluid (CSF) pleocytosis [CSF white blood cells (WBC) ≥ 5 cells/mm3] with negative bacterial and fungal cultures [4, 5, 6, 7]. Despite extensive efforts to identify a causative agent, approximately 50% to 70% of patients remain without an etiologic diagnosis [8, 9, 10]. According to a recent study in Qatar, viral meningitis is the most common form of CNS infection, with enteroviruses (EVs) being the most common cause. In the Persian Gulf, the prevalence of this phenomenon ranges from 7.2 to 40.4 percent, and it depends on the population's age, vaccination status, and seasonal and geographical pattern distribution [11, 12].

The reported incidence almost definitely underestimates the true rate, especially for enteroviral meningitis, the most prevalent infection detected. Herpes simplex virus (HSV) and flaviviral meningitis are the most serious remaining causes of viral meningitis and CNS infections in terms of morbidity and mortality [13]. EVs, notably EV-A71, is one of the most leading neurotropic viruses known, and it is responsible for approximately 85% to 90% of viral meningitis in western and south Asian countries [14, 15, 16]. Besides, the variable epidemiology of EV-A71 in Asia-Pacific countries has indicated patterns of repetition outbreaks every 2–3 years, with different frequency and clinical intensity [17, 18, 19, 20]. HSV was the second most common cause of ASM, after enteroviruses, and causes severe CNS disease with 70% fatality

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rate in untreated patients and 30% fatality rate with a high risk of permanent neurological sequelae even in treated cases [21].

Recent trends in viral meningitis in Asian countries have been published highlighting the significance of EVs and HSV in CNS infections, especially in pediatrics; therefore, the rapid and reliable identification of the etiology of ASM is critical for clinical care and treatment, as well as the implementation of adequate control measures [12, 17, 22]. Previously, real-time PCR-based methods and other molecular methods for detecting HSV-1/2 and enterovirus independently or simultaneously were identified; low-cost and low-tech methods are still required in resource-limited conditions. In this research, we implemented and tested a simple, fast, and low-cost diagnostic tool for screening three types of clinical samples to present the two viruses that are currently thought to be the significant viral causes of ASM in Asia. RT Multiplex PCR was used to screen clinical samples obtained from Iranian children with suspected viral meningitis.

## 1. Materials and Methods

### 1.1 Clinical specimens

This prospective cohort study was conducted in Imam Hussein Pediatric (IHP) Hospital, Isfahan, Iran. Stool, throat swabs, and cerebrospinal fluid (CSF) were collected from all children less than 16 years old with ASM criteria definition, including the onset of meningeal symptoms and fever, pleocytosis of the CSF, and no growth on routine bacterial culture [4, 23]. Specimens were collected within 24 hours of the onset of symptoms in a medium containing Eagle's minimum essential medium, 500 U/ml penicillin, 500 g/ml streptomycin, and 10% fetal bovine serum (Gibco, Carlsbad, CA) and quickly transported to the laboratory for further study. The specimens were held at -70 °C before further investigation.

Finally, a total of 303 samples consisting of 101 CSF samples, 101 stools, and 101 throat swabs were collected from 101 children admitted with suspected viral meningitis/meningoencephalitis from June 2019 to June 2020 in Imam Hussein Hospital, Isfahan, Iran.

### 1.2 Positive Viral Control

The following viruses were used as positive controls in both monoplex and multiplex PCR. DNA and cDNA preparation HSV (6.5 x $10^6$ copies/µL) and enterovirus (5.7 x $10^6$ copies/µL) were provided by Iranian children hospitalized for acute fever and then confirmed by sequencing.

### 1.3 Extraction of Viral Genome and cDNA Synthesis

TRIzol-LS (Gene all, Korea) was used for nucleic acid extraction according to the manufacturer's instruction.

For reverse transcription, 5 µl of extracted RNA was added to a reaction mixture consisting 4 µl of 5 x First-Strand Buffer (Invitrogen, Carlsbad, CA, USA), 1 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 1 µl of M-MLV (200 U/µL) (Roche, Mannheim, Germany), random primers (1 µg/µL) (Hexa-deoxyribonucleotide mixture) (Roche, Mannheim, Germany), 0.5 µl of RNase Inhibitor (40 U/µL) (Invitrogen, Carlsbad, CA, USA), and distilled water. Thus, the total volume of the reaction mixture was up to 15 µL. RT reactions were carried out at 37°C for 1 hour, followed by heat inactivation at 70°C for 5 minutes and cooling on ice.

### 1.4 Primers

All pairs of specific primers used in this study for target viral genomes were designed by other authors and published previously [24, 25].

### 1.5 RT-Monoplex PCR

For RT monoplex PCR, only one pair of primers was used to detect the target viral genomes. After adding 4 µL of cDNA to the PCR mixture containing 10 µL of Master mix (Amplicon, Denmark), 1 µL of each specific primer (20 µM), the mixture was brought to 25 µL with distilled water. The thermal cycling program was 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 7 minutes, then held at 4°C.

### 1.6 RT-Multiplex PCR

For RT multiplex PCR, two pairs of specific primers for detecting EVs and HSV were mixed in a single tube. The volume, concentration of primers, reagents, and thermal cycler program were the same as described above.

### 1.7 Specificity Testing of Two Pairs of Primer

The specificity of amplification using the two pairs of primers was tested with the positive control for HSV-1/2 and EVs. Specificity testing was also carried out for negative controls.

### 1.8 Sensitivity Testing of RT Multiplex and RT Monoplex PCR

To compare the sensitivity level of RT multiplex PCR and RT monoplex PCR, 10-fold serial dilution ($10^{-1}$ to $10^{-4}$) in MilliQ water of the two different viral cDNA, two positive controls of the HSV and EVs, were tested by RT multiplex-PCR and RT monoplex-PCR. Furthermore, with the same PCR machine, multiplex and monoplex RT-PCR were performed simultaneously for the same dilution series.

### 1.9 Electrophoresis and Sequencing Analysis

The PCR mixture was electrophoresed in a 1.5% agarose gel in 1X TBE (40 mM Tris-boric acid [pH 8.0], followed by staining with safe stain (Sinaclon Co, Tehran, Iran) for 20 minutes and then visualized under ultraviolet light. The results were recorded via photography. The RT-PCR products were purified and sequenced to confirm the specificity of amplification.

## 2. Results

### 2.1 Specificity Testing of the Two Primer Pairs

The specificity of the mixture of two pairs of specific primers used in the present study was tested; results are shown in Fig. 1. Each pair of primers amplified the viral genomes of positive controls and specifically and independently generated two different sizes of amplicons of 440 and 292 bp for EVs and HSV-1/2, respectively. No cross-reaction with non-targets was identified. For the negative controls, nuclease-free water and clinical samples positive for *E. coli* were used, and no amplicon was observed.

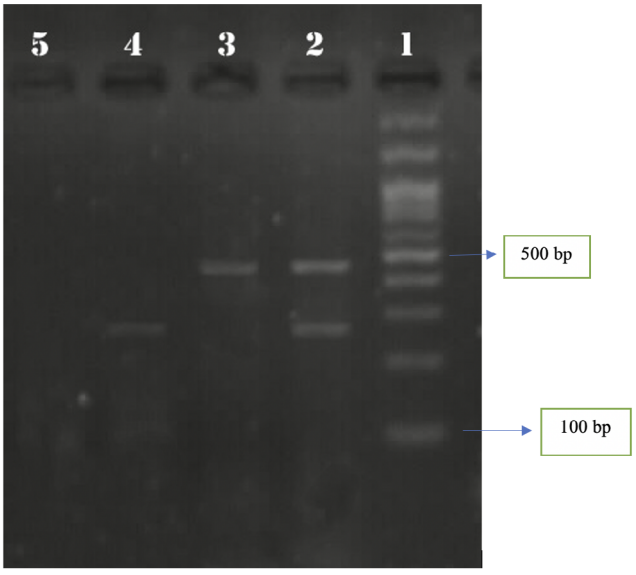


*Fig. 1. The multiplex PCR assay's specificity was examined using PCR mixes containing all two-primer pairs and a single template (cDNA/DNA) from the positive controls. Lane 1: standard 100-bp DNA ladder marker. Lane 2: the mixture of enterovirus (440 bp) and HSV (292 bp) positive controls. Lane 3: enterovirus (440 bp), Lane 4: HSV (292 bp), Lane 5: negative control (E. coli)*

### 2.2 Sensitivity Evaluation of RT- Multiplex and RT-Monoplex PCR

The RT-PCR sensitivity tests were carried out using 10-fold serial dilutions of the two positive controls. Taken together, the results indicated that the sensitivity of RT multiplex PCR was the same as RT monoplex PCR (Fig. 2).

### 2.3 Detection of Target Viruses in Clinical Specimens by RT-Monoplex PCR and RT-Multiplex PCR

A total of 303 clinical samples (negative bacterial culture) were collected from children less than 16 years old with meningitis/meningoencephalitis in Imam Hussein Hospital, Isfahan, Iran, during the period of June 2019 to June 2020. All clinical specimens, including CSF, throat swabs, and stool, were tested for the presence of EVs and HSV-1/2 by RT- monoplex PCR and RT-multiplex PCR with specific primers. The results presented that there was no difference in viral detection rate between two assays in clinical samples. HSV-1/2 and EVs were detected from 13 CSF samples from 13 patients and 47 clinical specimens (including 23 throat swabs, 23 stools simultaneously and one CSF sample), respectively.

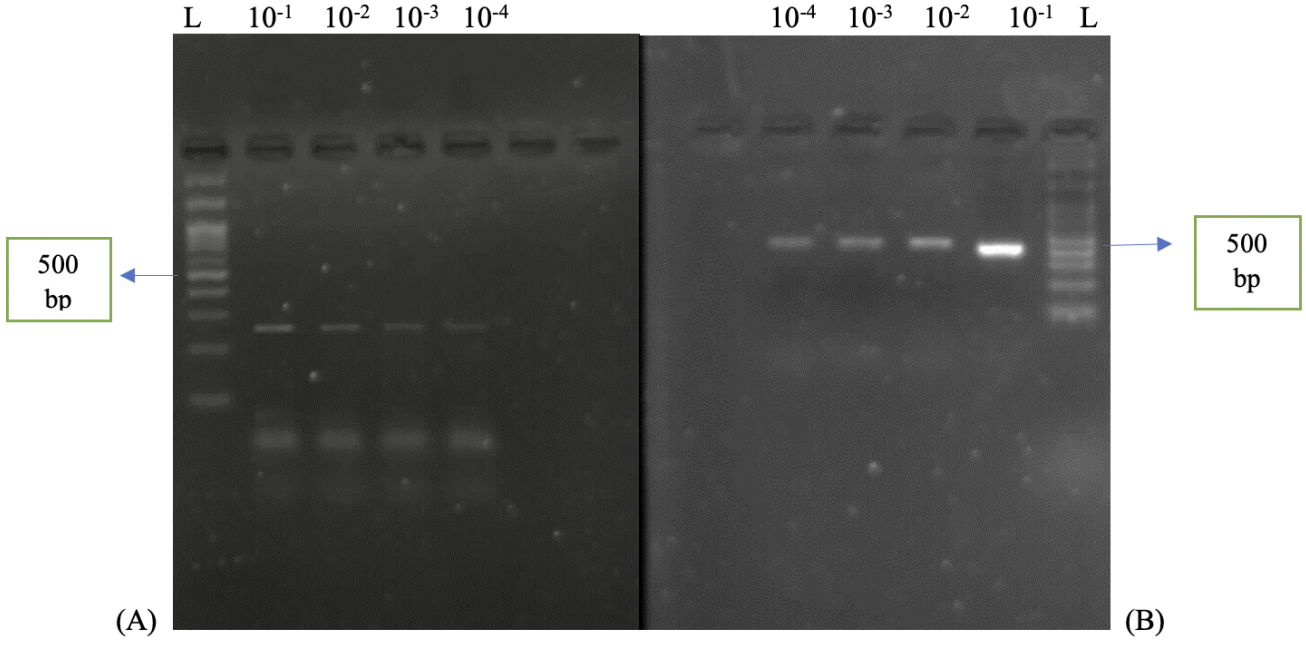


*Fig. 2. Comparison of the sensitivity between multiplex PCR and monoplex PCR for the 10-fold serial dilutions of positive controls. (A): HSV (292 bp) (B): Enteroviruses (440 bp)*

## 3. Discussion

To recognize pathogens in infectious CNS diseases, nucleic acid amplification techniques (NAATs) such as real-time PCR and conventional PCR have been extensively developed. PCR is now commonly used for the molecular diagnosis of pathogens in clinical specimens and is considered a convenient, helpful, and efficient process. Furthermore, the multiplex PCR assay is an easy method that has the potential to provide fast and cost-effective laboratory diagnosis [26].

A reverse transcription (RT) multiplex PCR assay detects EV and HSV-1/2 genomes with high sensitivity. It provides direct identification of HSV that may be present in clinical samples, eliminating the need to analyze clinical specimens separately for each virus. In addition, since the test uses a single aliquot of sample and a single PCR run, a quick diagnosis can be obtained while saving time, sample, and reagents [27].

Several parameters must be considered in an RT multiplex PCR assay that can influence primer-template affinities and competition between various primers. In this analysis, the sensitivity of this assay using random hexamers was compared to the sensitivity using two virus-specific primer pairs during the RT process, and the results showed that the sensitivity of RT multiplex PCR assays using random hexamers was higher; therefore, prior to PCR, random hexamers were chosen for the RT stage. This result is similar to previous research [26]. Additionally, the melting temperature of primer-template complexes has a significant impact on amplification efficiency. The importance of changing the primer sequence and concentration represents the competition between simultaneous DNA amplification occurring in the same tube [28]. Only by carefully selecting primers and adjusting MgCl2, dNTP, and primer concentrations was a sensitive test achieved. When one DNA amplification is less effective than another in a simultaneous reaction, two units of Taq DNA polymerase were used in each multiplex PCR to provide adequate enzymatic operation [29, 30].

Aseptic meningitis is a common syndrome associated with EV, especially in children; since it is difficult to differentiate viral from bacterial etiology and other viral etiology, the identification of enteroviral RNA can remove the need for unnecessary therapy. Furthermore, the possibility of treating EV infections with new antivirals creates new demand for detecting enterovirus RNA, which is especially important in agammaglobulinemia patients [7, 12]. According to previous study, because the sensitivity of PCR was 96% for fecal samples and 76% for CSF, the fecal PCR test was clinically the most sensitive to detect enterovirus during meningitis. Furthermore, the clinical sensitivity of stool PCR remained quite high throughout the disease [31]. Glimaker et al. revealed that stool PCR is highly useful for identifying enteroviral meningitis (sensitivity, 93%) [32]. Even in the absence of clinical symptoms, enteroviral replication in the gastrointestinal tract can last for several weeks following a clinical infection. As a result, the presence of enteroviral RNA in feces does not prove that enterovirus is the causal agent of meningitis, but it does suggest that this etiology is highly probable. Stool PCR was clinically the most sensitive for detecting enterovirus during enteroviral meningitis and might provide a tentative diagnosis throughout the disease course. CSF PCR provided a definitive diagnosis [33, 34, 35], but its value was clearly decreased for samples received more than two days after clinical onset. As a result, it is advised that, in addition to CSF PCR, fecal samples were taken from individuals with suspected enteroviral meningitis be evaluated by PCR, especially if the symptoms have lasted more than two days [31]. On the other hand, HSV diagnosis from CSF samples must be precise because acyclovir antiviral therapy is successful mainly when administered early in the disease phase [13]. This assay cannot distinguish HSV-1 from HSV-2 consequently, eliminating the need to monitor clinical specimens with separate PCR assays or two primer pairs. Most importantly, the same antiviral medications are used to treat infections caused by both of these viruses [13, 36].

Thus the current assay has the ability to provide clinical and epidemiological information in a timely and responsive manner.

The findings obtained using this RT multiplex PCR for EV targets were consistent with those obtained using monoplex PCRs for all types of clinical samples analyzed. Using such primer sequencing, Pham et al. found the sensitivity of multiplex PCR for throat swabs and CSF samples were less than monoplex PCR; however, Read et al. investigated the same sensitivity for EV detection in CSF specimens [26, 37].

Our study's analysis of target ratios showed that the sensitivity of monoplex RT-PCR and multiplex RT-PCR with HSV-1/2 targets in CSF samples were equal; additionally, the previous researches were also reported the same sensitivity between monoplex and multiplex PCR for HSV-1/2 in CSF specimens [24, 38, 39]. The RT multiplex PCR was found to be specific for HSV-1/2 and EVs. According to direct sequence analysis, the viruses that we observed and characterized were the target viruses, with no non-target virus amplification. Because of their high sensitivity and specificity, current methods are helpful diagnostic tools in laboratories. This is particularly true in patients with neurological infections, where only a few copies of the virus might be present in CSF samples. In this analysis, we used our test on early CSF samples from patients to detect enteroviral RNA or identify HSV within a few hours.

## 4. Conclusion

To conclude, the RT multiplex PCR assay used in this study was shown to be useful for the laboratory diagnosis of neurological diseases of presumed viral origin, with no unexplained amplifications compared with presumptive clinical diagnosis. In addition, the assay has the potential to provide a rapid diagnosis of EV and HSV infections on CSF samples taken soon after the onset

of disease, making it a valuable tool for patient care and antiviral therapy design. This assay is also helpful for investigating early cases of enteroviral aseptic meningitis in population outbreaks. As a result, combining the identification of multiple targets in a single reaction is particularly useful for adapting to inadequate CSF volumes received from some children with multiple microbiological test requests while also minimizing turnaround times and costs. Based on the results obtained, RT multiplex PCR could be a viable alternative to single-targeted PCR as inpatient treatment for children to prevent viral infection misdiagnosis.

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Consent to Participate: Written consent was obtained from all subjects participating in this study.

## Authors' Contribution

SM conceptualized and designed the study. Specimen collection, statistical analysis, were performed by HR, RS, and MA. Molecular testings and manuscript writing were done by MKh. BNE provided scientific support. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Conflict of Interest

The authors have no potential conflicts of interest to declare.

## Ethics Approval

The Ethics Committee of the Isfahan University of Medical Science and Imam Hussein Pediatric Hospital approved the protocol of this prospective study. The ethical code was IR.MUI.MED.REC.1398.336.

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