Comparative evaluation of Herpes Simplex Encephalitis cases by Shell Vial Culture and Real-Time PCR at Tertiary Care Hospital in North India

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Abstract:
Herpes Simplex Encephalitis (HSE) is the most common cause of fatal sporadic acute encephalitis occurring worldwide, contributing to 10-20% cases of viral encephalitis. The aim of this study was to Comparative evaluation of Herpes Simplex Encephalitis cases by Shell vial Culture and Real-time PCR at Tertiary Care Hospital in North India. Out of Seventy Five cases of suspected Herpes simplex virus (HSV) encephalitis, ten cases were positive by real-time PCR and six by shell vial culture. Maximum numbers of cases were seen in 13-20 years of age group. As compared to real-time PCR from shell vial culture was 60% sensitivity, 100% specificity with positive prediction value of 100% and negative prediction value of 94.5%.

Key words: Herpes Simplex Encephalitis, Herpes Simplex Virus, Shell Vial culture and Real-time PCR.

1. INTRODUCTION
Herpes simplex virus (HSV) is the most common cause of acute sporadic encephalitis in worldwide, accounting for 10–20% of all cases1, 2. Herpes Simplex Virus type I (HSV-1) and type II virus can be detected in the laboratory using a variety of methods. These range from standard and rapid culture (shell vial) to molecular amplification methods. More than 90% of the cases are caused by Herpes Simplex Virus type I (HSV-1) and 7% are caused by herpes simplex virus type 2 (HSV-2)1. There is bimodal age distribution of HSE, with one third of cases occurring in those less than 20 years old and two third cases in aged 50 years or more 2, 5. As per global data 2-4 individuals per million populations per year get affected by this disease1. In United States, prevalence equals one case per 250 000 population/year while in Sweden 2.5 per million population/year get affected 2. There are no documented studies from India, however in older study HSV-1 encephalitis constituted a very low proportion (1.1%) of acute viral encephalitis cases seen in Eastern Uttar Pradesh (India)2. In India, HSE appears to be under diagnosed, probably due to lack of awareness and sensitive diagnostic facility1. Epidemiological studies for virus are not available in many part of the country, due to lack of virology research laboratory1. The laboratory diagnosis of HSV infection has traditionally relied on virus isolation in cell culture1. This has the inherent disadvantage of requiring prompt specimen transportation to the laboratory to ensure viability of the virus, as well as the need for cell culture facilities, and has a minimum turn-around time of 24-72 hours. Other methods routinely used, which do not rely on the detection of viable virus, include direct fluorescent antigen (DFA) and enzyme immunoassays (EIA) 1. Thus percentage of cases of viral encephalitis and proportion of HSE are difficult to estimate. HSV can be detected in the laboratory using a variety of methods. These range from standard and rapid culture (shell vial) to a molecular amplification method6. CSF culture is of extremely low sensitivity for diagnosis of HSV encephalitis. Positive culture has been reported in less than 5% of cases3. Shell vial culture (SVC) is a modification of the conventional cell culture technique for rapid detection of virus in vitro2. A small vial, called 1-dram vial or a shell vial, has become popular for containment of cell culture monolayer. This system works on the principle that the low speed centrifugation enhances viral
infectivity to the susceptible cells \(^3,^4\). It is thought that the minor trauma to the cell surface produced as a result of low speed centrifugation mechanical force enhances the viral entry into the cells, which in turn reduces the total time taken for the virus to produce infection of the cell. The cost of cell cultures in shell vials is the same as that of cell culture techniques used conventionally. This has made SVC very popular in the field of virology.

Generally the introduction of PCR is recognized as the reference standard assay method for the sensitive and specific diagnosis of CNS infections caused by HSV \(^9\). Recent studies have suggested that detection of HSV DNA by PCR increases the sensitivity of viral infection detection compared to antigenic detection or cell culture methods \(^9\). Application of real-time PCR in our routine clinical laboratory allowed a 16% increase in detection rate over cell culture. Use of real-time PCR in another clinical laboratory setting demonstrated a 22% increase in sensitivity over shell vial culture with 100% specificity. Similar observations have been reported by others \(^2,^3,^4,^5\). In an effort to improve HSV detection, new technologies have been investigated, and real-time PCR technology has been found to offer excellent performance, surpassing other PCR detection methods \(^2\). The aim of this study was to comparative evaluation in Herpes Simplex Encephalitis cases by Shell vial Culture and Real-time PCR at Tertiary Care Hospital in Northern India Population.

2. MATERIALS AND METHODS

2.1. Sample

This study has been conducted at Sanjay Gandhi Post graduate Institute of Medical Sciences, situated at Lucknow, India. A total of 75 cases of >10 years of age with or without treatment history of suspected cases of Herpes viral encephalitis admitted between 1\(^{st}\) Nov 2006 to 1\(^{st}\) Nov 2008. All the patients were diagnosed by clinical presentations, biochemical findings and CT/MRI findings according to the universal standard for the diagnosis of viral encephalitis. Cerebrospinal fluid samples (CSF) were collected from these patients and further testing was performed on them. CSF samples from 75 cases of non-viral meningitis (with laboratory confirmed of other etiology) were taken as negative controls.

2.2. Shell Vial Method

A sterile tube with flattened lower end at one surface (shell vial) was taken. A thin sterile glass cover slip with size about 12mm in size, was taken and cleaned with spirit followed by flaming to insured complete sterility. 0.5ml Vero cells (4×10\(^5\) cells/ml) which is a African green monkey kidney continuous cell line and 2ml of 10% Eagle’s modified Minimal Essential Media (MEM) was taken in the vial. These were incubated at 37\(^0\)C till the confluent monolayer was formed. On formation of monolayer on the cover slip, about 200µl of filtered CSF was inoculated in the shell vial along with 2ml, 10% of MEM. These tubes were rotated at 500 rpm for 30 minutes. This was followed by incubation at 37\(^0\)C for 36 hours. They were examined for the presence of any cytopathic effect (CPE) i.e. rounding and clustering of cells. Maintenance medium was removed carefully form the shell vials and discarded. Monolayer was rinsed with Phosphate buffered saline (PBS), dried and then fixed with chilled acetone for 30 min and incubated in a humid chamber at 37\(^0\)C for 30 minutes. After washing with PBS, 1:10 rabbit anti-mouse Immunoglobulin G (IgG) Fluorescein Isothiocyanate (FITC) conjugated antibody was added to each of these smears and incubated at 37\(^0\)C in a humid box for 30 minutes. Smears were then dried and observed under fluorescent microscope.
(Olumpus, Japan) for intranuclear apple green fluorescence. Positive and negative controls were included in every run of the assay for completion.

2.3. Real Time PCR

The real-time quantitative Polymerase Chain Reaction (PCR) assay for HSV1 and 2 was carried out using Taqman PCR kit (Genome Diagnostic Pvt. Ltd.) using the primers and fluogenic probe based on the UL30 gene sequence. The DNA was extracted from 200µl of CSF , using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany), and eluted in 50 µl of Elution Buffer (AE). 20 µl of the DNA extracted from each sample were used for the real-time PCR assay, which can detect both HSV-1 and HSV-2 with almost equal efficiency. Following 5 cycles of 20 second at 97\textdegree{} C and 1 min at 58\textdegree{} C, 45 cycles of 20 second at 96\textdegree{} C and 1 min at 58\textdegree{} C were carried out using a Model Rotor Gene 2000/3000 (Corbett research Australia). Real-time fluorescence measurements were made and threshold cycle value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation for the base line). Standard curves were constructed using the values obtained from serially diluted 5 positive control ranging between \(2.5 \times 10^7\) to \(2.5 \times 10^3\) (Fig 1).

3. RESULTS

Out of 75 cases of suspected HSV encephalitis cases, 10 cases were positive by real-time PCR and 6 by shell vial culture method. None of the control sample was found to be positive by real-time PCR or shell vial culture. The maximum numbers of cases were seen in 13-20 years age group (Table-1). Table-1 shows that the incidence of confirmed HSV Encephalitis cases in the age group 13-20 years was significantly higher as compared to other age group (p=0.019). The sensitivity, specificity, positive predictive value and negative predictive values were calculated with 95% confidence interval (95% CI) using the standard formulas. Considering Real-Time PCR as gold standard, shell vial culture method showed a sensitivity of 60% (95% CI 0.3127-0.8318) and specificity of 100% (95% CI 0.9442-1) with positive prediction value of 100% and negative prediction value of 94.5% (Table-2). We showed our results in graphical presentation of sensitivity and specificity between Real-time PCR and Shell vial culture techniques for the detection of herpes simplex virus (Figure 1). All 3 shell vial culture negative sample which were positive by real-Time PCR had low HSV DNA copy number. Shell vial culture results were positive when copy count of HSV was \(\geq\)1429/ml (Table-1). All isolates were HSV type 2.

4. DISCUSSION

Shell vial culture works on the principle that on centrifugation mechanical force enhances the viral infectivity to the susceptible cells\(^2\). In different studies, shell vial culture technique has been shown to increase the rate of isolation of the viruses without any compromise on the specificity and also has been shown to significantly reduce the time taken to as less as 24 hours as compared to conventional cell culture technique. There have been no documented studies, which purely compare the sensitivity and specificity of shell vial assay and real-time PCR (as gold standard). In our study shell vial assay showed a sensitivity of 60%, specificity and positive prediction value of 100% and negative prediction value of 94.5%. The shell vial assay was positive once the HSV DNA copy number exceeded \(\geq\)1429 HSV DNA copies/ml. The real-time PCR offers more information than other tests currently available. However, this procedure should be used in conjugation with patient history and other laboratory data to support a clinical
Table 1

Characteristics of cerebrospinal fluid samples those were positive for Herpes Simplex Virus

<table>
<thead>
<tr>
<th>No</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Shell Vail Culture</th>
<th>Real Time PCR ( Copies /ml )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>F</td>
<td>SLE, Encephalopathy</td>
<td>Positive</td>
<td>1429</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>M</td>
<td>Viral Encephalitis</td>
<td>Negative</td>
<td>1122</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>F</td>
<td>Encephalopathy</td>
<td>Positive</td>
<td>15138</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>M</td>
<td>Herpes Encephalitis</td>
<td>Positive</td>
<td>4369</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>F</td>
<td>Herpes Encephalitis</td>
<td>Negative</td>
<td>970</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>M</td>
<td>Viral Encephalitis</td>
<td>Negative</td>
<td>77</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>M</td>
<td>Viral Encephalitis</td>
<td>Positive</td>
<td>8696849</td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>F</td>
<td>Encephalopathy</td>
<td>Positive</td>
<td>14123</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>M</td>
<td>Viral Encephalitis</td>
<td>Positive</td>
<td>8756</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>M</td>
<td>Herpes Encephalitis</td>
<td>Negative</td>
<td>57</td>
</tr>
</tbody>
</table>

Table 2.

Comparative analysis of sensitivity and specificity between Real-time PCR and Shell vial culture techniques for the detection of herpes simplex virus in cerebrospinal fluid.

<table>
<thead>
<tr>
<th>Shell Vial culture</th>
<th>Real Time PCR</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>6</td>
<td>60%</td>
<td>100%</td>
<td>100%</td>
<td>94.50%</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>65</td>
<td>100%</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

Sensitivity (Sen.), specificity (Spec.), Positive and Negative Predictive Values of Shell Vial Culture was calculated using Real Time PCR as the gold standard with 95% confidence interval (95% CI) using the standard formulas.

Figure 1:
Comparative graphical analysis of sensitivity and specificity between Real-time PCR and Shell vial culture techniques.
diagnosis. Real-time PCR with shell vial culture may prove to be practical in initiating the acyclovir treatment in most centers in India. Even though demerits of shell vial culture are low sensitivity, time taking, require high viral load, highly equipped viral laboratory. The rapid diagnosis of HSV infections can be extremely clinically useful, especially when a clinical diagnosis is uncertain in the immunocompromised patient, the pregnant female, and the neonate, where atypical lesions may be present, or when antiviral therapy is contemplated. HSV is the most frequently detected virus in most clinical laboratories. New rapid assays, such as immunofluorescence and EIA, have not improved the sensitivity of HSV detection over conventional cell culture. The benefit of direct detection by real-time PCR has proven to be cost-effective on a per-run basis, when implemented with a high-throughput laboratory, particularly when replacing conventional, culture-based approaches to microbial detection. Its also demonstrates high sensitivity and specificity with turnaround times of less than 2 hours from specimen receipt to a result being available in the diagnosis of HSE.

5. REFERENCES


[16] Anderson TP, Werno AM, Beynon KA, Murdoch DR. Failure to genotype herpes simplex virus by real-time PCR assay and melting curve analysis due to sequence variation.


