Abstract

Herpes simplex encephalitis (HSE) represents one of the most severe infectious diseases of the central nervous system (CNS). As effective antiviral drugs are available, an early, rapid and reliable diagnosis has become important. The objective of the present study was to develop a sensitive ELISA protocol for herpes simplex viruses (HSV) antigen detection and quantitation by assessing the usefulness of antipeptide antibodies against potential peptides of HSV glycoprotein B (gB). A total of 180 CSF samples of HSE and non-HSE patients were analyzed using a panel of antipeptide antibodies against synthetic peptides of HSV glycoprotein gB. The cases of confirmed and suspected HSE showed 80% and 51% positivity for antipeptide against synthetic peptide QLHDLRF and 77% and 53% positivity for antipeptide against synthetic peptide MKALYPLTT, respectively for the detection of HSV antigen in CSF. The concentration of HSV antigen was found to be higher in confirmed HSE as compared to suspected HSE group and the viral load correlated well with antigen concentration obtained using the two antipeptides in CSF of confirmed HSE group. This is the first report describing the use of antibodies obtained against synthetic peptides derived from HSV in diagnostics of HSE using patients' CSF samples.

Keywords

Herpes simplex encephalitis, synthetic peptides, antipeptide antibodies, viral load, antigen concentration

Background

Human herpes viruses are the best known and consist of a large group causing various acute, sub acute and chronic disorders of the central nervous system (CNS) and peripheral nervous systems in adults and children with no recent travel history, and contact with animals or insect bites. Among herpes viruses, herpes simplex virus 1 (HSV-1) and herpes simplex virus 2 (HSV-2) causes infections of the CNS which leads to herpes simplex encephalitis (HSE).[1,2] The diagnosis of HSE is always challenging as neurological syndromes are similar among different causes of encephalitis. For effective treatment of subjects with antiviral drugs, rapid diagnosis of HSV infections is of utmost importance.

The available diagnostic tests for HSE have certain inherent limitations which limit their use for rapid confirmation of HSV infection of the CNS. 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.[3,4,5] HSV antigen detection tests using ELISA have provided good specificity but less sensitivity for the detection of antigen directly from clinical specimens.[6, 7, 8, 9, 10] Earlier in our laboratory we have developed a method to detect HSV antigen by ELISA[11]. 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.[12, 13] The present report describes a method in which antipeptides 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 B, one of major immunogenic proteins of HSV on the basis of online software which uses Kolaskar and Tongaonkar method.[14] Peptides were designed having varying antigenicity and identified potential peptides were synthesized. Altogether 07 peptides consisting of type-common epitopes of glycoprotein gB of HSV-1 and -2 were synthesized for predicted putative regions. These synthetic peptides were then subjected for the production of antipeptides by immunizing the rabbits against the peptides conjugated to KLH. The antipeptides 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 our previous study.[15] Using HSV antigen concentration as a marker of HSV replication level, we 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.

Materials and Methods

Study subjects

We carried out study of patients with suspected encephalitis for a four year period. The study involves patients with suspicion of viral encephalitis based on symptoms viz., presence of fever, altered mental status (low level of consciousness, behaviour or personality changes) and other clinical manifestations (e.g. focal neurological deficits, seizures), CSF findings showing mild increase in protein, glucose often normal and mild pleocytosis. In the first week of hospitalization following diagnostic investigations were performed that included staining for bacterial culture, determination of the protein, sugar level and cell counts in CSF, CT scan and MRI of the brain. The samples of suspected viral encephalitis patients were further grouped into confirmed HSE (PCR positive) and suspected HSE (PCR negative). In suspected HSE cases, the clinical picture was suggestive of encephalitis, but HSE was not fully excluded by the use of PCR. All the samples were stored at -20°C until further analysis. The control subjects used in the study were those where a clear and unambiguous diagnosis was identified as other CNS infectious (bacterial, fungal etc.) and non-infectious neurological disorders (hypertension, status epilepticus, stroke etc.). The Institutional Ethics Committee of Central India Institute of Medical Sciences, Nagpur, India approved the study and an informed consent was obtained from all the patients enrolled in the study.

Samples

Approximately 2 ml of CSF (by standard lumbar puncture) were collected from patients with viral encephalitis and non-viral neurological disorders. A total of 180 CSF samples were taken for analysis of antigen in CSF.

Quantitative real-time PCR (qPCR) assay

Total 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. The amplification reactions were carried out using the primers for HSV which are described earlier by us using SYBR green chemistry.[15] The amplification reactions were carried out in a total volume of 10 µl, containing 1 µl of template DNA, 5 µl of Power SYBR® Green PCR master mix (Applied Biosystems, Foster City, USA), 1 µl each of (0.5 µM) forward and reverse primer, and 2 µl of sterile water. The amplification conditions consisted of preincubation at 95^{o}C for 10 min and two steps (40 cycles) at 95^{o}C for 15 s and 65^{o}C for 1 min. The quantification cycle (C_t) was calculated as the cycle number at which the concentration increase became exponential. The specific target amplification was analyzed by melt curve analysis of the Applied Biosystems StepOne Real-Time PCR systems, Foster City, USA, which consisted of first melting step at 95^{o}C for 15 s, annealing at 60^{o}C for 1 min, and second melting step at 95^{o}C for 15 s.

Antipeptides production

Synthetic peptides of 95% purity as determined from mass spectrometry and HPLC were used for the production of antipeptides. Rabbits were immunized by synthetic

peptides (Table 1) conjugated to KLH. The antibodies were affinity purified with the Protein A affinity matrix and then dialyzed against PBS buffer. Antipeptides were custom synthesized from Hongkong GenicBio BioTech Co., Limited.

ELISA

One hundred microliters of CSF 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, antipeptides 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–horseradish peroxides 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.

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.

Results

CSF samples of 180 patients admitted to the Neurological Department of CIIMS with suspected encephalitis were collected in a four year period with HSV PCR as a diagnostic service. The samples of patients were grouped into HSV and non-HSV infected groups on the basis of PCR assay results, clinical observations and biochemical and pathological analyses of CSF samples.

Figure 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 antipeptides 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 antipeptides. 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 2). However, the control group showed lesser positivity for the antigen in CSF thus suggesting the lower rates for cross reactivity of the antipeptides 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 2). For the preparation of standard curve, different concentration of peptides and their respective antipeptides at a particular dilution was taken. The sample assay values were obtained from the absorbance using the regression equation of the standard curve. Table 3 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 $4.1x10^{5}$ copies/ml of CSF. The correlation plots shows that HSV antigen concentration as determined in ELISA using AP-5 [Figure 3(a)] and AP-6 [Figure 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 antipeptide. 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, antigen using AP-5 and AP-6 antipeptide antibodies were determined as shown in Table 4.

Discussion

HSV cause acute and recurrent infections of the CNS in humans leading mostly to HSE. Diagnosis of HSE remains difficult because of overlapping clinical symptoms that appears in viral infections of the CNS other than caused by HSV. Viral culture from CSF sample is recognized as the gold standard technique for the diagnosis of HSE.[16, 17] HSV PCR test of the CSF is now considered as the standard for HSE diagnosis, but it requires sophisticated technology and well-trained personnel.[18] Antibody detection of the CSF has little diagnostic value due to the delay in antibody development and the requirement for immediate diagnosis of HSE.[19]

In a suspected case of HSE, the detection of viral antigen is an alternative to the detection of viral nucleic acids or antibodies against it. We have previously reported a sensitive and specific ELISA to capture and detect HSV antigen in the CSF.[11] In this study, we developed an antigen detection assay by utilizing the antipeptide antibodies obtained against potential peptides of HSV gB. To our knowledge not much has been reported about the diagnostic significance of antipeptide antibodies in HSE. However,

studies have been reported, based on the evaluation of antipeptide antibodies for antigen detection in clinical samples for the diagnosis of certain infections.[13]

We 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 antipeptide 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 antipeptides 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, we have 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.[20, 21, 22]

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, antipeptide 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.

Conflict of Interest

Shradha S. Bhullar, Nitin H. Chandak, Neeraj N. Baheti, Hemant J. Purohit, Girdhar M. Taori, Hatim F. Daginawala and Rajpal S. Kashyap declare that they have no conflict of interest.

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Table 1. Sequence of peptides of HSV glycoprotein gB used for the production of antipeptides

S. No	Peptide sequence	Antipeptide
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

Table 2. Positivity for HSV antigen using antipeptides 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%)

Table 3. Concentration of HSV antigen using antipeptides 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

Table 4. Detection of HSV and antigen using the two antipeptide 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

Figure 1 ELISA values for the detection of HSV antigen using antipeptides in CSF from subjects with and without HSV infection

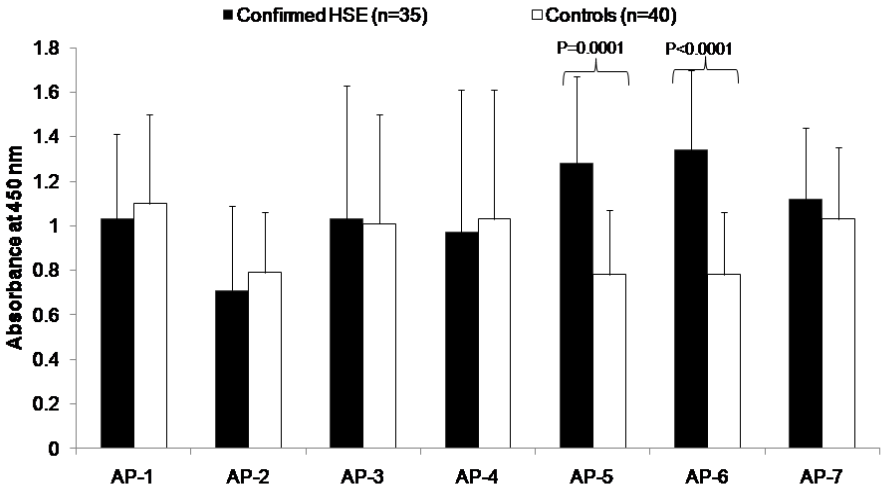


Figure 2 Third order regression standard curve with regression equations for the determination of antigen concentration using antipeptides in ELISA

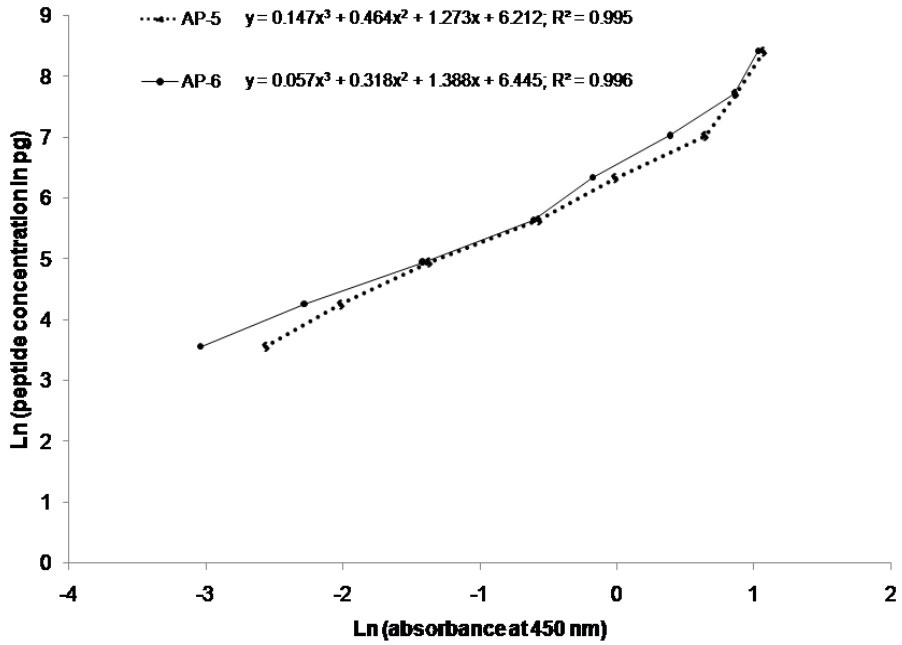
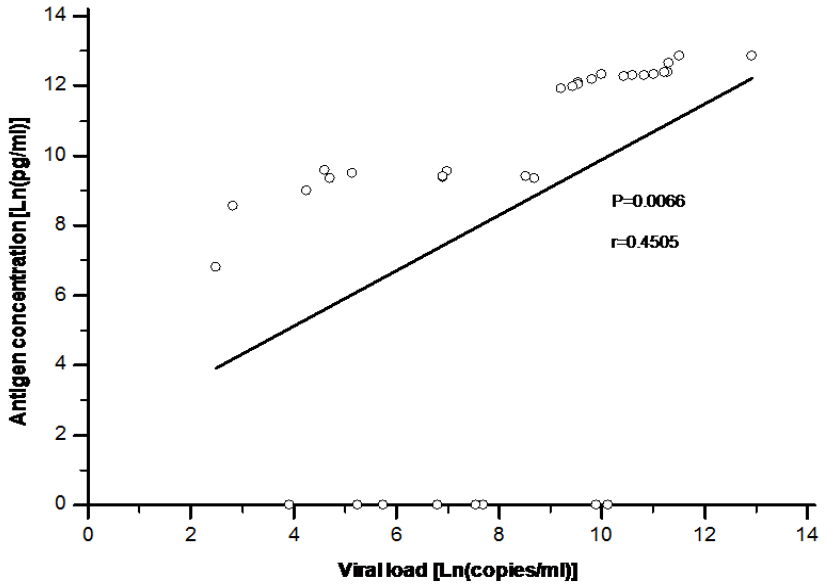
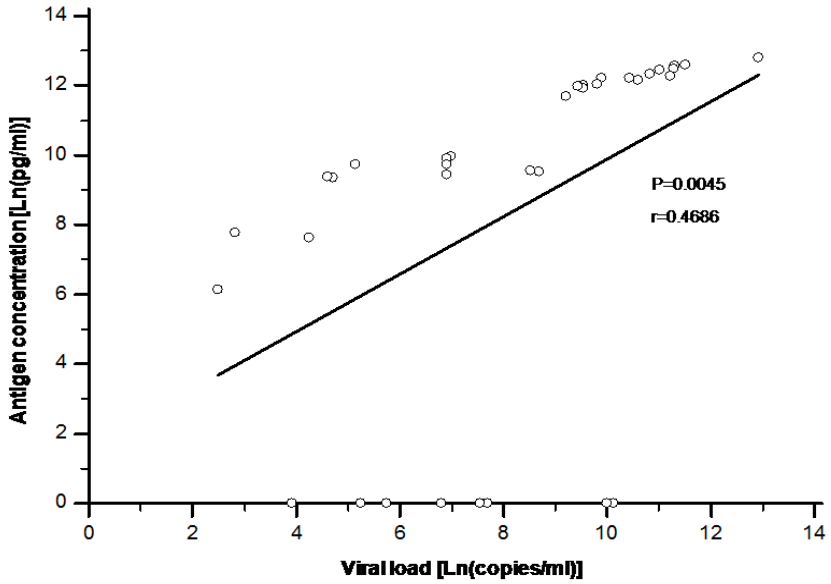


Figure 3 Correlation between concentrations of HSV antigen and HSV DNA in CSF of confirmed HSE cases using AP-5 (a) AP-6 (b)





Development of a single dilution ELISA for determination of antibody titer using synthetic peptide as antigen in cerebrospinal fluid of herpes simplex encephalitis patients

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ABSTRACT

Determination of antibody response is widely adopted to diagnose herpes simplex encephalitis (HSE). The objective of the present study was to determine antibody titer against an immunodominant synthetic peptide of envelope glycoprotein of herpes simplex virus (HSV) -1 and -2 in CSF of HSE patients. The method of single CSF dilution ELISA was adopted to determine the IgM and IgG antibody titer values in CSF of HSE patients. The antibody titers obtained in ELISA were correlated with HSV DNA levels. The titer values between 5-10 and 5-20 were obtained for IgM and IgG antibody analysis, respectively using synthetic peptide in CSF of confirmed and suspected HSE group. A weak positive correlation of IgM antibody titer and a weak negative correlation of IgG antibody titer were obtained with viral load in the CSF of HSE patients. This assay gave reproducible results and helped to save sample, time and cost of reagents. This is the first report describing the use of synthetic peptide of HSV for determining antibody titer in CSF of HSE patients.

KEYWORDS

Herpes simplex virus, herpes simplex encephalitis, synthetic peptides, single dilution ELISA, viral load

INTRODUCTION

Herpes simplex virus (HSV) infection of central nervous system (CNS) occurring in the neonatal period (<1month of age) causes neonatal herpes, while in older children & adults it can manifest as either life threatening herpes simplex encephalitis (HSE) or the more benign aseptic meningitis (Puchhammer-Stöckl E et al., 1990; Riancho et al., 2013; Sili et al., 2014; Jain et al., 2014). Herpes simplex encephalitis (HSE) is a severe viral infection of the human central nervous system (CNS) and the most common cause of non-epidemic encephalitis contributing to 10-20% cases of viral encephalitis (Banatvala, 2011). There are no specific clinical symptoms in HSE that differ from other encephalitides (Whitley et al., 1982; Kohl, 1988). Diagnosis of HSE is based on medical history and examination followed by analysis of cerebrospinal fluid (CSF) for the identification of the infecting organism by viral cultivation, brain biopsy, PCR and serology. However, due to inherent limitations of these tests the diagnostics of HSE is always challenging (Bhullar et al., 2011).

Molecular methods such as PCR for the diagnosis of viral CNS infections are now recognized as the standard laboratory method for the diagnosis of viral infections. Studies using PCR protocols have been reviewed and have demonstrated the utility of the laboratory molecular diagnosis of HSE and other CNS infections (Aurelius et al., 1993; Yerly et al., 1996; Read et al., 1997; Tang et al., 1999). Several studies have reported that, PCR as well as detection of specific antibodies in CSF samples should be retrospectively analyzed for the diagnosis of HSE (Akçali et al., 2008). Detection of specific IgM and IgG in CSF have been widely used for disease diagnosis (Petersen and Marfin, 2002; Holzmann, 2003) using several

immunological methods such as neutralization, complement incorporation reaction, haemagglutination, indirect immunofluorescence, "radioimmunoassay" and enzyme linked immunosorbant assay (ELISA) (Griffin, 2000; Serter, 2002). Immunodiagnostic tests have been developed for the detection of viral, bacterial and parasitic diseases based on synthetic peptides derived from antigenic proteins (Gómara and Haro, 2007).

We have previously identified an immunogenic epitope for detecting IgM and IgG antibody in CSF of HSE patients using synthetic peptide of the antigenic envelope glycoprotein B (gB) of HSV-1 and -2 (Bhullar et al., 2014). In this study, we aimed to determine the IgM and IgG antibody titer using this synthetic peptide in CSF of HSE patients. The antibody titers were determined using the identified potential peptide by the method of single dilution as reported earlier (Ramadass et al., 2008). The antibody titers obtained in ELISA were correlated with HSV DNA levels to determine whether there is a correlation between the magnitude of DNA load and specific seroresponsiveness. To our knowledge there is no report available for the determination of antibody titers using peptides of the envelope glycoproteins of the two viruses in analysis of CSF of HSE patients using the method of single dilution ELISA.

MATERIALS AND METHODS

Study subjects and samples

The study involves patients with suspicion of viral encephalitis based on symptoms viz., presence of fever, altered mental status (low level of consciousness, behavior or

personality changes) and other clinical manifestations (e.g. focal neurological deficits, seizures), CSF findings showing mild increase in protein, glucose often normal and mild pleocytosis. Diagnostics performed in the first week of hospitalization included staining for bacterial culture, determination of the protein, sugar level and cell counts in CSF, computed tomography scan and magnetic resonance imaging of the brain. The 40 control subjects used in the study had other CNS infections (bacterial, fungal etc.) and non-infectious neurological disorders (hypertension, epilepsy, stroke etc.). The samples of viral encephalitis patients were further grouped into 35 confirmed HSE (PCR positive) and 105 suspected HSE cases (based on clinical symptoms). The study has been carried out at Central India Institute of Medical Sciences, Nagpur, India, and was ethically approved.

Real-time PCR

Total DNA was extracted from 200 µl of CSF samples from patients by using a ZR viral DNA kit (Zymo Research, USA), according to the manufacturer's protocol. The amplification reactions were carried out using SYBR green and the primers for HSV which were described earlier (Bhullar *et al.,* 2013). The amplification reactions were carried out in a total volume of 10 µl, containing 1 µl of template DNA, 5 µl of Power SYBR® Green PCR master mix (Applied Biosystems, Foster City, USA), 1 µl each of (0.5 µM) forward and reverse primer, and 2 µl of sterile water. The amplification conditions consisted of preincubation at 95°C for 10 min and two steps (40 cycles) at 95°C for 15 s and 65°C for 1 min. The quantification cycle (Ct) was calculated as the cycle number at which the concentration increase became exponential. The specific target amplification was analyzed by melt curve analysis of the Applied Biosystems StepOne Real-Time PCR systems, Foster City, USA, which consisted of first melting

step at 95°C for 15s, annealing at 60°C for 1 min, and second melting step at 95°C for 15s.

Standard curve

To quantitate viral DNA, a standard curve was obtained for each experiment by co-amplification of known amounts of HSV DNA (determined spectrophotometrically). Seven consecutive dilutions (dilution factor 1:10) were prepared containing 10^6-1 copies/reaction. The amounts of HSV DNA in samples were obtained by plotting C_t values onto the standard curve.

Indirect ELISA

Hundred microlitres of 5 ng/µl of different peptides were coated in microtiter wells and incubated for 3 hrs at 37°C. The wells were then blocked with 0.5% BSA after washing once and kept for 2 hrs at 37°C. The wells were washed and stored at 4°C overnight. Next day, diluted CSF (two fold serially diluted up to 1:640) samples of 100 µl were added to the wells and incubated for 1 hr at 37°C. After washing the wells with PBS, 100 µl of secondary antibody (Goat-anti-human IgM/IgG -HRP conjugated antibody, 1:5000 and 1:10000 respectively) was added and incubated for 45 min at 37°C. 100 µl of 3, 3', 5, 5'-tetramethylbenzidine/H_2O_2 substrate solution was then added and incubated for 10 min. This colour development reaction was stopped by adding 100 µl of 2.5N H_2SO_4. Absorbance of the colour was read at 450 nm.

Positive/negative threshold (PNT) baseline and observed antibody titers

The titer values were determined using PNT baseline method. For the construction of PNT baseline 15 different CSF were taken from patients of Non-infectious neurological disorders group in whom the CSF was found to be negative for HSV DNA by real-time PCR assay and serum anti-HSV IgG was found to be negative using the Platelia HSV (1+2) ELISA Kit, Bio-Rad Laboratories, USA. The absorbance of serially diluted 35 PCR positive CSF was plotted onto the PNT baseline and the observed antibody titer was defined as the point at which the plotted lines intersect the PNT baseline.

Regression analysis and predicted antibody titer

The regression analysis was carried out with $\log_{10}$ values of absorbance at each dilution of 35 PCR positive CSF with their corresponding $\log_{10}$ observed antibody titers to obtain regression equation. The regression equation with the best correlation coefficient value was henceforth used for the determination of antibody titers in CSF of HSE patients.

Statistical analysis

All the statistical analysis was done using the MedCalc statistical software (version 10.1.2). The level of significance was set at $P<0.5$.

RESULTS

Patients admitted to the Neurological Department of Central India Institute of Medical Sciences with suspicion of viral encephalitis were confirmed for HSE on the basis of

PCR assay, clinical observations and biochemical and pathological analyses of CSF samples. The IgM and IgG antibody response was determined against the synthetic peptide of envelope glycoprotein of the virus.

The mean plus three standard deviation (Mean+SD) values of the samples of a total of 15 patients of the control group taken for the construction of PNT baseline were found to be 0.15±0.02 for IgM analysis and 0.21±0.09 for IgG analysis. The observed antibody titers of IgM and IgG antibodies of the 35 PCR positive CSF were determined from the PNT baseline (Figure 1). The highest correlation was obtained at dilutions 1:10 and 1:20 for IgM and IgG analysis respectively in CSF samples (Table 1). On the basis of these values, the regression equations were hence obtained (Table 1). The absorbance values of samples at this dilution were directly converted into predicted antibody titers using the regression equations.

The titer values between 5-10 and 5-20 were obtained for IgM and IgG antibody analysis, respectively against peptide in CSF of both confirmed and suspected HSE group. Geometric mean titer values of 8 for IgM; 12 and 10 for IgG were obtained in CSF of confirmed HSE and suspected HSE group respectively (Table 2).

The antibody titers obtained in ELISA were correlated with HSV DNA levels. A weak negative correlation of IgM (r^2=-0.3105; P=0.0695) antibody titer and a weak positive correlation of IgG (r^2=-0.5076; P=0.0019) antibody titer were obtained with viral load in the CSF of HSE patients [Figure 2 (a) and (b)].

DISCUSSION

CSF antibody measurements may be useful in retrospective diagnosis or in cases in which CSF was obtained at a later period following illness onset and PCR is negative. Therefore, analysis of IgG and IgM response in CSF may be useful alternative tool for retrospective diagnosis of HSE. There are only two Indian studies that determined the serological profile of patients with HSE (Ratho et al., 1999; Panagariya et al., 2001). We earlier described the use of synthetic peptide of HSV for determining serological response in HSE patients (Bhullar et al., 2014). Studies have been reported describing the possibility of HSV serology by using synthetic peptides of gB. There is only one reported study available which determine immunity induced by peptides corresponding to the antigenic sites of gB (Mester et al., 1990).

After identification of the immunodominant epitope for determination of antibodies in the CSF of HSE patients (Bhullar et al., 2014), we next aimed for the development of single CSF dilution method for determination of antibody titer against this epitope. Determination of antibody titer using synthetic peptide has not been adopted in HSE. However, studies have been made to determine titer of antibodies against anti-citrullinated peptide in subjects (Bizzaro et al., 2013). Several studies have been done to develop single dilution ELISAs for the determination of antibody titer against antigenic proteins of organisms (Kumar et al., 2003; Dey et al., 2008; Miura et al., 2008).

Correlation between the magnitude of HSV DNA load and peptide specific seroresponsiveness was determined. In this context, correlation between viral load

and antigen specific antibody titers has been reported for several viruses including BK virus and JC polyomavirus infections (Bohl et al., 2005; Lundstig et al., 2007; Randhawa et al., 2008) and influenza virus H1N1 (Hung et al., 2010). Pastrana et al., found substantially stronger correlation between Merkel cell polyomavirus viral load and capsid-specific antibody titer (Pastrana et al., 2012). Among herpes viruses, positive correlation between Epstein-Barr virus viral load and anti-viral capsid IgG titers and human herpesvirus-6 viral load and antibody titer has been reported earlier (Besson et al., 2006; Behzad-Behbahani et al., 2011). However, no report is available determining the correlation between antibody titer and viral load in the CSF of HSE patients. In this study, we observed a significant positive trend, with anti-peptide IgG titers increasing with viral load. The rise in HSV IgG antibody titer together with increase in HSV DNA in the CSF samples of patients suggests possible association between the virus and disease progression. Since the presence of higher amount of HSV DNA in the CSF would be an indicator of acute and active infection, the viral load correlated well with IgG antibody titer values. However, a weak negative correlation of IgM antibody titer with viral load was obtained in the CSF of HSE patients. The duration of onset of disease before the CSF had been collected was almost two times more in the patients with higher viral loads which suggest the lower titer values of IgM antibodies in the CSF of these patients.

The findings of studies for determination of antigen titer may provide new insights into viral pathogenesis and have practical implications for the use of quantitative serology as a clinical biomarker in HSE. As far as we know, this is the first report describing the use of synthetic peptide of HSV for determining serological response and its titer values in the CSF of HSE patients. With this method, more precise

decisions can be made with less time and labor. Similarly, the test can be repeated to obtain the reproducibility of the data at different times with minimal consumption of sample and reagents.

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Table 1. CSF and serum dilutions with their highest correlation coefficient value and the regression equation for calculation of antibody titers

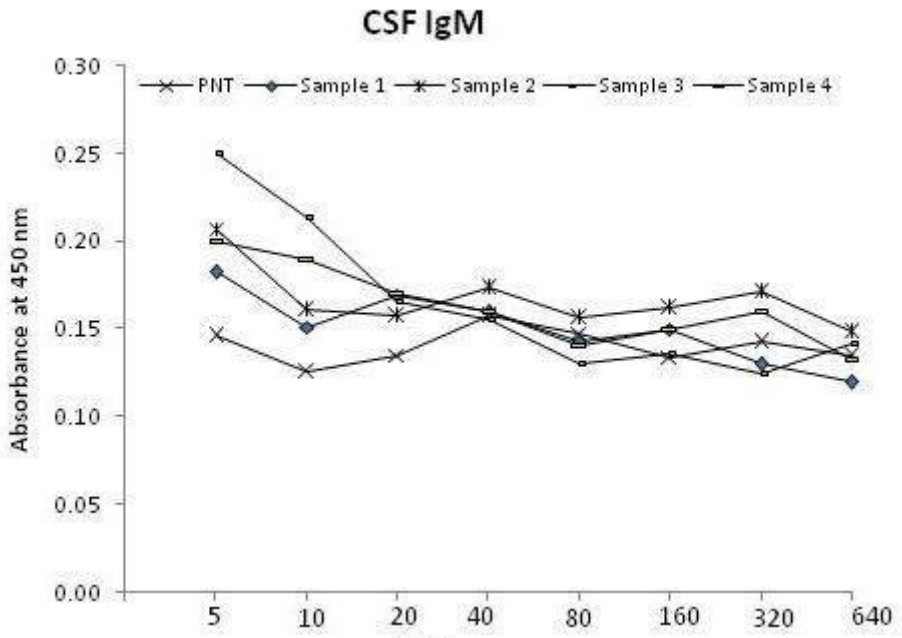
Sample	Antibody	Dilution	Correlation coefficient	Equation [y=C+mx]
CSF	IgM	1:10	0.9098	y = -0.1048 + 0.1048 x
	IgG	1:20	0.8129	y = -0.1731 + 0.2346 x

R^2= Correlation Coefficient
C= Intercept
m= Slope
y= Log_{10} ($Absorbance_{450nm}$)
x= Slope X Log_{10} titer

Table 2. Range along with geometric mean titer of IgM and IgG antibody titer against P-16 in CSF of confirmed and suspected HSE cases

Antibody class	Group	Range (titer)	Geometric mean titer
IgM	**Confirmed HSE**	5-10	8
	Suspected HSE	5-10	8
IgG	**Confirmed HSE**	5-20	12
	Suspected HSE	5-20	10

Figure 1. PNT baseline for determination of (a) IgM and (b) IgG antibody titer using serially diluted CSF and serum samples



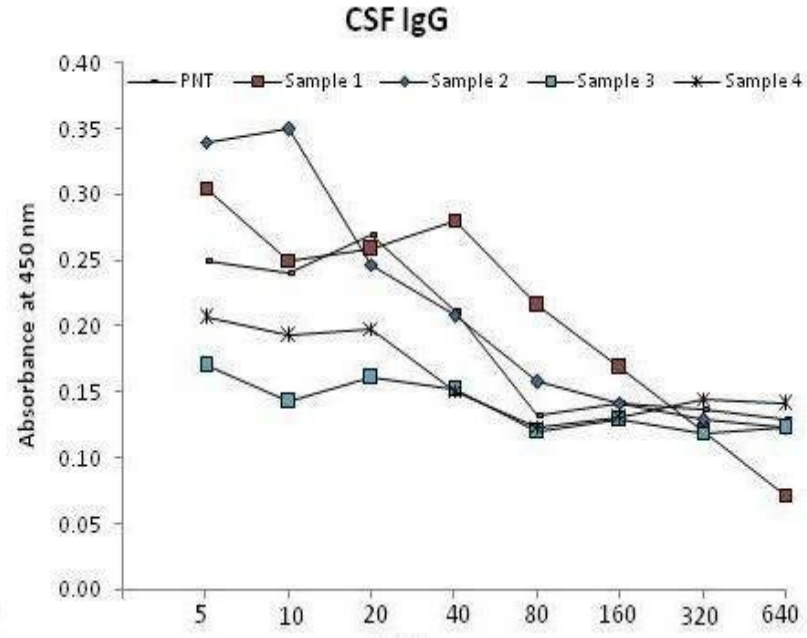


Figure 2. Correlation of viral load and (a) IgM and (b) IgG antibody titer in CSF of HSE patients