1. 1. BACKGROUND

PCR amplification of HSV DNA in CSF specimens is now the recognized reference standard assay for the sensitive and specific diagnosis of CNS infections caused by HSV ***(Mitchell et al., 1997; Ito et al., 1998)***. PCR assay of CSF samples for detection of HSV DNA had been used to confirm the diagnosis of HSE ***(Rowley et al., 1990; Aurelius et al., 1991; Kimura et al., 1991; Aslanzadeh et al., 1992; Aslanzadeh et al., 1993)***. The widespread use of PCR amplification of HSV DNA has led to a change in our understanding of the signs and symptoms of HSE and has dramatically broadened the observed clinical symptoms of HSV related infections of the CNS ***(Koskiniemi et al., 1996; Domingues et al., 1997(a); Domingues et al., 1997(b))***. Therefore, prospective studies using this technique may be useful for defining the observed clinical manifestations of HSE.

A suitable target for amplification is very important to obtain good sensitivity and specificity of the PCR assay. The choice of target sequence of DNA and the design of primers within the sequence are equally important. Seven major loci, distributed throughout the unique long (UL) ***(Aslanzadeh et al., 1992)*** and unique short (US) ***(Anderson et al., 1993)*** regions of the 152-kb genome of HSV, have been reported for the detection of viral DNA in clinical specimens ***(Tang et al., 1999)***. Among these regions, DNA polymerase gene (UL30) is the most widely used target to amplify sequences of HSV ***(Troendle-Atkins et al., 1993; Kessler et al., 1994; Vrioni et al., 2007)***. This target is well conserved in the genome of both HSV-1 and -2 and hence can

be used for the type common diagnosis of infection caused by either HSV-1 or -2.

Differentiation of the two types of HSV is often useful for epidemiologic purposes. HSV-1 has been almost exclusively associated with focal, necrotizing forms of encephalitis typically localized to the temporal lobes of the brain, whereas, HSV-2 generally causes meningitis and milder forms of CNS disease. Molecular methods for genotype distinction have included unique enzyme restriction sites, nested amplification using type-specific primers, multiplex PCR, type-specific probes, direct sequence analysis, and allele-specific PCR ***(Kimura et al., 1990; Lakeman and Whitley, 1995; Casas et al., 1996; Cassinoti et al., 1996)***. PCR of HSV DNA in CSF specimens has not yielded technically convenient methods for HSV genotype designation ***(Shoji et al., 1994; Abraham et al., 2009)***. Thus, because of the lack of variability in the nucleotide sequence of the HSV genes coding for structural components of the virus, differentiation of the two genotypes has not been routinely obtained. US3 gene is found conserved in the US region of HSV genome containing certain stretches of DNA that show variation in sequence among the two viruses. This gene encodes a serine/threonine kinase. Therefore, to determine the prevalence of the type of HSV in the study population primers targeting US3 gene of HSV-1 and HSV-2 can be designed to differentiate between the two on the basis of PCR.

The aim of this study was thus to ascertain the usefulness of PCR methodology for diagnosis of HSV infections of the CNS in clinical specimens.

All CSF samples submitted for HSV PCR analysis from September 2008 through August 2013 were included in the study. Prior to the development of in-house PCR assays, the samples were analyzed for HSV by PCR using commercially available kit. The in-house PCR assays were developed by optimizing the conditions for the detection of HSV and the results were compared with the commercial kit. Two sets of target were used for the development of in-house PCR assay, one targeting the UL30 gene (DNA polymerase gene) for type-common detection of HSV and the other targeting the US3 gene (serine/thereonine kinase gene) of HSV-1 and HSV-2 to differentiate between the two on the basis of PCR.

Target loci in the HSV genome for detection of DNA in CSF specimens ***(Adapted from Tang et al., 1999)***

HSV gene/ transcriptional unit	Designation of protein	Reference(s)
UL23	TK	*Kimura et al., 1990; Nicoll et al., 1991; Dennett et al., 1997; Mitchell et al., 1997; Tang et al., 1998; Behzad-Behbahani et al., 2003*
UL27	Glycoprotein B	*Pohl-Koppe et al., 1992; Lakeman and Whitley, 1995*
UL30	DNA polymerase	*Rowley et al., 1990; Kimura et al., 1991; Rozenberg and Lebon, 1991; Aslanzadeh et al., 1992; Ando et al., 1993; Aslanzadeh et al., 1993; Troendle-Atkins et al., 1993; Guffond et al., 1994; Kessler et al., 1994; Nakajima et al., 1995; Tyler et al., 1995; Casas et al., 1996; Koskiniemi et al., 1996; Mitchell et al., 1997; Revello et al., 1997; Fomsgaard et al., 1998; Ito et al., 1998; Minjolle et al., 1999; Simko et al., 2002; Kamei et al., 2004; Benjamin et al, 2013; Lévêque et al., 2014*
UL42	DNA binding	*Puchhammer-StoÈckl et al., 1990; Cassinotti et al., 1996*
UL44	Glycoprotein C	*Shoji et al., 1994*
US4	Glycoprotein G	*Aurelius et al., 1991; Cassinotti et al., 1996; Cinque et al., 1998*
US6	Glycoprotein D	*Powell et al., 1990; Anderson et al., 1993; Uren et al., 1993; Kudelova et al., 1995; Cassinotti et al., 1996; Cinque et al., 1998; Danise et al., 1997; Jeffery et al., 1997; Read et al., 1997; Read et al., 1999; Rodrigues et al., 2013*

1. 2. MATERIALS AND METHODS

1. 2. 1. Patient selection and samples collection

The Central India Institute of Medical Sciences, Nagpur, is a tertiary referral center. Patients with suspected cases of HSE who were admitted were enrolled in this study. Neurological diagnostic investigations were performed during the first week of hospitalization; these investigations included the acid fast bacilli (AFB), India ink and Gram stain, microbial culture, HIV status, estimation of protein and sugar and cell counts in CSF, CT scan and MRI of the brain. In CT plain/contrast imaging of the brain was done, whereas, for MRI T1 & T2-weighted, DWI and FLAIR images were taken.

CSF was collected under all aseptic precautions by standard lumbar puncture technique. Approximately 5 ml of CSF was obtained. The collected samples were divided into two fractions, one fraction was subjected for antigen/IgM/IgG/PCR analysis and the other fraction was used for microbial analysis along with cell count, protein and sugar content. The initial samples were drawn from all the patients before treatment. CSF samples were obtained from some of the patients whenever possible during treatment.

Clinical data of patients was prospectively collected on case record forms. The clinical outcome for each patient was assessed. Patients were re-examined at the time of completion of treatment. The outcome was assessed in terms of normal clinical recovery; mild, moderate or severe impairment; or death.

Clinically, the patients were divided into the categories of those with and without HSV infection of the CNS as discussed below-

1. 2. 1. 1. HSE (n=140)

HSE was suspected on the basis of observations that included the presence of fever, altered mental status (low level of consciousness, disorientation, behaviour or personality changes) and other clinical manifestations (e.g. focal neurological deficits, seizures etc.). All patients received intravenous acyclovir (30 mg/kg of body weight/day) as empirical therapy.

(a) Confirmed HSE

Acute case of HSV infection was defined based on clinical and/or MRI features consistent with HSE and in whom HSV infection was confirmed by conventional PCR assay. All patients diagnosed with HSE were treated intravenously with acyclovir for 8 - 21 days.

(b) Suspected HSE

This group included patients with acute onset of fever and clinical features similar to viral encephalitis. CSF findings showed mild increase in protein, glucose often normal and mild pleocytosis. Patients showed good clinical response to acyclovir treatment.

1. 2. 1. 2. Non- HSE group (n=179)

This group included patients with tuberculous, pyogenic or fungal meningitis and non-infectious neurological disorders such as hypertension, status epilepticus, stroke etc.

(a) Other infectious cases (n=87)

Patients included in this group were of tuberculous meningitis (TBM), pyogenic and fungal meningitis.

TBM: Diagnosis of TBM was based on clinical features including sub-acute or chronic fever with features of meningeal irritation such as headache, neck stiffness and vomiting, with or without other features of CNS involvement. CSF findings in these patients included increased proteins, decreased glucose (CSF:blood glucose ratio<0.5), and/or pleocytosis with lymphocytic predominance. All these patients showed good clinical response to antituberculous drugs.

Non-tuberculous infectious meningitis: This group included patients having pyogenic or fungal meningitis. Pyogenic meningitis was suspected in patients who had acute high-grade fever with features of meningitis. These patients often had altered sensorial, as well as CSF findings of increased proteins, very low sugar (CSF:blood glucose ratio<0.2), and pleocytosis with polymorphonuclear predominance. Response to broad-spectrum antibiotics was also considered as one of the diagnostic criteria for pyogenic meningitis. Fungal meningitis showed CSF profiles similar to TBM; however India ink staining showed the presence of potential etiological agents such as

Cryptococcus. Fungal meningitis was further confirmed by culturing on selective media.

(b) Non-Infectious neurological disorders (n=92)

All other patients who had no evidence of CNS or extra CNS bacterial, fungal or viral infections were grouped in the non-infectious neurological disorders group. Patients included in this group had hypertension, status epilepticus, stroke or other disorders.

Institutional Ethics Committee

An informed consent was obtained from all the patients. The Institutional Ethics Committee of Central India Institute of Medical Sciences, Nagpur, India approved the study.

1. 2. 2. DNA Extraction

The genomic DNA was extracted from 200 µl of CSF samples from patients by using a ZR Viral DNA kit (Zymo Research, CA, USA), according to the manufacturer's protocol. In each 1.5 ml microcentrifuge tube, 800 µl ZR viral DNA buffer was added to 200 µl of CSF sample. Tubes were mixed briefly by vortexing and allowed to stand at room temperature for 5-10 minutes. The mixture was then transferred to a Zymo-Spin™ IC column in a collection tube and centrifuged at 8000 rpm for 1 min. The flow through was discarded from the collection tube. 300 µl of DNA wash buffer was added to the column and tubes were centrifuged at 8000 rpm for 1 min. The flow through was discarded and this step was repeated for one more time. The columns were then placed

into a microcentrifuge tube. The viral DNA was eluted from the Zymo-Spin IC columns in a volume of 20 µl of elution buffer and was then used for PCR protocol. DNA was stored at -20°C for long term storage.

1. 2. 3. Primer designing

The designing and analysis of the primers was done with the help of online software Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were checked and analyzed for secondary structures, hairpins and dimmers using NetPrimer software (http://www.premierbiosoft.com/netprimer/) and optimal primer pairs were selected for synthesis. The cross reactivity of primers was checked with other organisms using National Centre for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST).

1. 2. 4. Primer synthesis

The primers were synthesized by Sigma-Genosys at a scale of 5 OD. Lyophilized primers were dissolved in Tris- ethylene diamine tetra acetic acid (EDTA) [TE] buffer at a concentration of 100 µM.

1. 2. 5. Positive and negative controls of PCR

With each run, both a positive and negative controls were included. The positive control was the DNA obtained from culture of HSV-1 and HSV-2 provided by Dr. Shankar Madhavan, Shankara Nethralaya, Chennai as a kind gift. The negative control was PCR grade water instead of sample DNA. To prevent cross-contamination, different sets of pipettes and distinct work areas were used for DNA template preparation, PCR mixture preparation, DNA

amplification and gel analysis. Moreover, one positive and negative control was used during DNA extraction and PCR with every set of samples.

1. 2. 6. PCR protocols for different assays and primers

1. 2. 6. 1. Amplification using commercial kit

PCR was performed by using commercially available kit for detection of HSV-1 and -2 as per manufacturer's protocol (Cinnagen, Inc., Iran). The amplification reactions consisted of 1X PCR mix, Taq DNA polymerase in each tube with 5 µl DNase free deionized water in negative control, 5 µl (1pg/µl) of standard in positive control and 5 µl of sample DNA in sample reaction tube. The final volume of each PCR reaction was 25 µl. The PCR reaction was performed in a thermal minicycler (Peqlab Biotechnology GmbH, Erlangen, Germany). Each cycle consisted of initial denaturation at 94°C for 1min, followed by 50 cycles each consisting of 94ºC for 1 min, 72°C for 40 s, and further primer extension at 72ºC for 7 min. The presence of 256 bp fragment indicated a positive test.

1. 2. 6. 2. Amplification of UL30 gene target

The set of primers used for HSV has been described earlier ***(Lakeman and Whitley, 1995; Cunningham et al., 1996)*** (5'-ATC AAC TTC GAC TGG CCC TT-3' and 5'-CCG TAC ATG TCG ATG TTC AC-3') which generate a 179 bp product of the UL30 (DNA polymerase) gene after amplification. A 50 µl reaction contained 10X assay buffer (Bangalore Genei, Bangalore, India), 2mM of $MgCl_2$ (Bangalore Genei, Bangalore, India), 0.8 mM deoxynucleotide

triphosphates (dNTP) (Bangalore Genei, Bangalore, India), 1 µM of each primer (Sigma-Genosys, USA), 2 units of Taq DNA Polymerase (Bangalore Genei, Bangalore, India) and 5 µl of extracted DNA. Amplification was carried out in a Veriti thermal cycler (Applied Biosystems, Foster City, USA), which involved 40 cycles of denaturation at 94°C for 1 min, annealing of primers at 65°C for 1 min, and primer extension at 72°C for 1 min.

1. 2. 6. 3. Amplification of US3 gene target for differentiation of HSV-1 and HSV-2

US3 gene is found conserved in the US region of HSV genome. This gene encodes a serine/threonine kinase. Primers targeting US3 gene of HSV-1 and HSV-2 were designed to differentiate between the two on the basis of PCR. The set of primers used for HSV-1 were (5' AACGCGTCCTTGTTCTCGGC-3' and 5'-TGAGGCGCGATTCTGGATGC-3') and that of HSV-2 were (5'-ATAGCAGCCACCCGAACTACCC-3' and 5'-TGACCCCAGAAACGACGTGC-3') which generate 127 bp and 144 bp product for HSV-1 and -2 respectively upon amplification. The details of primers are shown in tables below. Reaction mixtures for amplification is same as described in the earlier paragraph except the primer concentration i.e. 0.5 µM of each primer (Sigma-Genosys, USA) and the annealing temperature of 58°C for 1 min.

HSV-1 primers

	Sequence (5'->3')	Strand on template	Length	Start	Stop	Melting temperatu -re (Tm)	GC %
Forward primer	AACGCGTCCTTG TTCTCGGC	Plus	20	1393	1412	57.9	60%
Reverse primer	TGAGGCGCGATT CTGGATGC	Minus	20	1519	1500	57.5	60%
Product length	127						

HSV-2 primers

	Sequence (5'->3')	Strand on template	Length	Start	Stop	Tm	GC %
Forward primer	ATAGCAGCCACCCGAA CTACCC	Plus	22	620	641	58.11	59%
Reverse primer	TGACCCCAGAAACGAC GTGC	Minus	20	763	744	57.22	60%
Product length	144						

1. 2. 7. Analysis of PCR products

The amplified DNA was detected after electrophoresis in 2% agarose gel stained with 6µl of 10mg/ml Ethidium Bromide (for 50ml of agarose gel) and gel was visualized on a Gel documentation system (Bio-Rad Laboratories, CA).

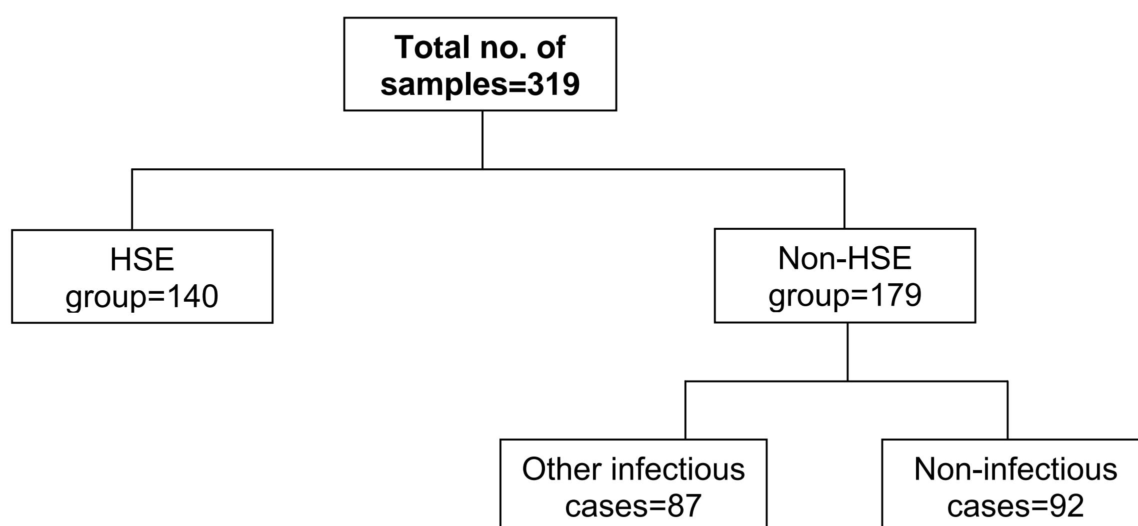
1. 2. 8. Statistical analysis

The statistical analysis was done by the Chi-square test using the Med-calc software (version 10.1.2).

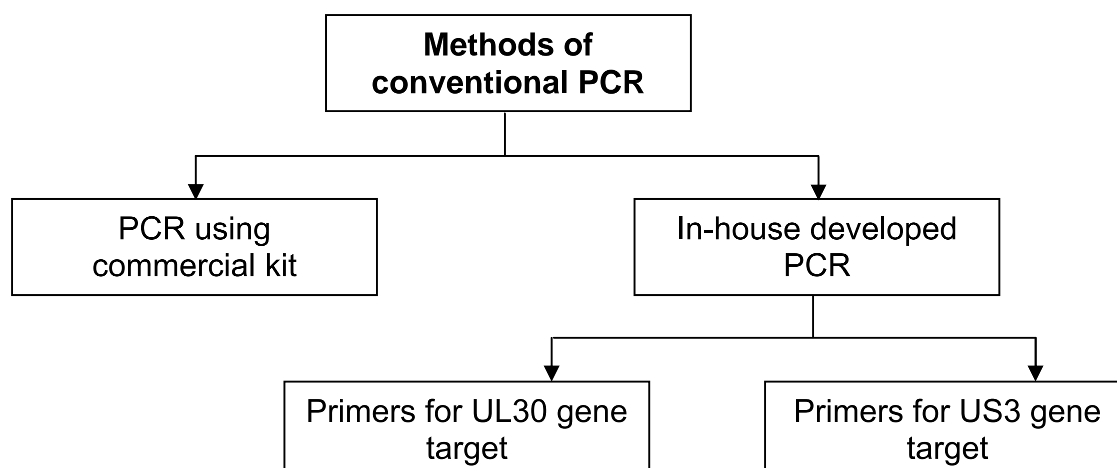
1. 3. RESULTS

In the present study, prospective detection of HSV DNA in CSF specimens from September 2008 through August 2013 was carried out by the commercial available kit and by using the literature based primers of the UL30 gene target. The samples were also tested by the primers targeting US3 gene of HSV-1 and that of HSV-2.

Flow chart for classification of patients in different groups



Flow chart for analysis of samples of HSE and non-HSE groups by the conventional PCR assays



Type-common detection of HSV-1 and HSV-2 was done using the commercial available kit and by using the literature based primers of the UL30 gene target (Figure 1. 1 and 1. 2). In a further extension of the study, the samples positive by assay of the UL30 gene target were also tested by the primers targeting US3 gene of HSV-1 and that of HSV-2. The cross reactivity of primers of both the viruses was checked by subjecting HSV-1 to be amplified in presence of HSV-2 primers and vice versa, respectively (Figure 1. 4 and 1. 6). A known amount of HSV DNA was taken in 10-fold serial dilutions from 1ng-1fg. The limit of detection (LOD) was found to be 10fg of HSV DNA to be detected by the assay of both UL30 and US3 gene targets (Figure 1. 3, 1. 5 and 1. 7).

Table 1. 1 shows prospective detection of HSV DNA in CSF from September 2008 through August 2013 in total of 319 CSF specimens. Among the 319 cases, out of a total of 140 CSF samples of patients initially suspected for HSE, 36 CSF samples were found to be positive for the presence of HSV by PCR using commercial kit, whereas primers targeting the UL30 gene detected 35 cases positive for HSV. Approximately 105 cases suspected for HSE were found to be negative by the both the assays. In addition to that, 179 CSF samples from patients with other CNS infectious or non-infectious neurological disorders were found to be negative by the assays. 35 samples positive by assay of the UL30 gene target were also found to be positive by HSV-1 PCR, whereas none of the samples were positive by HSV-2 PCR. In the 179 CSF samples from patients with other CNS infectious or non-infectious neurological disorders tested by both the assays, none was found to be positive for the presence of HSV-1 or HSV-2. There was no major

difference between the results obtained by the commercial and in-house PCR assays detecting the presence of HSV in CSF.

In an analysis of 319 CSF samples, 192 (60%) were male whereas 127 (40%) were females. Out of 35 patients positive for HSV DNA, 20 (57%) were male and 15 (43%) were females. However, the proportion of males to females positive for HSV DNA was more or less similar throughout the study period (Table 1. 2).

Table 1. 3 shows age distribution in HSE and non-HSE subjects. Greater number of PCR positive patients belonged to age groups between 41-50 [07 (20%)] and 51-60 [11 (31.42%)].

Evaluation of possible relationships was done between presence of HSV DNA in CSF with that of patients clinical and laboratory manifestations (Table 1. 4). There were no significant clinical differences between confirmed HSE (PCR positive) cases and suspected HSE (PCR negative) cases. Although a reduced level of consciousness was more commonly associated with PCR positive cases, this difference was not statistically significant (P=0.0967). No significant differences were found on neurological examination of these patients in motor or cranial nerve deficit and sensory abnormality.

CSF tests. White blood cell counts in CSF samples except for polymorphs were similar in PCR positive and PCR negative cases.

Neurodiagnostic findings. CT/MRI showed abnormalities in more number of patients in PCR positive as compared to PCR negative group; however, the

difference was not statistically significant.

Clinical outcome. Most of the patients of the PCR positive group had mild to moderate or severe impairment after completion of treatment. The mortality rate associated with CNS infection in patients with PCR negative cases was more than PCR positive cases.

Figure 1. 1. Gel showing the results of PCR using the commercial kit. M shows the 100 bp molecular weight marker. L6 contain positive control DNA, L1-L5 contains DNA obtained from CSF sample (L1 negative by PCR; L2-L5 positive by PCR) and L7 contains no-template control (NTC).

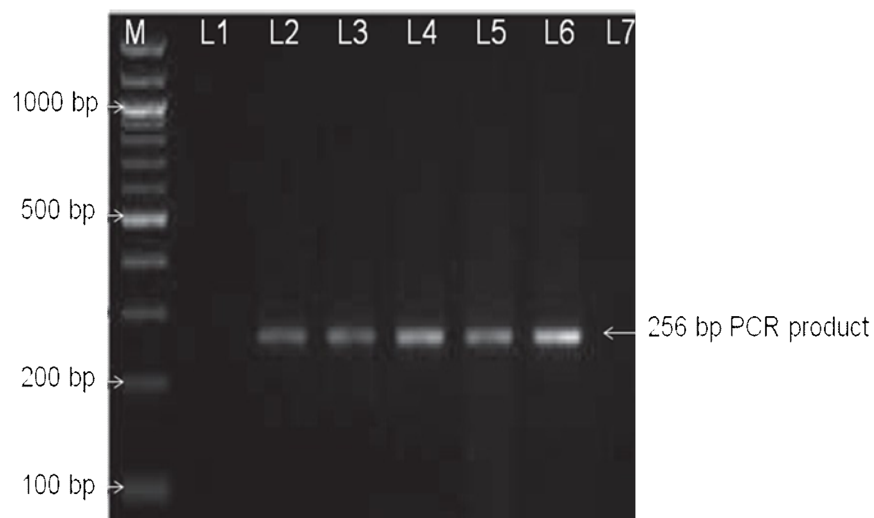


Figure 1. 2. Gel for optimized HSV PCR using the primers targeting the UL30 gene target. M shows the 100 bp molecular weight marker. L1 and L2 contain positive control DNA of HSV-1 and HSV-2 respectively, L6 contains NTC. L3-L5 contains DNA obtained after extraction from CSF sample (L4 negative by PCR; L3 and L5 positive by PCR).

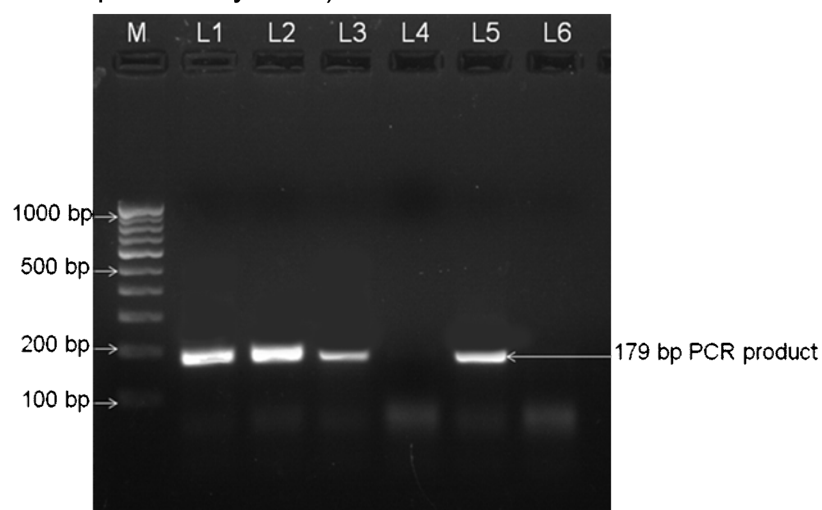


Figure 1. 3. Gel shows the detection limit of UL30 gene target. The detection limit of PCR was checked by making 1:10 dilutions of positive control. Dilution series was prepared of HSV DNA from 1ng-1fg. M shows the 100bp molecular weight marker. L1 contains 1 ng DNA, L2 contains 100 pg DNA, L3 contains 10 pg DNA, L4 contains 1 pg DNA, L5 contains 100 fg DNA, L6 contains 10 fg DNA, L7 contains 1 fg DNA

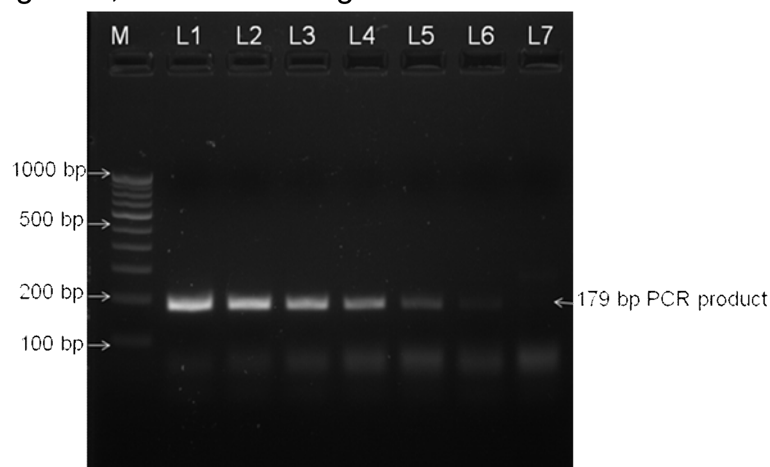


Figure 1. 4. Gel for optimized HSV PCR using the primers targeting the US3 gene target of HSV-1. M shows the 100bp molecular weight marker. L1 and L4 contain positive control DNA of HSV-1 and HSV-2 respectively, L5 contains NTC. L2 & L3 (positive by PCR) and L6 & L7 (negative by PCR) contain DNA obtained after extraction from CSF sample.

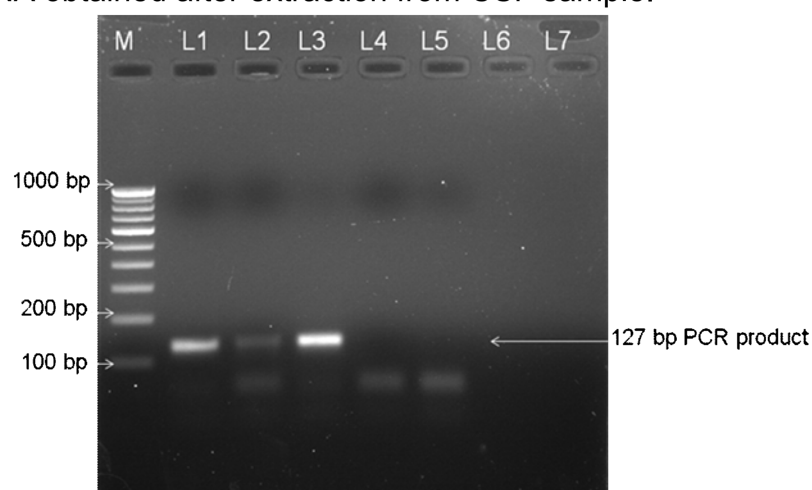


Figure 1. 5. Gel shows the detection limit of US3 gene target of HSV-1. The detection limit of PCR was checked by making 1:10 dilutions of positive control. Dilution series was prepared of HSV-1 DNA from 1ng-1fg. M shows the 100bp molecular weight marker. L1 contains 1 ng DNA, L2 contains 100 pg DNA, L3 contains 10 pg DNA, L4 contains 1 pg DNA, L5 contains 100 fg DNA, L6 contains 10 fg DNA, L7 contains 1 fg DNA.

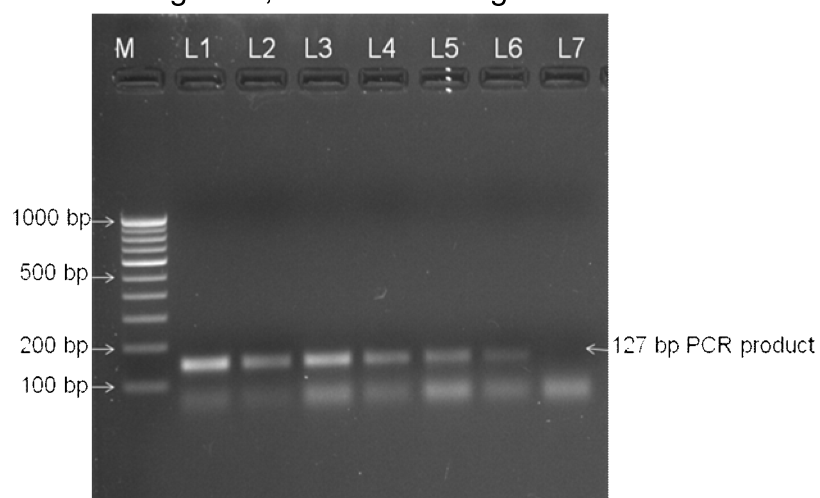


Figure 1. 6. Gel for optimized HSV PCR using the primers targeting the US3 gene target of HSV-2. M shows the 100bp molecular weight marker. L1 and L4 contain positive control DNA of HSV-2 and HSV-1 respectively, L5 contains NTC. L2 and L3 contain DNA obtained after extraction from CSF sample (negative by PCR).

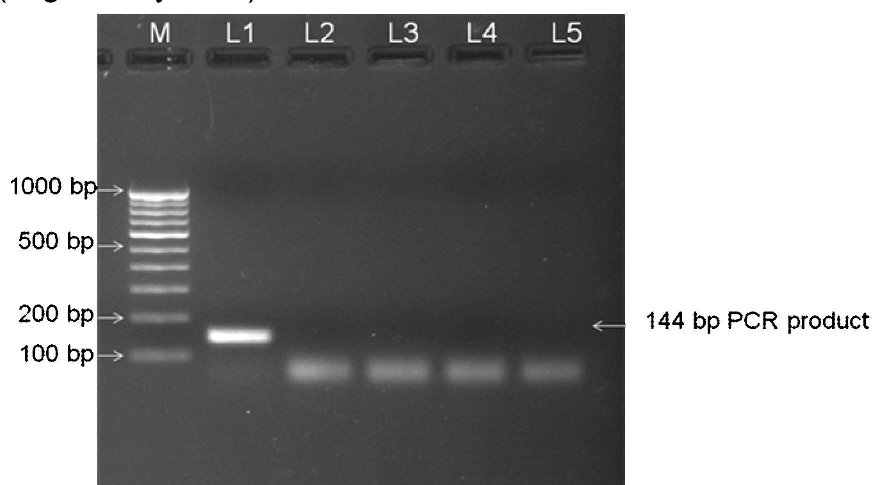


Figure 1. 7. Gel shows the detection limit of US3 gene target of HSV-2. The detection limit of PCR was checked by making 1:10 dilutions of positive control. Dilution series was prepared of HSV-2 DNA from 1ng-1fg. M shows the 100bp molecular weight marker. L1 contains 1 ng DNA, L2 contains 100 pg DNA, L3 contains 10 pg DNA, L4 contains 1 pg DNA, L5 contains 100 fg DNA, L6 contains 10 fg DNA, L7 contains 1 fg DNA.

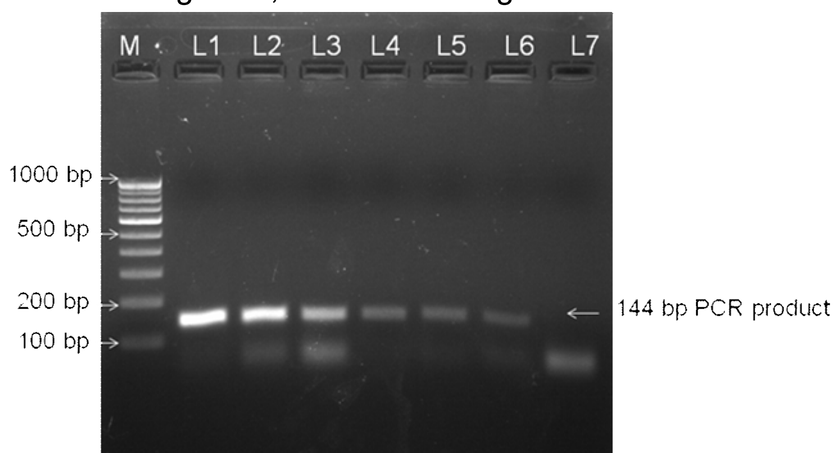


Table 1. 1. Detection of HSV DNA from CSF specimens from September 2008 through August 2013

Duration	No. of specimens tested	No. of positive specimens by commercial kit	No. of positive specimens by in-house PCR		
			DNA pol gene target	US3 gene target	
				HSV-1	HSV-2
Sep 2008-Aug 2009	75	13	12	12	0
Sep 2009-Aug 2010	58	4	4	4	0
Sep 2010-Aug 2011	63	7	7	7	0
Sep 2011-Aug 2012	64	4	4	4	0
Sep 2012-Aug 2013	59	8	8	8	0
Total	**319**	**36**	**35**	**35**	**0**

Table 1. 2. Male and female prevalence in total number of subjects and in patients with HSV positive CSF specimens by in-house PCR protocol

No. of total subjects		Total male/female ratio	No. of subjects positive		HSV-positive male/female ratio
Male	Female		Male	Female	
44	31	1.42	8	4	2.00
30	28	1.07	2	2	1.00
43	20	2.15	3	4	0.75
38	26	1.46	2	2	1.00
37	22	1.68	5	3	1.67
192	**127**	**1.51**	**20**	**15**	**1.33**

Table 1. 3. Age distribution in total number of suspected HSE subjects and in patients with HSV positive CSF specimens

Age (years)	Total subjects n (%)	No. of subjects positive n (%)
<10	17 (5.32%)	03 (8.57%)
10-20	39 (12.22%)	04 (11.42%)
21-30	52 (16.30%)	02 (5.71%)
31-40	49 (15.36%)	03 (8.57%)
41-50	38 (11.91%)	07 (20%)
51-60	68 (21.31%)	11 (31.42%)
>60	56 (17.55%)	05 (14.28%)

Table 1. 4. Assessment of clinical symptoms in the confirmed HSE and suspected HSE group

		Confirmed HSE (n=35)	Suspected HSE (n=105)	P value
Clinical symptoms n (%)	Motor deficit	20 (57.14%)	35 (33.33%)	0.2059
	Sensory abnormality	13 (37.14%)	29 (27.61%)	0.3939
	Cranial nerve deficit	22 (62.85%)	51 (48.57%)	0.2044
	Visual field disorder	0	3 (2.85%)	0.7377
	Reduced level of consciousness	23 (65.71%)	50 (47.61%)	0.0967
	Seizures	17 (48.57%)	46 (43. 81%)	0.7687
Imaging	CT/MRI lesions n (%)	19 (54.28%)	37 (35.23%)	0.0729
CSF tests	TLC	0-500	0-500	--
	Lymphocyte (%)	0-100	0-100	
	Polymorphs (%)	1-2	0-50	
	Sugar (mg/dl)	32.48-118.2	26-128	
	Protein (mg/dl)	23.44-187.46	20.64-301.4	
	AFB negative	35 (100%)	105 (100%)	
	Gram stain negative	35 (100%)	105 (100%)	
	Bacterial culture negative	35 (100%)	105 (100%)	
	HIV negative	35 (100%)	105 (100%)	
Clinical Outcome	Normal	12 (34.28%)	37 (35.23%)	0.0879
	Mild to moderate impairment	16 (45.71%)	34 (32.38%)	
	Severe impairment	5 (14.28%)	3 (2.85%)	
	Death	2 (5.71%)	12 (11.43%)	

1. 4. DISCUSSION AND CONCLUSIONS

Several studies, which have been reviewed, have demonstrated the utility of the laboratory molecular diagnosis of HSE and other CNS infections ***(Tang et al., 1999; Read et al., 1997; Yerly et al., 1996; Aurelius et al., 1993)***. PCR has been considered to revolutionize the HSE diagnosis by amplifying viral nucleic acid from CSF ***(Aurelius et al., 1991; Koskiniemi et al., 1996; Baringer, 2000)***. Therefore, prospective studies using this technique may be useful for defining the spectrum of clinical manifestations of HSE.

HSV PCR has become one of the most commonly performed tests of CSF in viral infections of the CNS. At our institution, there has been an increased demand of PCR tests ordering as part of the evaluation of suspected CNS infections, often without prioritization in order of diagnostic likelihood. All CSF samples submitted for HSV PCR analysis from September 2008 through August 2013 were included in the study. The aim was thus to ascertain the usefulness of PCR methodology for routine diagnostic testing of HSV infections of the CNS in clinical specimens which was done by optimizing the conditions for the detection of HSV-1 and -2 by PCR.

I prospectively analyzed CSF samples submitted to our laboratory for HSV detection by PCR. These samples were also analyzed for HSV-1 and -2 by PCR assay using commercially available kit and in-house developed protocols and the results were compared. The present study thus focused on the

preliminary evaluation of target genes of HSV in the samples of suspected HSE patients for diagnostic purpose.

During this study, CSF samples of suspected HSE cases were screened by PCR assay. Type-common detection of HSV-1 and HSV-2 was done using the available commercial kit and by using the literature based primers of the UL30 gene target. Out of a total of 140 CSF samples of patients initially suspected for HSE, 36 CSF samples were found to be positive for the presence of HSV by PCR using commercial kit, whereas primers targeting the UL30 gene detected 35 cases positive for HSV. Approximately 105 cases suspected for HSE were found to be negative by the both the assays. In addition to that, 179 CSF samples from patients with other CNS infectious or non-infectious neurological disorders were found to be negative by the assays. Thus, the primers for the UL30 gene target detected almost all the samples positive for HSV in comparison to the available commercial kit.

Thirty five samples positive by assay of the UL30 gene target were also found to be positive by in-house HSV-1 PCR, whereas none of the samples were positive by in-house HSV-2 PCR that suggest the prevalence of HSV-1 genotype in the study population. In the 179 CSF samples from patients with other CNS infectious or non-infectious neurological disorders tested by both the assays, none was found to be positive for the presence of HSV-1 or HSV-2.

Koskiniemi et al., 1996, showed that HSE is a disease of the middle-aged and elderly. Similar findings were obtained in this study where greater number of PCR positive patients belonged to age group in between 41-50 [07 (20%)] and 51-60 [11 (31.42%)]. There was no significant difference in the number of males and females of the HSE group. Gender based differences have not been reported in HSE ***(Riera-Mestre et al., 2009)***.

Reduced level of consciousness was more commonly associated with PCR positive cases as compared to PCR negative cases. This has previously been shown by some workers ***(Domingues et al., 1998)***. CSF profile usually does not show any significant differences in HSV infections of the CNS ***(Hanson et al., 2007)***. Neurodiagnostic findings have shown differences in PCR positive and PCR negative cases of HSE ***(Hatipoglu et al., 2008; Sili et al., 2014)***. CT/MRI showed abnormalities in more number of patients in PCR positive as compared to PCR negative group. In all the cases of HSE, abnormalities revealed by CT included hypodensities involving temporal lobe and occipital lobes, parafalcine frontal cortex, cerebellum, thalami and corona radiata. MRI showed gyral edema, hyperintensities in cingulate gyrus, hippocampus, insular/parietal/ parasaggital/ parafalcine/ perisylvian cortex and temporal or frontal lobe lesions. MRI revealed hyperintensity lesions in one or both inferomedian regions of the temporal lobes, which usually extended to the insular cortex.

In this study it was observed that, most of the patients of the PCR positive group had mild to moderate or severe impairment after completion of

treatment. The age of the patient and the level of consciousness at initiation of therapy have been identified as major determinants of prognosis and have been shown to affect the clinical outcome of patients ***(Whitley et al., 1986)***. Studies have assessed the clinical outcome of patients in HSE and have suggested the involvement of several factors affecting the clinical outcome of HSE patients ***(Raschilas et al., 2002; Hsieh et al., 2007)***.

The results obtained with in-house PCR assays were similar to the results obtained by the commercial kit for detecting the presence of HSV DNA in CSF. The developed technique presented in the study can be used in the identification of HSV infections of the CNS.