5. 1. BACKGROUND

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 in diagnostics of viral infections of the CNS ***(Levine et al., 1978; Elitsur et al., 1983; Porter-Jordan et al., 1990)***. HSV antigen detection tests using ELISA have provided good specificity but less sensitivity for the detection of antigen directly from clinical specimens ***(Coleman et al., 1983; Morgan and Smith, 1984; Nerurkar et al., 1984; Warford et al., 1984; Ho and Hirsch, 1985)***. Recent reports on assessment of HSV antigen in the CSF of HSE patients are lacking. To improve sensitivity and specificity of the ELISA based HSV antigen detection, it is needed to develop better methods to capture viral antigen.

Anti-peptide antibodies are excellent tools for biologic research and discovery and studies have shown anti-peptide antibody as a potential diagnostic candidate ***(Saravanan et al., 2004; Pattnaik et al., 2006)***. The present report describes a method in which anti-peptides against synthetic peptides was evaluated for the detection of antigen from clinical specimens by an in-house ELISA method. Antigenic determinant analysis was carried out on glycoprotein gB, one of major immunogenic proteins of HSV on the basis of online software which uses Kolaskar and Tongaonkar method ***(Kolaskar and Tongaonkar, 1990)***. Peptides were designed having varying antigenicity and identified potential peptides were synthesized. Altogether 07 peptides consisting of type-common epitopes of gB of HSV-1 and -2 were synthesized

for predicted putative regions. These synthetic peptides were then subjected for the production of anti-peptides by immunizing the rabbits against the peptides conjugated to KLH. The anti-peptides were screened in clinical samples of suspected and confirmed HSE patients for the development of antigen based ELISA. The concentration of HSV antigen was also determined using the developed ELISA protocol. HSV DNA was measured by real-time PCR and viral load was obtained as per the previous study (Chapter 2). Using HSV antigen concentration as a marker of HSV replication level, it was determined whether quantitative HSV antigen correlates with HSV DNA levels in CSF of HSE patients. This study aim for diagnosis of HSE by detecting presence of the viruses using antibodies against peptides corresponding to type common epitopes of HSV-1 and -2.

5. 2. MATERIALS AND METHODS

5. 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-

5. 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.

5. 2. 1. 2. Non- HSE group (n=40)

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=20)

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=20)

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.

5. 2. 2. DNA extraction and PCR

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

5. 2. 3. Anti-peptides production

Synthetic peptides of 95% purity as determined from mass spectrometry and HPLC were used for the production of anti-peptides. Rabbits were immunized by synthetic peptides with an either N-terminal or C-terminal cysteine residue (Table A) conjugated to KLH. The anti-peptide antibodies were affinity purified with the Protein A affinity matrix and then dialyzed against PBS buffer. Purified antibody concentrations were determined with Coomassie brilliant blue G-250. Anti-peptides were custom synthesized from Hongkong GenicBio BioTech Co., Limited.

Rabbit Custom Antibody Production Protocol:

Protocol Day	Procedure	Description	Remarks
Week 1~3	Peptide Design and Synthesis	A Cys was added to either end of the peptide for conjugation	
Week 3	Conjugation	Conjugate the peptide to a carrier protein (KLH)	
Week 4	Pre-bleed	Pre-immune serum collection	~2 ml serum/rabbit
Week 4	1st Immunization	400ug/rabbit in Complete Freund's Adjuvant (CFA)	
Week 6	2nd Immunization	200ug/rabbit in Incomplete Freund's Adjuvant (IFA)	
Week 8	3rd Immunization	200ug/rabbit in Incomplete Freund's Adjuvant (IFA)	
Week 9	Bleed	1st test bleed	5 ml serum/rabbit
Week 10	4th Immunization	200ug/rabbit in Incomplete Freund's Adjuvant (IFA)	
Week 11	Bleed	2nd test bleed	5 ml serum/rabbit
Week 11~12	Final Bleed	Production bleed (Protein A/G affinity purification)	40~60 ml serum/rabbit

Protein A/G affinity purification: Approximately 25~30 ml anti-sera from the rabbit that showed higher ELISA titer were purified. About 30 mg purified antibody was obtained.

Sequence of peptides of HSV gB used for the production of anti-peptides

S. No	Peptide sequence	Anti-peptides
1	QPRRCPT	AP-1
2	DRAPVPFEEVID	AP-2
3	DHEFVPLEVYT	AP-3
4	DSGLLDYTE	AP-4
5	QLHDLRF	AP-5
6	MKALYPLTT	AP-6
7	RYMALVS	AP-7

5. 2. 4. ELISA for anti-peptide titer assay

Peptide was diluted to 5μg/ml PBS. ELISA plate (Greiner-Bione, USA) was coated by 100μL of each peptide (5μg/ml) per well over night at 4°C. The wells were blocked with 0.5% BSA, 200μL per well, for 1h at 37°C. The immune sera (1:375, 1:1500, 1:3000, 1:6000, 1:24000, 1:48000, 1:96000, 1:192000, 1:384000, 1:768000), 100μL per well were added in the plate and incubated for 45 min at 37°C. The plate was then washed with PBS for 3 times. Goat anti-rabbit IgG labeled with HRP, 100μL per well, was added and the plate was incubated for 30 min at 37°C. After incubation, the plate was again washed with PBS for 3 times. The substrate solution was added, 100μL per well, avoiding light for 15 min at 37°C. 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 and positive/negative>2.1 was considered as the positive criterion.

5. 2. 5. ELISA for HSV antigen detection in CSF

One hundred microliters of CSF samples from HSV-infected patients at 1:5 dilutions was separately added to the microtiter wells. The wells were

incubated at 37°C for 90 min. Blocking with 0.5% BSA in PBS was done for 45 min at 37°C. After washing with PBS, anti-peptides antibodies (1:20,000 dilutions) were added and the plates were incubated at 37°C for 45 min. After incubation, the wells were washed and goat anti-rabbit IgG–HRP secondary antibody was added (1:10,000 dilution). The wells 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 and 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. Negative reference control was selected from pooled CSF of non-HSV infected patients who had never been exposed to HSV, and the absence of HSV antibodies in serum was demonstrated by BioRad HSV1+2 ELISA kit. Synthetic peptides were used as positive reference control. A single dilution of the positive stock was made in the negative reference CSF to achieve a defined reactivity, which was within the accurate detection limits of the ELISA. ELISA was run with the positive CSF control, negative CSF control and sample blank (PBS). Three replicates of positive and negative controls were included on each ELISA plate along with the test sample.

5. 2. 6. ELISA for HSV antigen concentration using AP-5 and AP-6

For the calibration curve, the peptides at different concentration were used as standards. These standards were diluted with CSF of non-HSV infected patients and assayed at the same time as the specimens. The absorbance log A_{450} was plotted versus log peptide concentration and concentrations in each specimen were calculated from the calibration curve.

5. 2. 7. Statistical analysis

All statistical analyses were done using MedCalc software (version 10.1. 2).

5. 3. RESULTS

The present report describes a method in which anti-peptides obtained against synthetic peptides was evaluated for the detection of antigen from clinical specimens by an in-house ELISA method. Altogether 07 peptides consisting of type-common epitopes of glycoprotein gB of HSV-1 and -2 were selected. These synthetic peptides were then subjected for the production of anti-peptides by immunizing the rabbits against the peptides conjugated to KLH. The anti-peptides were screened in clinical samples of suspected and confirmed HSE patients for the development of antigen based ELISA. The concentration of HSV antigen was also determined using the developed ELISA protocol. HSV antigen concentration was correlated with HSV DNA levels in CSF of HSE patients.

Flow chart for analysis of samples of HSE and non-HSE groups by anti-peptide based antigen detection assays

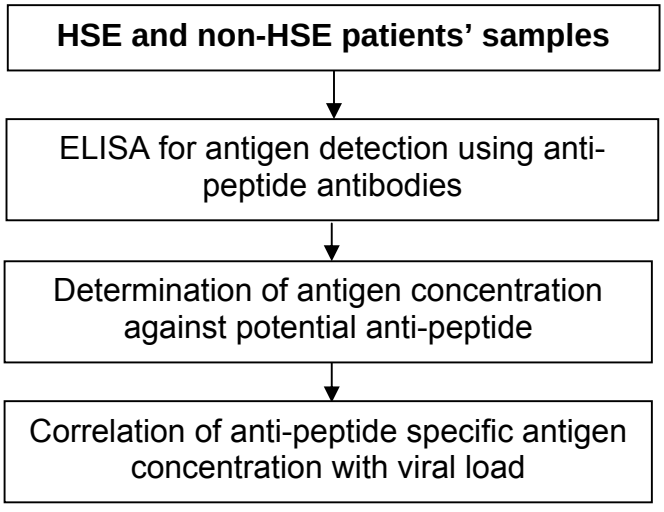


Figure 5. 1 shows the mean±SD for the occurrence of HSV antigen in CSF from the HSV-infected and non-HSV infected groups as determined by indirect ELISA method along with their P values. The significant P values were obtained for anti-peptides against synthetic peptides 16 and 17 (hereby referred to as AP-5 and AP-6) ($P=0.001$ and $P<0.0001$, respectively). The mean absorbance value for the HSV antigen in the HSV infected group was 1.28±0.39 for AP-5 whereas 1.34±0.36 for AP-6.

The rates of positivity for HSV antigen in CSF were determined from the mean absorbance value for the two anti-peptides. The positivities obtained with confirmed HSE group was 80% and 77% and 51% and 53% in suspected HSE group for AP-5 and AP-6, respectively (Table 5. 1). However, the control group showed lesser positivity for the antigen in CSF thus suggesting the lower rates for cross reactivity of the anti-peptides in fluid of patients with non-viral infectious or other disorders of the CNS.

The concentration of antigen in the CSF samples was also determined by plotting the standard curve with synthetic peptides as antigens (Figure 5. 2). For the preparation of standard curve, different concentration of peptides and their respective anti-peptides at a particular dilution was taken. The sample assay values were obtained from the absorbance using the regression equation of the standard curve. Table 5. 2 shows the mean concentration of antigens against AP-5 and AP-6 in CSF of confirmed and suspected HSE cases.

The number of HSV DNA copies detected in clinical samples determined by real-time PCR assay as reported earlier varied from $1.2x10^1$ to $4.1x10^6$ copies/ml of CSF. The correlation plots shows that HSV antigen concentration as determined in ELISA using AP-5 [Figure 5. 3 (a)] and AP-6 [Figure 5. 3 (b)] well correlated with HSV viral load. The HSV antigen concentration varied in accordance to HSV DNA level with r=0.4505 for AP-5 and r=0.4686 for AP-6.

In addition to that, approximately 20% of the cases positive by PCR were not found to be positive for the presence of antigen against the particular anti-peptide. Follow up CSF specimens were hence obtained for 12 patients on day 1, 7, 14 and 21 of admission and the positivity and negativity of PCR and antigen using AP-5 and AP-6 anti-peptide antibodies were determined as shown in Table 5. 3.

Figure 5. 1. ELISA values for the detection of HSV antigen using anti-peptides in CSF from subjects with and without HSV infection

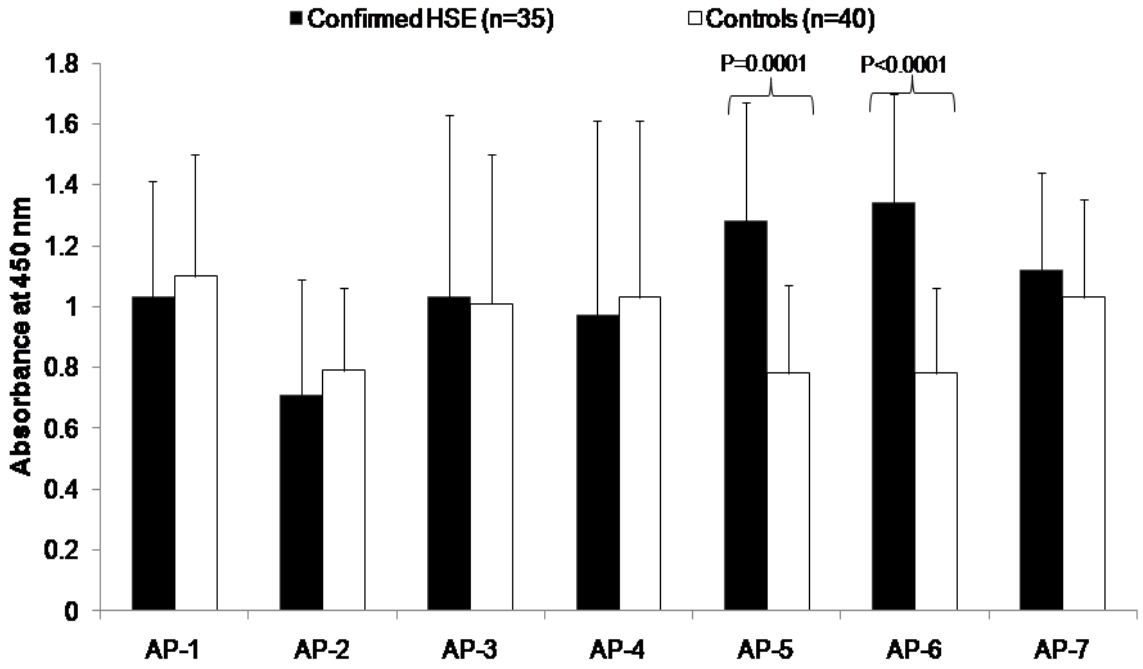


Table 5. 1. Positivity for HSV antigen using anti-peptides in ELISA in CSF of patients with confirmed and suspected HSE and control subjects

Subjects	AP-5	AP-6
Confirmed HSE (n=35)	28 (80%)	27 (77%)
Suspected HSE (n=105)	54 (51%)	56 (53%)
Control (n=40)	3 (7.5%)	1 (2.5%)

Figure 5. 2. Third order regression standard curve with regression equations for the determination of antigen concentration using anti-peptides in ELISA

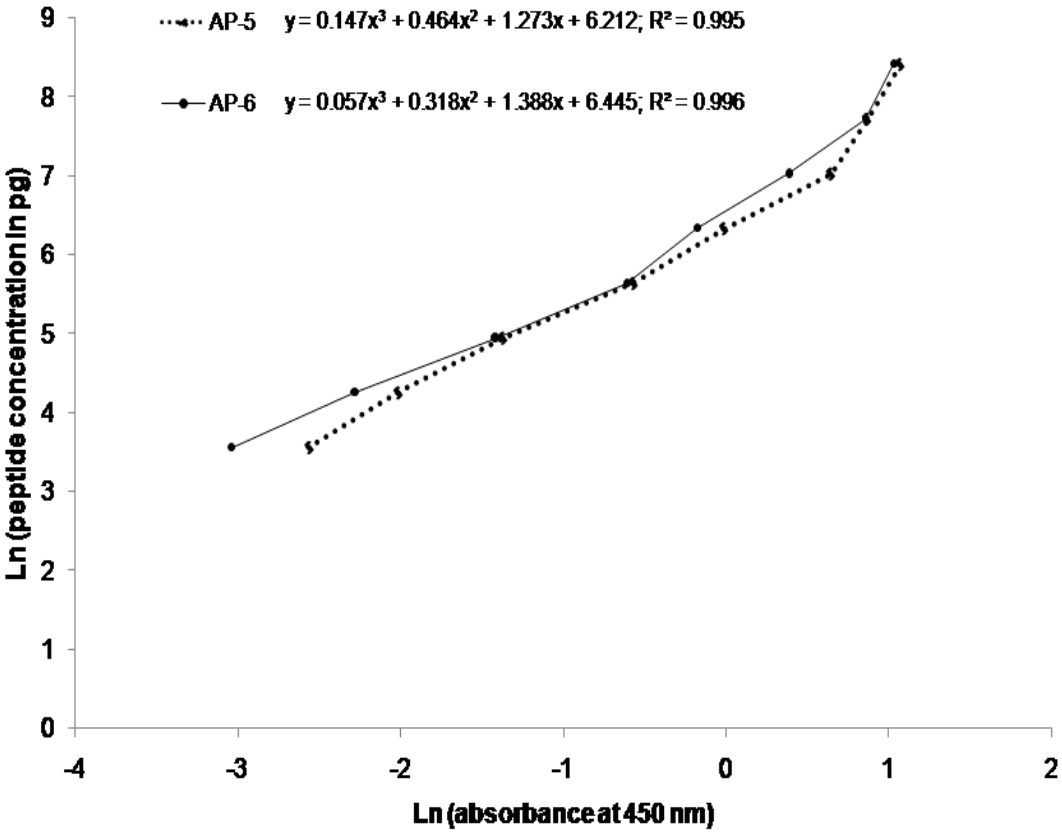
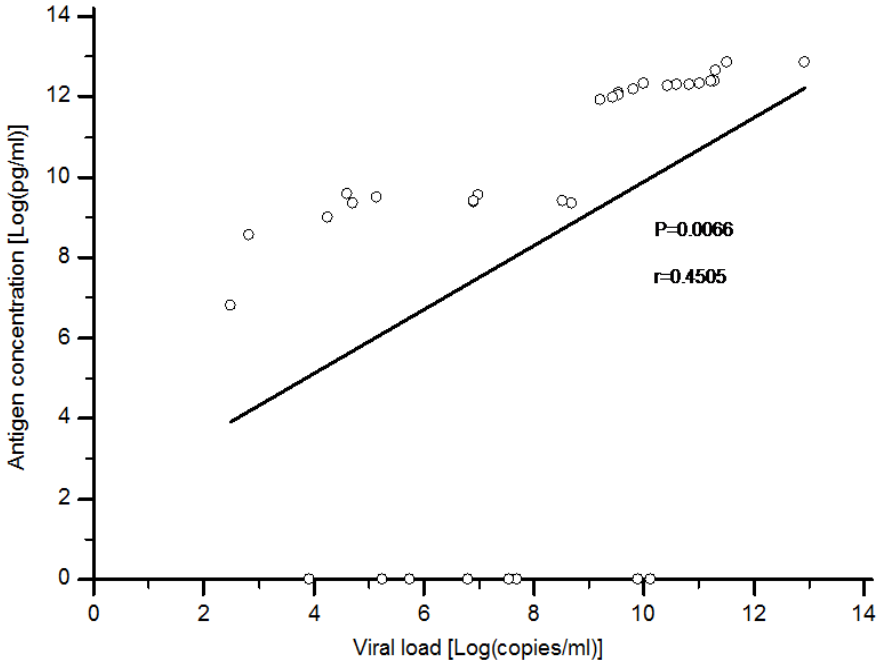


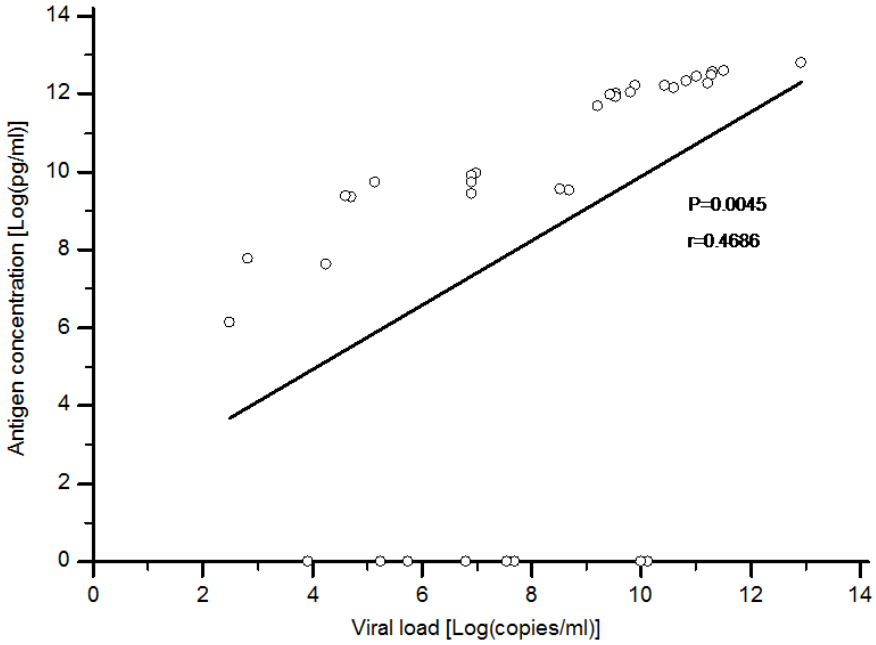
Table 5. 2. Concentration of HSV antigen using anti-peptides in ELISA in CSF of patients with confirmed and suspected HSE

Subjects	Range (pg/ml)		Mean antigen (pg/ml)	
	AP-5	AP-6	AP-5	AP-6
Confirmed HSE (n=35)	470-381700	900-358000	104651	97258
Suspected HSE (n=105)	600-172200	1210-164400	69136	77031

Figure 5. 3. Correlation between concentrations of HSV antigen and HSV DNA in CSF of confirmed HSE cases using AP-5 (a) AP-6 (b)



(a)



(b)

Table 5. 3. Detection of HSV and antigen using the two anti-peptide antibodies i.e., AP-5 and AP-6 in ELISA in patients on various days after admission

Patient No.	Sample collection interval (days)	HSV PCR	AP-5 antigen	AP-6 antigen
1	1	Positive	Positive	Positive
	7	Negative	Negative	Negative
2	1	Positive	Positive	Positive
	7	Negative	Negative	Negative
3	1	Positive	Positive	Positive
	7	Negative	Positive	Positive
4	1	Positive	Positive	Positive
	7	Positive	Positive	Positive
5	1	Negative	Positive	Positive
	7	Positive	Positive	Positive
6	1	Positive	Positive	Positive
	14	Negative	Negative	Negative
7	1	Positive	Positive	Positive
	14	Negative	Negative	Negative
8	1	Positive	Positive	Positive
	14	Negative	Negative	Negative
9	1	Positive	Positive	Positive
	7	Positive	Positive	Positive
	14	Negative	Negative	Negative
10	1	Positive	Positive	Positive
	7	Positive	Positive	Positive
	14	Negative	Negative	Negative
11	1	Positive	Positive	Positive
	14	Positive	Positive	Negative
	21	Negative	Negative	Negative
12	1	Positive	Positive	Positive
	14	Positive	Negative	Negative
	21	Negative	Negative	Negative

5. 4. DISCUSSION AND CONCLUSIONS

In this study, an antigen detection assay was developed by utilizing the anti-peptide antibodies obtained against potential peptides of HSV gB. To our knowledge not much has been reported about the diagnostic significance of anti-peptide antibodies in HSE. However, studies have been reported, based on the evaluation of anti-peptide antibodies for antigen detection in clinical samples for the diagnosis of certain infections ***(Saravanan et al., 2004)***.

I assessed the usefulness of antigen detection assay on the basis of indirect ELISA method for the detection of HSV infection in CSF from patients with suspected HSE with additional information on concentration of antigen. Our results indicate that the antigen detection assay had reliable sensitivity (80% and 77% for AP-5 and AP-6, respectively). This observation proves that anti-peptide antibody has the ability to detect presence of virus in samples and hence can be used to develop virus capture assay. In our study, out of 105 cases negative by PCR, approximately 51% and 53% of the cases were positive for antigen detectable by AP-5 and AP-6. Therefore, antigen detection using AP-5 or AP-6 can be pursued in cases where there is high suspicion of HSV infection of the CNS and PCR of the CSF has been found to be negative.

The antigen concentrations obtained using the two anti-peptides in ELISA were well correlated with HSV DNA levels. The strong correlation between antigen and HSV DNA concentrations suggests that levels of HSV antigen is

produced proportionately to virus load and hence can be used as virus load marker. At present, there is no other evidence to support this study as reports are not available to clarify the mechanisms behind this observation. To confirm these preliminary observations, additional clinical and diagnostic studies of much larger populations are required. However, antigen concentrations have been well correlated with virus load by several workers ***(Kimura et al., 2002; Ganji et al., 2011; Marchetti et al., 2011)***.

The antigen was also detected in follow up CSF samples of 12 patients in whom the CSF were obtained on day 1, 7, 14 and 21 of admission. The antigen detectable by AP-5 and AP-6 were obtained on day 1 and 7 of admission whereas, it was not found on day 14 and 21 of admission. All the 12 cases showed positivity by PCR in CSF collected at day 1 except one case where the antigen was detectable by both AP-5 and AP-6. Thus, in cases where CSF shows negativity for HSV DNA, the antigen could be detected which helps in improved diagnosis of the disease and early administration of antiviral therapy.

ELISA has some advantages over nucleic acid amplification assays. ELISA is a relatively simple method and provides a low-cost and quantitative analysis with high reproducibility. Peptides are as effective as proteins in raising antibodies and therefore, anti-peptide antibodies can be a powerful tool for their use in ELISA. 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 during the course of illness where there is a suspicion of HSV infection.