4. 1. BACKGROUND

Human herpesviruses cause various acute, subacute, and chronic disorders of the CNS and peripheral nervous systems in adults and children. Among herpesviruses, HSV-1 and -2 cause a wide spectrum of clinical manifestations like meningitis, encephalitis, myelitis etc. in the CNS of infants as well as adults. However, clinical criteria (symptoms) are not reliable enough to differentiate between different causes of encephalitis, as numerous neurological syndromes may mimic HSE. Therefore, accurate and rapid diagnosis of HSV infections is of utmost importance for effective therapy with antiviral drugs.

Molecular diagnostic assays using PCR are widely used for detecting herpesvirus infections of the CNS but can be negative when CSF is obtained at late times after the onset of disease ***(Aurelius et al., 1993; Landau et al., 2005)***. The IgM ELISA method, which provides evidence of HSV infection, is used to lend support to clinical findings in the assessment of patients with suspected HSV infection. However, the sensitivity of the IgM ELISA is low in the acute stage of illness. Additionally, IgG cannot be detected in HSV-infected patients in the acute stage ***(Whitley and Lakeman, 1995; Yamada et al., 2003)***. A rapid antigen detection test that uses ELISA for the detection of HSV infection may be a more accurate diagnostic method for patients in the acute stage of infection.

ELISA systems are specific (specificities ranging from 96 to 100%) but lack sensitivity (sensitivities ranging from 46 to 76%) in detecting HSV antigen directly from clinical specimens ***(Morgan and Smith, 1984; Warford et al., 1984)***. Immunologic analyses of CSF for the presence of specific antigens have not yielded sensitive tests for the laboratory diagnosis of HSV-related CNS disease ***(Coleman et al., 1983; Nerurkar et al., 1984; Ho and Hirsch, 1985)***. As the ELISA technology continues to improve, better methods have been developed to capture viral antigen and therefore improve sensitivity. The objective of this study was to develop a sensitive and specific ELISA protocol to detect HSV antigen. The present report describes a method in which hyperimmune sera from HSV seropositive patients was evaluated for the detection of antigen by an in-house ELISA method. The CSF and paired sera were simultaneously screened for HSV infection from patients with HSE.

4. 2. MATERIALS AND METHODS

4. 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 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-

4. 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 (n=35)

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 (n=105)

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.

4. 2. 1. 2. Non- HSE group (n=199)

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.

(c) Healthy controls (n=20): Additionally, serum samples of healthy volunteers from the working staff of the institute with no signs and symptoms of clinical impairment were included as controls.

4. 2. 2. DNA extraction and PCR

As discussed in Materials and Methods section of Chapter 1 and Chapter 2.

4. 2. 3. Anti-HSV IgG detection in sera

Anti-HSV IgG was detected in sera of HSE patients by Platelia HSV (1+2) ELISA Kit, Bio-Rad Laboratories, USA, according to the manufacturer's instructions. The method uses qualitative determination of IgG class antibodies to HSV in human serum. The antigen, composed of purified & inactivated HSV-1 and -2, were bound to the solid phase (8-well strips). The specific immunoglobulins were bound to the antigen after incubation with dilute human serum. After washing, incubation was done with the conjugate, composed of human IgG monoclonal antibodies labeled with Peroxidase. After washing, the substrate was added. The intensity of the color was read at 450 nm in an ELISA reader (Robonik, India Pvt. Ltd.).

4. 2. 4. Preparation of anti-HSV

Pooled sera from patients infected with HSV (positive for anti-HSV IgG as demonstrated by Platelia HSV (1+2) ELISA Kit, BioRad) was collected, and IgG was purified by protein G affinity column chromatography (IgG purification kit; Bangalore Genei, India), according to the manufacturer's instructions. The IgG bound to the column was eluted in different fractions of 1 ml each and the

fraction showing maximum absorbance at 280 nm was used at a dilution of 1:4000.

4. 2. 5. HSV antigen detection by ELISA

One hundred microliters of CSF (1:50) and paired serum samples (1:200) from HSV-infected patients was separately added to the microtiter wells (Greiner Bio-One, USA), and then the wells were blocked with 0.5% BSA in 1XPBS for 45 min. After the wells were washed with PBS, anti-HSV IgG was added (1: 4,000) and the plates were incubated at 37°C for 45 min. After incubation, the wells were washed and goat anti-human IgG–HRP secondary antibody (Banglore Genei, Banglore, India) (1:10,000) was added. The samples were then incubated for 45 min at 37°C. After another wash with PBS, 100 µl of the TMB-H_2O_2 substrate solution was added to the wells, which were incubated at room temperature for approximately 10 min. The reaction was then stopped with 100 µl of 2.5 N H_2SO_4. The absorbance of colour in each well was read at 450 nm.

4. 2. 6. HSV antigen detection by ELISA with Protein A

In order to improve the specificity of the method, a modification was done by incorporating protein A in the above mentioned protocol for ELISA. The wells of ELISA plate were separately coated with 100 µl of CSF (1:50) and paired serum (1:200) samples. Protein A (1: 4,000) was added after blocking with 0.5% BSA in PBS for 45 min and incubated for 45 min at 37°C. After incubation, the wells were washed and anti-HSV IgG was added (1: 4,000) and the plates were incubated at 37°C for 45 min. The wells were washed and

goat anti-human IgG–HRP secondary antibody (1:10,000) was added and incubated for another 45 min at 37°C. After another wash with PBS, 100 µl of the TMB-H_2O_2 substrate solution was added to the wells, which were incubated at room temperature for approximately 10 min. The reaction was then stopped with 100 µl of 2.5 N H_2SO_4. The absorbance of colour in each well was read at 450 nm.

4. 2. 7. Statistical analysis

The results are expressed as mean ± SD together with the range. Cut-off values for the absorbance of the HSV antigen were calculated by using mean ± SD of the absorbance of the HSV antigen for the control group. The sensitivity (true-positive rate) for the test was calculated as [the number of samples in the HSV-infected group with an absorbance of greater than or equal to the (mean ± SD) of the absorbance for the control group divided by the total number of samples for the HSV-infected group] x 100. The specificity (true-negative rate) for the test was calculated as [the number of samples in the HSV-infected group with an absorbance of less than the (mean ± SD) of the absorbance for the control group divided by the total number of samples for the group] x 100.

4. 3. RESULTS

CSF and paired sera samples of 319 patients admitted to the Neurological Department of CIIMS were grouped into HSE and non-HSE groups on the basis of PCR, clinical observations and biochemical and pathological analyses of CSF samples. The objective of the present study was to develop a sensitive ELISA protocol for HSV antigen detection by assessing the usefulness of hyperimmune sera isolated from HSV seropositive patients. To improve the specificity of the method, protein A was incorporated in the protocol for ELISA. The CSF and paired sera were simultaneously screened for HSV infection from patients with HSE.

Table 4. 1 shows the occurrence of HSV antigen in CSF from the HSE and non-HSE groups as determined by indirect ELISA method, along with the mean and range of the absorbance. The mean absorbance value for the HSV antigen in the HSE group was 1.19 ± 0.62 (range, 0.78 – 2.37). The cut-off value for absorbance at 450nm for a positive result of antigen detection was 0.87. The rate of positivity for HSV antigen with CSF for confirmed HSV infection was 68.57%, while the rate of positivity for patients in the suspected HSE and non-HSE group (other infectious and non-infectious groups) was 53.33% and 51.40% respectively. This higher rate of positivity in non-HSE groups could possibly be explained as the presence of IgG in the samples which might be cross-reacting with goat anti-human IgG–HRP secondary antibody.

Table 4. 2 depicts that with the use of protein A in the ELISA protocol, there was a significant decrease in the rates of positivity for non-HSE group **(p<0.028),** thereby decreasing false positivity and resulting in enhanced specificity. The mean absorbance value for the HSV antigen in the HSE group was 0.53 ±24 (range, 0.29 – 0.90) which was higher than that of non-HSE group, 0.31 ± 0.19 (range, 0.21 – 0.51). The cut-off value for absorbance at 450nm for a positive result of antigen detection was 0.33.

Table 4. 3 shows the presence of HSV antigen in the paired sera samples of HSE and non-HSE groups. The mean absorbance value for the HSV antigen in the HSE group was 1.80 ± 0.53 (range, 0.79 – 2.47). The cut-off value for absorbance at 450nm for a positive result of antigen detection was 1.34. The rates of positivity obtained were 64.37%, 43.48% and 20% for other infectious, non-infectious and healthy control groups, which were still higher than that of HSV antigen detection in CSF, owing to the presence of significantly raised level of immunoglobulin in the sera from all the subjects.

However, in the protein A based ELISA, there was a significant decrease in the rates of positivity with sera for non-HSE group **(p<0.001)** (Table 4. 4). The cut-off value for absorbance at 450nm for a positive result of antigen detection was 0.44. The mean absorbance value for the HSV antigen in the HSE group was 0.63 ± 0.22 (range, 0.41 – 1.13) which was higher than that of non-HSE group, 0.34 ± 0.24 (range, 0.16 - 1.08). However, number of positive cases has declined in sera from HSE group, giving a positivity of 74.29% (26/35), whereas antigen detection was positive for 82.86% (29/35) in CSF of HSE

group. The HSV antigen was not found in the healthy control group except 1 case (negativity, 95%).

The concordance for antigen detection in sera and CSF of HSE (n=35) patients was 62.85% whereas concordance for negative ELISA results was 91% between CSF and sera samples of non-HSE (n=179) group (Table 4. 5).

Table 4. 1. Demonstration of HSV antigen in CSF from subjects with and without HSV infection by ELISA along with mean absorbance and range

Patient group	Positivity for HSV Ag	Negativity for HSV Ag	Absorbance Mean ± SD	Range
HSE (n=35)	24 (68.57%)	3 (31.43%)	1.19 ± 0.62	0.78 – 2.37
Suspected HSE (n=105)	56 (53.33%)	49 (46.67%)	0.87 ± 0.47	0.31 – 1.67
Non-HSE (n=179)	92 (51.40%)	87 (48.60%)	0.81 ± 0.43	0.39 – 1.45
Other Infectious (n=87)	56 (64.36%)	31 (35.63%)	0.85 ± 0.28	0.33 – 1.32
Non-Infectious (n=92)	36 (39.13%)	56 (60.86%)	0.59 ± 0.20	0.32 – 0.97

Ag=antigen

Table 4. 2. Demonstration of HSV antigen in CSF from subjects with and without HSV infection by ELISA using Protein A along with mean absorbance and range

Patient group	Positivity for HSV Ag	Negativity for HSV Ag	Absorbance Mean ± SD	Range
HSE (n=35)	29 (82.86%)	6 (17.14%)	0.53 ±24	0.29 – 0.90
Suspected HSE (n=105)	54 (51.43%)	51 (48.57%)	0.36 ± 0.10	0.19 – 0.58
Non-HSE (n=179)	29 (16.2%)	150 (83.8%)	0.31 ± 0.19	0.21 – 0.51
Other Infectious (n=87)	19 (21.84%)	68 (78.16%)	0.29 ± 0.13	0.21 – 0.46
Non-Infectious (n=92)	10 (10.87%)	82 (89.13%)	0.27 ± 0. 08	0.19 – 0.35

Ag=antigen

Table 4. 3. Demonstration of HSV antigen in paired sera from subjects with and without HSV infection by ELISA along with mean absorbance and range

Patient group	Positivity for HSV Ag	Negativity for HSV Ag	Absorbance Mean ± SD	Range
HSE (n=35)	29 (82.86%)	6 (17.14%)	1.80 ± 0.53	0.79 – 2.47
Suspected HSE (n=105)	68 (64.76%)	37 (35.24%)	1.79 ± 0.33	1.42 – 2.33
Non-HSE (n=179)	97 (54.19%)	82 (45.81%)	1.59 ± 0.51	0.63 – 2.36
Other Infectious (n=87)	56 (64.37%)	31 (35.63%)	1.63 ± 0.43	1.09 – 2.24
Non-Infectious (n=92)	40 (43.48%)	52 (56.52%)	1.56 ± 0.53	0.87 – 1.85
Healthy Controls (n=20)	4 (20%)	16 (80%)	1.31 ± 0.42	0.6 – 1.31

Ag=antigen

Table 4. 4. Demonstration of HSV antigen in paired sera from subjects with and without HSV infection by ELISA using Protein A along with mean absorbance and range

Patient group	Positivity for HSV Ag	Negativity for HSV Ag	Absorbance Mean ± SD	Range
HSE (n=35)	26 (74.29%)	9 (25.71%)	0.63 ± 0.22	0.41 – 1.13
Suspected HSE (n=105)	42 (40%)	63 (60%)	0.36 ± 0.31	0.11 – 1.08
Non-HSE (n=179)	35 (19.55%)	144 (80.45%)	0.34 ± 0.24	0.16 – 1.08
Other Infectious (n=87)	19 (21.84%)	68 (78.16%)	0.34 ± 0.21	0.22 – 0.89
Non-Infectious (n=92)	15 (16.3%)	77 (83.7%**)**	0.35 ± 0.18	0.16 – 0.62
Healthy Controls (n=20)	1 (5%)	19 (95%)	0.26 ± 0.12	0.16 – 0.44

Ag=antigen

Table 4. 5. Concordance of ELISA results between sera and CSF for HSE and non-HSE groups

Category	ELISA results			Concordance (%)
		Serum positive	Serum negative	
HSE (n=35)	CSF positive (n=29)	21+	5-	62.85
	CSF negative (n=6)	5+	1-	
Non-HSE (n=179)	CSF positive (n=29)	24+	5-	91
	CSF negative (n=150)	11+	139-	

4. 4. DISCUSSION AND CONCLUSIONS

The detection of the viral antigen can be an alternative to isolation of the virus or the detection of viral nucleic acids, IgG, or IgM. After screening of HSE cases using the PCR methodology in our laboratory, I next developed an antigen detection assay by utilizing the hyperimmune sera from patients with HSE. Pooled sera from HSV IgG positive HSE patients were collected, IgG (antibody) was purified by protein G affinity column chromatography, and purified antibodies were used for the detection of HSV antigen. To our knowledge not much has been reported about the diagnostic significance of hyperimmune sera in HSE. However, studies have been reported, based on the evaluation of hyperimmune sera for antigen detection in clinical samples for the diagnosis of certain infections ***(Kashyap et al., 2010)***. I assessed the usefulness of antigen detection assay on the basis of indirect ELISA method for the detection of HSV infection in sera and CSF from patients with

suspected HSE. Our results indicate that the antigen detection assay had a higher sensitivity (83% and 69% in sera and CSF, respectively). However, a lower specificity was obtained with this method. The reason for low specificity was possibly due to cross-reactivity of goat anti-human IgG-HRP with host IgG's which are mildly to moderately increased in response to an infection. The higher positivity obtained with ELISA assay in other infectious cases as compared to that of non-infectious disorders, also supports the above mentioned possibility.

The so called cross reactivity with IgG of CSF and paired sera was ameliorated by introducing an additional step of protein A in the ELISA protocol. Protein A is a surface protein originally found in the cell wall of the bacteria Staphylococcus aureus. It binds proteins from many of mammalian species, most notably the Fc portion of IgG's. When CSF or sera sample is added to microtitre wells, the host IgGs already present in the sample get bound to wells along with the antigens. This IgGs are then detected by anti-human IgG (used as secondary antibody used in the protocol). The protein A included in the ELISA protocol, binds to those host IgGs, thus rendering them unavailable to be detected by anti-human IgG. The inclusion of protein A in the ELISA protocol resulted in enhancing the overall specificity for the detection of HSV infection. The higher positivity obtained with other infections was significantly lowered. The ELISA method utilizing hyperimmune sera along with protein A for antigen detection yielded good sensitivity (83% and 74%) and specificity (84% and 81%) in CSF and sera, respectively, for the diagnosis of HSV infection.

HSE can occur following primary infection or by reactivation of latent virus in the presence or absence of clinically apparent manifestations ***(Sekizawa et al., 1984; Yamada et al., 2003; Landau et al., 2005)***. The high concordance (63%) for positive ELISA results for antigen detection in sera and CSF of HSE (n=35) patients suggests a high probability for the presence of HSV in both the biological fluids in HSE. Presence of virus in both the CSF and sera of (21/35) individuals could be due to three possibilities i) it is a part of primary episode with the virus infecting the CNS via the olfactory route and thus seeding the blood by crossing the blood barrier ii) the infection could be due to reactivation of endogenous latent virus iii) or infection by an exogenous virus in the CNS in an already latently infected individual. Of the non-HSE cases, out of 150 ELISA negative CSF samples, HSV antigen was found to be positive in 11 serum samples which could be due to reactivation of HSV without clinical relevance in those cases.

The findings of this study suggest that the diagnostic sensitivity of ELISA based system utilizing hyperimmune sera along with protein A is high when evaluated in both the biological fluids i.e., CSF and sera of HSE patients. The results indicate that the indirect ELISA method used in this study is sensitive, specific and cost-effective and if confirmed further, can be adopted in any clinical laboratory with minimal requirements. The test can not only be useful for initial screening purposes but can also be repeated in sera during the course of illness where there is a suspicion of HSV infection.