Diagnostic Performance of Polymerase Chain Reaction in Suspected Cases of Herpes Simplex Encephalitis

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ABSTRACT

Introduction: One of the prime diagnostic goals in suspected cases of viral encephalitis is to identify a specific etiologic agent, with emphasis on disease that requires therapeutic intervention, such as Herpes simplex encephalitis. Diagnosis of viral infections of the central nervous system has been revolutionized by the advent of new molecular diagnostic technologies, such as the standard Polymerase chain reaction/Real time polymerase chain reaction. The technique can be performed rapidly and inexpensively, with a turnaround time of 24 h or less.

Materials and Methods: A total of 54 patients suspected of HSV encephalitis in adult or paediatric patient with fever (≥38°C), altered mental status, whether with or without headache, seizures or focal neurologic abnormalities were selected for study. The PCR reactions were optimized with the same set of primers and reaction conditions both for the known HSV control strain and all the 54 patients CSF samples.

Results: Overall, 6 (11.1%) of the CSF samples were positive for HSV DNA. Negative CSF controls yielded negative results for HSV DNA, thus confirming the efficiency and efficacy of the amplification process.

Conclusion: In conclusion, detection of Viral DNA in CSF by PCR for the diagnosis of suspected Herpes simplex virus encephalitis patients should clearly be the standard diagnostic modality.

Key words: Herpes Simplex Encephalitis, Cerebrospinal Fluid, Polymerase Chain Reaction.

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INTRODUCTION

Over 100 viruses are known to cause acute viral encephalitis in humans.¹ The cardinal clinical and laboratory findings are largely similar regardless of the inciting agent and consist of fever, headache, and altered mental status, which are often accompanied by seizures and focal neurologic abnormalities. From a practical point of view, a clinician confronted with a patient having fever, headache, and altered mental status must distinguish encephalitis from non-infectious causes of brain dysfunction (encephalopathy). The goal in cases of encephalitis is to identify a specific etiologic agent, wherever essential, with particular emphasis on diseases where effective therapeutic modalities are available, such as herpes simplex encephalitis.²⁻⁶ Diagnosis of viral infections of the central nervous system (CNS) has been revolutionized by the advent of new molecular diagnostic technologies, such as the Polymerase chain reaction (PCR) for the amplification of viral nucleic acid from CSF.⁷⁻⁸

The technique can be performed rapidly and inexpensively, with a turnaround time of 24 h or less rather than the standard minimum of 1 to 28 days required for culture. PCR is also preferable to serologic testing, which often requires 2 to 4 weeks after acute infection for development of a diagnostic rise in antibody titers. Positive CSF PCR test results have been noted up to 4 weeks after onset of clinical symptoms, depending on the pathogen.⁹ In India, Herpes Simplex Encephalitis (HSE) appears to be under diagnosed, probably due to lack of awareness and limited diagnostic facilities. This was a method development study to evaluate the performance feasibility of PCR in identification of the etiological agent of acute sporadic encephalitis on a limited number of clinical samples. The primary focus of the study was to identify etiologic agent in cases of suspected HSV encephalitis.
MATERIALS AND METHODS
The clinical criteria for suspicion of encephalitis in any adult or pediatric patient was defined as fever (≥38°C) and altered mental status, whether with or without headache, seizures or focal neurological abnormalities. It also included diagnostic imaging abnormalities observed on computed tomography (CT) or MRI scans, wherever performed.

Thus, 54 patients suspected of encephalitis were included in this study. Patients were excluded from the study if they had a proven bacterial, fungal, parasitic, or mycobacterial etiology demonstrated by Gram stain, ZN stain, India ink staining, culture, serology or PCR.

Virus and Sample Controls
A known HSV strain (76716) obtained from National institute of Virology; Pune was used as positive control. Ten CSF samples collected from patients with unrelated diseases (non-infectious CNS diseases) were used as negative controls.

Collection of Samples and Extraction of Viral DNA
All CSF samples were collected preferably within a week of the onset of neurological illness. The samples were collected aseptically and transported immediately to the laboratory and stored at -70°C until processed. DNA was extracted from 200 μl of the CSF sample and the control strains by using a QIAamp DNA Mini Kit (QIAGEN) as per the manufacturer’s instructions.

PCR Amplification and Optimization
The PCR reactions were first optimized and carried out by using the known viral control strain (HSV 76716). The same set of primers and reaction conditions were then applied to the clinical samples. The set of primers used to amplify HSV DNA is as shown in Table 1. A final PCR reaction volume of 50 μl consisted of 45 μl of mix containing 5 μl of PCR buffer (10X), dNTPs (0.2 mM of each) forward and reverse primers (0.5 μM each), Taq DNA polymerase (0.25 U), and MgCl₂ (1.5 mM) with 5 μl of template DNA. Cycling conditions were: hot start at 94°C for 10 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds and final extension step at 72°C for 10 minutes. Amplified products were demonstrated on 2% agarose gel. Electrophoresis of the amplified products, controls and molecular weight markers was done and observed under a UV trans-illuminator. Carryover contamination of the amplified products was avoided by strict physical separation of pre and post amplification processes with general precautions against contamination.

RESULTS
This was a method development study, done to evaluate the performance feasibility of PCR on a limited number of clinical samples. With use of strict inclusion and exclusion criteria for patient selection, sample size was limited to 54 CSF samples. The results of Neuro-radiоimaging were available for limited number of patients only. Age of the patients ranged between 06 months to 87 yrs. Twenty four (44%) cases were females and thirty cases (56%) were males. The maximum numbers of suspected encephalitis cases were under 20 years of age.

The initial optimisation of the PCR was carried out by using serial dilutions of suspensions of known strain of HSV. All dilutions were prepared in normal CSF samples. The selected concentration for primers was 0.5 μM, and the concentration of each dNTPs was 0.2 mM (maximum band intensity and minimal background). The annealing temperature (60°C) was chosen to be as high as possible, taking care not to reduce the sensitivity of the assay. Therefore, stringent annealing temperatures helped to increase specificity (Fig 1). Among the 54 samples collected over a period of one and half year, 6 (11.1%) cases turned out to be positive for the presence of HSV DNA in the CSF whereas 48 (89.9%) cases were negative (Fig 2). Forty (40/54) cases in the study were immunocompetent individuals, whereas 11 out of 54 cases were HIV infected individuals. All the HIV positive cases in this study were negative for HSV virus. Non-infectious negative CSF controls yielded Negative results for HSV DNA, confirming the efficiency of pre and Post amplification contamination preventive measures.

Table 1: Primer sequences and gene amplified for Herpes simplex virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>Gene amplified</th>
<th>Primer sequence</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpes simplex virus</td>
<td>Glycoprotein D region</td>
<td>Forward-5’-ATCCGAAAGCGACCCCGCTG-3</td>
<td>396 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse-5’-TCCGGCGGACAGGGTGCT-3-3'</td>
<td></td>
</tr>
</tbody>
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Fig 1: Gel electrophoresis of Amplified glycoprotein D gene products for HSV; Lane1- Positive Control, Lane2- Negative control; MWM -100bp Molecular weight marker

Fig 2: Gel electrophoresis of Amplified Glycoprotein D gene products HSV; Lane1-100 bp Molecular weight marker; Lane2-Control strain of HSV, Lane3 Negative control; Lane 4, 5, 8 Positive CSF samples; Lane 6, 7 Negative sample.
DISCUSSION

Over the past several years, the laboratory diagnosis of CNS diseases due to neurotropic group of viruses has been facilitated greatly by PCR. A Study by Levitz et al states that herpes simplex encephalitis contributes to 10-20% cases of viral encephalitis.10 The Viral DNA can be detected in the CSF as early as 24 hours after the onset of neurological symptoms. Out of 54 suspected encephalitis cases, six (11.1%) patients turned out to be positive for HSV DNA for which CSF was collected within seven days of onset of symptoms. However, sometimes PCR results can be negative during the early stages of disease11 and hence the timing of sample collection can play a crucial role in establishing the diagnosis. With modern neuroimaging techniques, HSV encephalitis is only rarely confused with abscess, tumour or infection. In 3 out of 6 PCR positive HSV encephalitis (HSVE) patients whose MRI results were available, also had lesions at the infero-medial region of one or both temporal lobes. In One patient MRI showed diffuse involvement of the brain.

The sensitivity of HSV IgM is as low as 16.7%, thus it has limited use in laboratories. At the same time lack of CSF IgM does not rule out herpes encephalitis. In this study, five cases positive for HSV DNA, did not show the presence of HSV specific IgM in CSF, which could be due to the collection of the CSF samples early in the course of the disease.

Although HSVE is not regarded as an opportunistic infection, the occurrence of encephalitis in immunocompromised patients has been documented and the suggestion made that unusual clinical and neuropathological features characterize the disorder in this population. In this study there were 14 immunocompromised cases amongst which 11 were HIV positive with CD4 counts below 200 cells/mm³, and one case each of post renal transplant, Non-Hodgkin’s lymphoma (NHL) and Burkitt’s lymphoma on chemotherapy. HSV PCR was positive in the NHL and Burkitt’s lymphoma case but was found to be negative in all cases of HIV. Herpes Simplex Encephalitis appears to be under diagnosed, probably due to lack of awareness and diagnostic facilities. Curiously, till 1992 only occasional case reports were available.12 In 1993, Satish Chandra et al.13 reported 9 cases of HSE and later in 1996 compiled data on 51 cases.14 However, serological and clear epidemiological studies for viruses are not available in this part of the country, due to lack of virology research laboratories. Thus, percentage of cases of viral encephalitis and proportion of HSE are difficult to estimate.10

One of the most frequently cited study has been conducted by Panagariya et al.15 in North- West India in 2001, covering the population of States of Rajasthan, Haryana, Punjab and parts of Uttar Pradesh. All patients admitted with provisional diagnosis of an encephalitic illness over a period of 30 months, were studied. Special investigations included CSF analysis, EEG, CT scan and MRI. Herpes simplex virus (HSV) antibody estimation in CSF and blood was done simultaneously using ELISA. No PCR was carried out. Twenty eight patients showed electroencephalographic, serologic and/or neuro-radiological evidence of Herpes simplex encephalitis. The main clinical features included altered sensorium (100%) and seizures (89%). Serological test for HSV antibody in CSF and blood was positive in 14 patients. In a study from Eastern Part of the country by A Mukherjee16 only sixteen cases of Herpes simplex Encephalitis (HSE) from Eastern India were seen over a period of five years (1996-2001). Selection criteria included clinical features, characteristic MRI changes and positive immunological test. However there was no nucleic amplification test performed on these samples. Preliminary findings of fever, headache with altered sensorium with the pattern of CSF cell and differential counts along with MRI findings could be sufficient to perform HSV-PCR, which could ultimately result in a rapid diagnosis of herpes simplex virus encephalitis.17,18 Many negative results in certain cases with strong suspicion of encephalitis could also be attributed to other viral etiological agent not tested for by PCR in this study. The diagnostic value of CSF PCR may be compromised by the presence of inhibitors leading to false-negative results. Elevated CSF protein levels that are observed in acute encephalitis may contribute to this inhibitory effect.15

Limitations of the present study is the limited sample size attributable to factors like limited population under study, low prevalence of sporadic encephalitis in adult population in the selected community, low suspicion index, strict inclusion criteria for selection of patient suspected to be having viral encephalitis and probably more reliability to radiologic techniques like MRI for diagnosis of encephalitis.

CONCLUSION

In conclusion detection of Viral DNA in CSF is clearly the emerging gold standard for the laboratory diagnosis of suspected acute sporadic encephalitis due to Herpes simplex virus.

REFERENCES

Satish Kumar et al. Diagnostic Performance of PCR in Herpes Simplex Encephalitis


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