Chapter-3

Designing and synthesis of antigenic peptides by targeting envelope glycoproteins of herpes simplex virus, development of synthetic peptide based IgM/IgG antibody detection ELISA and evaluation of the developed immunoassay in clinical samples of patients

3. 1. BACKGROUND

Detection of HSV DNA in CSF by PCR is now recognized as the standard laboratory investigation ***(Puchhammer-Stöckl et al., 1990; Rowley et al., 1990; Aurelius et al., 1991)*** and serological diagnosis has widely been adopted to determine antibody response against HSV ***(MacCallum et al., 1974; Koskiniemi and Vaheri, 1982; Hanada et al., 1988)***. Several studies have reported that, PCR as well as detection of specific antibodies in sera and CSF samples should be retrospectively analyzed for the diagnosis of HSE, so as to understand pathogenesis and follow the course of the disease ***(Akçali et al., 2008)***. PCR is although recognized as the standard laboratory technique for HSE diagnosis, however, it remains negative in cases where CSF has been obtained at a later period following onset of illness or when the viral load is low in CSF ***(Fomsgaard et al., 1998; Puchhammer-Stöckl et al., 2001; Akçali et al., 2008)***. CSF antibody measurements may be useful in retrospective diagnosis in such cases. Therefore, analysis of IgM and IgG response in CSF may be a useful alternative tool for diagnosis of HSE.

HSV-1 infections elicit strong antibody responses to the HSV-2 and vice versa due to high sequence homology between the two viruses ***(McClung et al., 1976; Eberle and Courtney, 1981)***. The type common antibody response against HSV is mostly attributed to glycoprotein B (gB), a 904-aa protein and a major component of infected cell membranes and virion envelopes ***(Vestergaard, 1980; Cai et al., 1988)***. Antibodies to HSV-specific polypeptides, particularly to gB, have been used to detect CSF antibodies

(Kahlon et al., 1987). Although the aa similarity between HSV-1 and HSV-2 is high, the envelope glycoprotein G (gG), a 238-aa long chemokine-binding protein, is the only HSV antigen known to induce type-specific antibody responses and is therefore used in serological assays to discriminate between HSV-1 and HSV-2 infections ***(Ashley, 1998; Görander et al., 2003)***.

The approach of peptide synthesis has advanced the use of synthetic peptides to study protein-protein interactions ***(Merrifield, 1963)***. Several immunodiagnostic tests for the detection of antibodies have been developed for the detection of viral, bacterial and parasitic diseases based on synthetic peptides derived from antigenic proteins ***(Gómara and Haro, 2007)***. Studies have been done to detect antibodies using peptides designed with the aid of online software ***(Morey et al., 2010)***. The antigen sequences can be used to obtain peptides with antigenic epitopes on the basis of prediction analysis by Kolaskar and Tongaonkar method ***(Kolaskar and Tongaonkar, 1990)***. To our knowledge not much has been reported regarding the use of peptides of the envelope glycoproteins of HSV-1 and HSV-2 in analysis of CSF of HSE patients by ELISA protocol. The aim of this study was to identify an immunodominant epitope determining the antibody response to HSV gB and gG in CSF of HSE patients. For this purpose synthetic peptides resembling type-common as well as type-specific domains of HSV gB and gG, respectively were prepared and tested in ELISA with IgM and IgG antibodies in CSF of HSE-confirmed patients, HSE-suspected patients and control subjects.

Determination of antibody titer against peptide has not been adopted in HSE. I aimed to determine the IgM and IgG antibody titer against immunodominant peptide in CSF of HSE patients. The antibody titers were determined against the identified potential peptide using the method of single dilution as reported earlier ***(Ramadass et al., 2008)***. To our knowledge not much has been reported regarding the use of peptides of the envelope glycoproteins of the two viruses in analysis of CSF of HSE patients by ELISA protocol. Therefore, in this study, the antibody titers were determined against the identified potential peptide using the method of single dilution as reported earlier.

3. 2. MATERIALS AND METHODS

3. 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-

3. 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.

3. 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.

3. 2. 2. DNA extraction and PCR

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

3. 2. 3. Selection and designing of peptides

The sequences of envelope glycoproteins (structural proteins) gB and gG of different strains of HSV were retrieved from EXPASY proteomic server - UniProtKB/Swiss-Prot. Strains which were used to obtain gB sequence from HSV-1 were strain 17: P10211; strain F: P06436; strain Patton: P08665; strain KOS: P06437 and from HSV-2 were strain 333: P06763; strain HG52: P08666; strain SA8: P24994. The consensus sequence of gB of HSV-1 and HSV-2 was obtained by multiple sequence alignment by CLC sequence viewer (version 6.6.2). The gG sequences were obtained from strain 17: P06484 of HSV-1 and strain HG52: P13290 of HSV-2. The antigenic peptides were identified from the consensus sequence of gB and from sequences of HSV-1 and HSV-2 gG on the basis of online software which uses Kolaskar and Tongaonkar method. Peptides were designed having varying antigenicity. The sequence homology of peptides was checked with other organisms using NCBI BLAST. Both type-common and type-specific peptides were selected.

The sequences of glycoproteins used for the selection of peptides are as shown below-

HSV-1 (type1 / strain 17: P10211; strain F: P06436; strain Patton: P08665; strain KOS: P06437

HSV-2 (type 2 / strain 333: P06763; strain HG52: P08666; strain SA8: P24994

Consensus sequence of gB (Gene name: UL27) of HSV-1 and HSV-2, 903 aa long

MRQGAARGCRWFVVWALLGLTLGVLVASAAPSSPGTPGVAAATQAANGGPATPAPPAPGP
APTGDTKPKKNKKPKNPPPPRPAGDNATVAAGHATLREHLRDIKAENTDANFYVCPPPTGA
TVVQFEQPRRCPTRPEGQNYTEGIAVVFKENIAPYKFKATMYYKDVTVSQVWFGHRYSQFM
GIFEDRAPVPFEEVIDKINAKGVCRSTAKYVRNNLETTAFHRDDHETDMELKPANAATRTSR
GWHTTDLKYNPSRVEAFHRYGTTVNCIVEEVDARSVYPYDEFVLATGDFVYMSPFYGYREG
SHTEHTSYAADRFKQVDGFYARDLTTKARATAPTTRNLLTTPKFTVAWDWVPKRPSVCTMT
KWQEVDEMLRSEYGGSFRFSSDAISTTFTTNLTEYPLSRVDLGDCIGKDARDAMDRIFARRY
NATHIKVGQPQYYLANGGFLIAYQPLLSNTLAELYVREHLREQSRKPPNPTPPPPGASANAS
VERIKTTSSIEFARLQFTYNHIQRHVNDMLGRVAIAWCELQNHELTLWNEARKLNPNAIASAT
VGRRVSARMLGDVMAVSTCVPVAADNVIVQNSMRISSRPGACYSRPLVSFRYEDQGPLVEG
QLGENNELRLTRDAIEPCTVGHRRYFTFGGGYVYFEEYAYSHQLSRADITTVSTFIDLNITML
EDHEFVPLEVYTRHEIKDSGLLDYTEVQRRNQLHDLRFADIDTVIHADANAAMFAGLGAFFEG
MGDLGRAVGKVVMGIVGGVVSAVSGVSSFMSNPFGALAVGLLVLAGLAAAFFAFRYVMRLQ
SNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKLAEAREMIRYMALVSAMERTEHKA
KKKGTSALLSAKVTDMVMRKRRNTNYTQVPNKDGDADEDDL

HSV-1 (type 1 / strain 17) Swiss-Prot: P06484

gG (Gene Name: US4), Strain 17, 238 aa long

MSQGAMRAVVPIIPFLLVLVGVSGVPTNVSSTTQPQLQTTGRPSHEAPNMTQTGTTDSPTAI
SLTTPDHTPPMPSIGLEEEEEEEGAGDGEHLEGGDGTRDTLPQSPGPAFPLAEDVEKDKPN
RPVVPSPDPNNSPARPETSRPKTPPTIIGPLATRPTTRLTSKGRPLVPTPQHTPLFSFLTASP
ALDTLFVVSTVIHTLSFLCIGAMATHLCGGWSRRGRRTHPSVRYVCLPSERG

HSV-2 (type 2 / strain HG52) Swiss-Prot: P13290

gG (Gene Name: US4), Strain HG52, 699 aa long

MHAIAPRLLLLFVLSGLPGTRGGSGVPGPINPPNSDVVFPGGSPVAQYCYAYPRLDDPGPLG
SADAGRQDLPRRVVRHEPLGRSFLTGGLVLLAPPVRGFGAPNATYAARVTYYRLTRACRQP
ILLRQYGGCRGGEPPSPKTCGSYTYTYQGGGPPTRYALVNASLLVPIWDRAAETFEYQIELG
GELHVGLLWVEVGGEGPGPTAPPQAARAEGGPCVPPVPAGRPWRSVPPVWYSAPNPGFR
GLRFRERCLPPQTPAAPSDLPRVAFAPQSLLVGITGRTFIRMARPTEDVGVLPPHWAPGALD
DGPYAPFPPRPRFRRALRTDPEGVDPDVRAPRTGRRLMALTEDTSSDSPTSAPEKTPLPVS
ATAMAPSVDPSAEPTAPATTTPPDEMATQAATVAVTPEETAVASPPATASVESSPLPAAAAA
TPGAGHTNTSSASAAKTPPTTPAPTTPPPTSTHATPRPTTPGPQTTPPGPATPGPVGASAAP
TADSPLTASPPATAPGPSAANVSVAATTATPGTRGTARTPPTDPKTHPHGPADAPPGSPAP
PPPEHRGGPEEFEGAGDGEPPEDDDSATGLAFRTPNPNKPPPARPGPIRPTLPPGILGPLAP
NTPRPPAQAPAKDMPSGPTPQHIPLFWFLTASPALDILFIISTTIHTAAFVCLVALAAQLWRGR
AGRRRYAHPSVRYVCLPPERD

3. 2. 4. Synthesis of peptides

Peptides were custom synthesized from Hongkong GenicBio BioTech Co., Limited using the standard Fmoc-solid phase peptide synthesis strategy with 95% purity and quantity of 10 mg each with no modification and were finally dissolved in concentration of 1 mg/ml of phosphate buffered saline (PBS).

Peptide purification:

Specified purities refer to high performance liquid chromatography (HPLC) chromatograms obtained with linear gradients of 0.1% trifluoroacetic/100% acetonitrile with 0.1% trifluoroacetic/100% water and detection at 220 nm. The peptides were checked for purity using a highly discriminating 4.6*250mm, kromasil C18-5 column HPLC column, and the molecular mass was confirmed at the monoisotopic level using a sensitive nanospray mass spectrometry.

3. 2. 5. ELISA

Hundred microlitres of 5 ng/µl of different peptides were coated in microtiter wells and incubated for 3 h at 37°C. The wells were washed and blocked with 0.5% bovine serum albumin (BSA) and kept for 2 h at 37°C. The wells were washed and stored at 4°C overnight. Next day, diluted CSF (1:5 in PBS) samples were added to the wells and incubated for 1 h at 37°C. After washing the wells with PBS, 100 µl of secondary antibody [Goat-anti-human IgM/IgG-horse radish peroxidase (HRP) conjugated antibody, 1:10000, 1:5000 respectively] was added and incubated for 45 min at 37°C. 100 µl of 3,3',5,5'-tetramethylbenzidine(TMB)/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 solution. The results were read at A_{450}.

3. 2. 6. 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.

3. 2. 7. 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.

3. 2. 8. Statistical analysis

The cut off values for IgM and IgG were determined using the receiver operating curve (ROC) analysis. In t-test, differences with $P \leq 0.05$ were considered significant. All the statistical analyses were done with the help of MedCalc software (version 10.1.2).

3. 3. RESULTS

Infections with virus HSV-1 and -2, elicit, to a large extend, a type-common immune response with broad cross reactivity. Thus, it is not possible to serologically discriminate between these viruses with protein based assays. Therefore, peptide sequences were selected that resembled type-common as well as type-specific domains of different virus proteins. The objective of the present study was to identify an immunodominant epitope to determine antibody response against synthetic peptides of the envelope glycoprotein of the two viruses in CSF of HSE patients. Peptide sequences were selected that resembled type-common as well as type-specific domains of virus glycoprotein gB and gG, respectively. The selected peptides were screened in clinical samples for determining their potential as target antigens for humoral IgM and IgG immune response in patients with HSE.

Flow chart showing analysis of samples of HSE and non-HSE groups by antibody detection assays

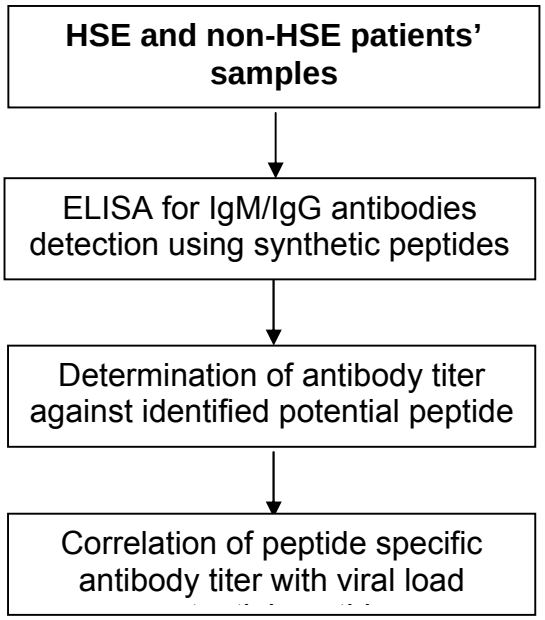


Figure 3. 1 shows the consensus sequence of gB with type-common peptide sequences in bold. Figure 3. 2 and Figure 3. 3 depict the sequences of gG of HSV-1 and HSV-2, respectively with type-specific peptide sequences in bold. Figure 3. 4 shows the antigenicity plot of the two glycoproteins gB and gG with their average antigenic propensities as determined by the software. Table 3. 1 shows the antigenic peptides designed from HSV glycoproteins.

Table 3. 2 and 3. 3 shows the mean value of absorbance for the binding of synthetic peptides to IgM and IgG antibodies in CSF from the HSV-infected (confirmed HSE) and non-HSV infected groups (control group) as determined by indirect ELISA method. The mean absorbance value for both IgM and IgG antibodies detectable by P-16 in confirmed HSE group was found to be significantly elevated as compared to control group. No significant difference was obtained for the mean absorbance values of confirmed HSE in comparison to control group for peptides of gG of both HSV-1 and HSV-2.

Table 3. 4 shows the occurrence of IgM and IgG antibodies detectable by P-16 in confirmed HSE, suspected HSE and control group as determined from the cut-off value of >0.322 and >0.42 for IgM and IgG estimation in CSF samples of HSE patients. The positivity for IgM antibodies detectable by P-16 was highest in confirmed HSE group (25.71%), whereas it was 22.85% in suspected HSE and 2.5% in control group. 42.85%, 37.14% and 5% of the cases of confirmed HSE, suspected HSE and control group respectively, were positive for IgG antibodies detectable by P-16. Considering the presence of both the classes of antibodies together, 17.14% cases of confirmed HSE and

15.23% of the suspected HSE group were positive, whereas control group cases showed the absence of both the antibodies together as detectable by P-16. The combined positivity showed highest percentage of 85.71% in confirmed HSE and 75.23% in suspected HSE group for the determination of IgM and IgG antibodies detectable by P-16 in HSV infected patients.

Follow up CSF specimens were also obtained for 12 patients on day 1, 7, 14 and 21 of admission and the positivity and negativity of PCR, IgM and IgG antibodies detectable by P-16 were determined as shown in Table 3. 5. The IgM or IgG antibodies in initial or follow up CSF were detectable in all except one case. The IgM antibodies detectable by P-16 were obtained on day 1 and 7 but not on day 14 and 21 of admission. The IgG antibodies detectable by P-16 were obtained in CSF up to day 21 of admission. In one case PCR of the CSF was negative, whereas the IgM antibodies were detectable by P-16.

Figure 3. 1. Consensus sequence of gB (904 aa) with peptide sequences in bold

MRQGAPARGXRWFVVWALLGLTLGVLVASAAPSSPGTPGVAAATQAANGG
PATPAPPAPGXAPTGDPKPKKNKKPKNPXPPRPAGDNATVAAGHATLREHL
RDIKAENTDANFYVCPPPTGATVVQFE**QPRRCPT**RPEGQNYTEGIAVVFKE
NIAPYKFKATMYYKDVTVSQVWFGHRYSQFMGIFE**DRAPVPFEEVID**KINAK
GVCRSTAKYVRNNLETTAFHRDDHETDMELKPANAATRTSRGWHTTDLKY
NPSRVEAFHRYGTTVNCIVEEVDARSVYPYDEFVLATGDFVYMSPFYGYRE
GSHTEHTSYAADRFKQVDGFYARDLTTKARATAPTTRNLLTTPKFTVAWDW
VPKRPSVCTMTKWQEVDEMLRSEYGGSFRFSSDAISTTFTTNLTEYPLSRV
DLGDCIGKDARDAMDRIFARRYNATHIKVGQPQYYLANGGFLIAYQPLLSNT
LAELYVREHLREQSRKPPNPTPPPPGASANASVERIKTTSSIEFARLQFTYN
HIQRHVNDMLGRVAIAWCELQNHELTLWNEARKLNPNAIASATVGRRVSAR
MLGDVMAVSTCVPVAADNVIVQNSMRISSRPGACYSRPLVSFRYEDQGPLV
EGQLGENNELRLTRDAIEPCTVGHRRYFTFGGGYVYFEEYAYSHQLSRADIT
TVSTFIDLNITMLE**DHEFVPLEVYT**RHEIK**DSGLLDYTE**VQRRN**QLHDLRF**AD
IDTVIHADANAAMFAGLGAFFEGMGDLGRAVGKVVMGIVGGVVSAVSGVSS
FMSNPFGALAVGLLVLAGLAAAFFAFRYVMRLQSNP**MKALYPLTT**KELKNP
TNPDASGEGEEGGDFDEAKLAEAREMI**RYMALVS**AMERTEHKAKKKGTSA
LLSAKVTDMVMRKRRNTNYTQVPNKDGDADEDDL

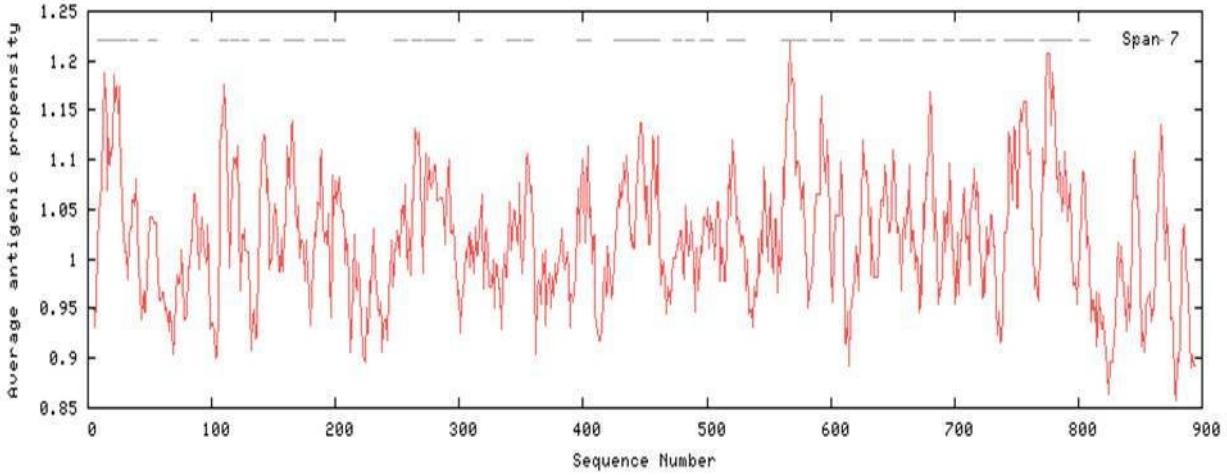
Figure 3. 2. Sequence of gG of HSV-1 (238 aa) with peptide sequences in bold

MSQGAMRAVVPIIPFLLVLVGVSGVPTNVSSTTQPQLQTTGRPSHEAPNMT
QTGTTDSPTAISLTTPDHTPPMPSIGLEEEEEEEGAGDGEHLEGGDGTRDT**L
PQSPGPAFPLAE**DVEKDKP**NRPVVPS**PDPNNSPARPETSRPK**TPPTIIGPLA**
TRPTTRLTSKGRPLVPTPQ**HTPLFSFLASPALDTLF**VVSTVIHTLSFLCIGA**M
ATHLCG**GWSRRGRR**THPSVRYVCLP**SERG

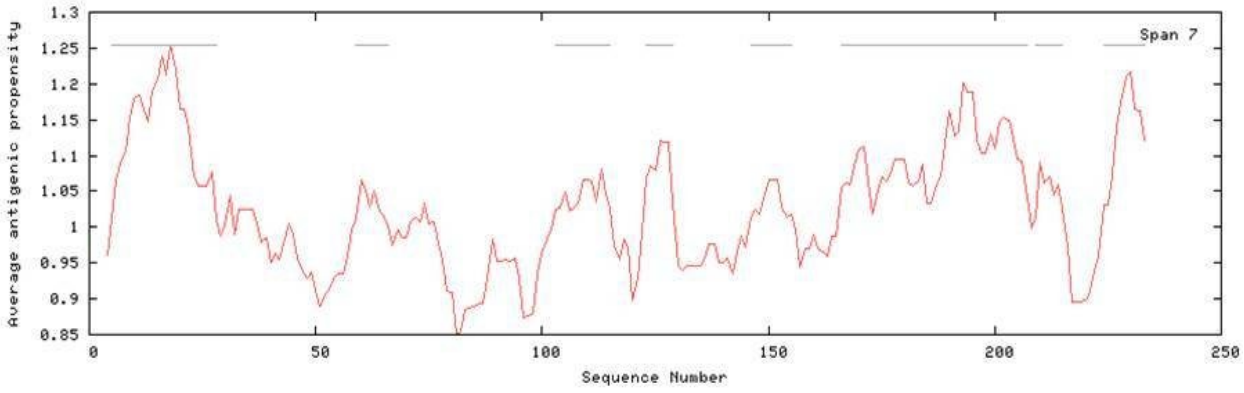
Figure 3. 3. Sequence of gG of HSV-2 (238 aa) with peptide sequences in bold

MHAIAPRLLLLFVLSGLPGTRGGSGVPGPINPPNSDVVFPGGSPVAQYCYA
YPRLDDPGPLGSADAGRQD**LPRRVVRH**EPLGRSFLTGGLVLLAPPVRGFG
APNATYAARVTYYRLTRACRQPILLRQYGGCRGGEPP**SPKTCGSYTY**TYQG
GGPPTRYALVNASLLVPIWDRAAETFEYQIELGGELHVGLLWVEVGGEGPG
PTAPPQAARA**EGGPCVPPVPA**GRP**WRSVPPVWYSA**PNPGFRGLRFRERC
LPPQTPAAPSDLPRVAFAPQSLLVGITGRTFIRMARPTEDVGVLPPHWAPGA
LDDG**PYAPFPP**RPRFRRALRTDPEG**VDPDVRA**PRTGRRLMALTEDTSSDS
PTSAPEKTPLPVSATAMAPSVDPSAEPTAPATTTPPDEMATQAATVAVTPEE
TAVASPPATASVESSPLPAAAAATPGAGHTNTSSASAAKTPPTTPAPTTPPP
TSTHATPRPTTPGPQTTPPGPATPGPVGASAAPTADSPLTASPPATAPGPS
AANVSVAATTATPGTRGTARTPPTDPKTHPHGPADAPPGSPAPPPPEHRG
GPEEFEGAGDGEPPEDDDSATGLAFRTPNPNKPPPARPGPIRPTLPPGILG
PLAPNTPRPPAQAPAKDMPSGPTPQHIPLFWFLTASPALDILFIISTTIHTAAF
VCLVALAAQLWRGRAGRRRYAHPSVRYVCLPPERD

Figure 3. 4. Antigenicity plot of gB and gG. (a) average antigenic propensity for consensus sequence of gB is 1.0199 (b) average antigenic propensity for sequence of gG of HSV-1 is 1.0285 (c) average antigenic propensity for sequence of gG of HSV-2 is 1.0240



(a)



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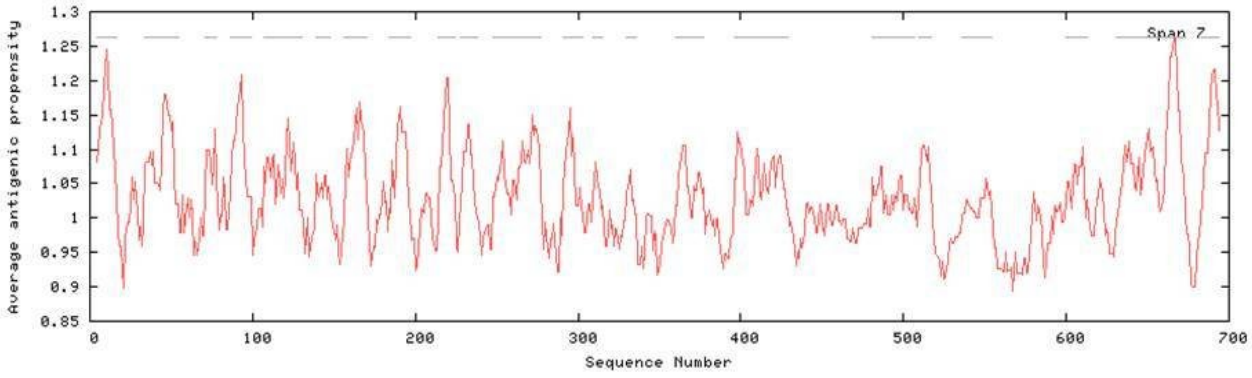


(c)

Table 3. 1. Shows list of peptides with their amino acid sequence

Glycoprotein	S. No	Peptide Name	Peptide designation	Sequence
gG of HSV-1	1	Peptide 2	P-2	LPQSPGPAFPLAE
	2	Peptide 3	P-3	NRPVVPS
	3	Peptide 4	P-4	TPPTIIGPLA
	4	Peptide 5	P-5	MATHLCG
	5	Peptide 6	P-6	THPSVRYVCLP
	6	Peptide 8	P-8	HTPLFSFLTASPALDTLF
gB of both HSV-1 and HSV-2	7	Peptide 10	P-10	QPRRCPT
	8	Peptide 13	P-13	DRAPVPFEEVID
	9	Peptide 14	P-14	DHEFVPLEVYT
	10	Peptide 15	P-15	DSGLLDYTE
	11	Peptide 16	P-16	QLHDLRF
	12	Peptide 17	P-17	MKALYPLTT
	13	Peptide 18	P-18	RYMALVS
gG of HSV-2	14	Peptide 19	P-19	LPRRVVRH
	15	Peptide 20	P-20	SPKTCGSYTY
	16	Peptide 22	P-22	EGGPCVPPVPA
	17	Peptide 23	P-23	WRSVPPVWYSA
	18	Peptide 25	P-25	PYAPFPP
	19	Peptide 26	P-26	VDPDVRA

Table 3. 2. Mean±SD and *P* value along with cut-off value determined by ROC analysis of gB (P-10 to P-18) and gG (P-2 to P-26) peptides for IgM analysis

	Confirmed HSE (n=35)	**Control (n=40)**		
	Mean±SD		***P* value**	**Cut-off value**
P-10	0.35±0.18	0.30±0.08	P=0.1718	>0.408
P-13	0.32±0.15	0.30±0.08	P=0.4148	>0.37
P-14	0.31±0.15	0.26±0.11	P=0.0897	>0.355
P-15	0.31±0.14	0.31±0.07	P=0.8179	>0.398
P-16	0.34±0.12	0.25±0.05	**P=0.0001**	>0.322
P-17	0.34±0.20	0.30±0.08	P=0.2535	>0.398
P-18	0.35±0.17	0.29±0.13	P=0.1686	>0.478
P-2	0.31±0.14	0.28±0.11	P=0.3119	>0.316
P-3	0.29±0.12	0.30±0.15	P=0.7601	>0.319
P-4	0.30±0.13	0.28±0.12	P=0.5005	>0.345
P-5	0.29±0.12	0.27±0.14	P=0.5235	>0.293
P-6	0.29±0.12	0.26±0.13	P=0.3176	>0.305
P-8	0.28±0.11	0.24±0.10	P=0.1112	>0.292
P-19	0.33±0.18	0.30±0.19	P=0.4979	>0.417
P-20	0.30±0.12	0.28±0.11	P=0.4640	>0.333
P-22	0.28±0.11	0.27±0.12	P=0.7166	>0.307
P-23	0.29±0.11	0.31±0.17	P=0.5667	>0.294
P-25	0.36±0.17	0.33±0.16	P=0.4445	>0.384
P-26	0.34±0.17	0.28±0.14	P=0.1049	>0.401

Table 3. 3. Mean±SD and *P* value along with cut-off value determined by ROC analysis of gB (P-10 to P-18) and gG (P-2 to P-26) peptides for IgG analysis

	Confirmed HSE (n=35)	**Control (n=40)**		
	Mean±SD		***P* value**	**Cut-off value**
P-10	0.40±0.23	0.36±0.13	P=0.3778	>0.487
P-13	0.38±0.19	0.36±0.13	P=0.5984	>0.47
P-14	0.37±0.21	0.31±0.12	P=0.1352	>0.454
P-15	0.39±0.21	0.36±0.13	P=0.3778	>0.487
P-16	0.44±0.18	0.32±0.11	**P=0.0012**	>0.42
P-17	0.37±0.23	0.31±0.13	P=0.1748	>0.459
P-18	0.36±0.21	0.30±0.17	P=0.1706	>0.546
P-2	0.43±0.12	0.39±0.21	P=0.3410	>0.488
P-3	0.39±0.14	0.34±0.17	P=0.1848	>0.438
P-4	0.40±0.14	0.36±0.21	P=0.3582	>0.476
P-5	0.40±0.14	0.39±0.20	P=0.8113	>0.464
P-6	0.39±0.12	0.36±0.20	P=0.4575	>0.446
P-8	0.45±0.18	0.40±0.31	P=0.4212	>0.521
P-19	0.39±0.13	0.33±0.19	P=0.1323	>0.45
P-20	0.39±0.14	0.37±0.18	P=0.6076	>0.476
P-22	0.37±0.12	0.35±0.18	P=0.5912	>0.433
P-23	0.38±0.12	0.34±0.18	P=0.2843	>0.403
P-25	0.42±0.09	0.37±0.17	P=0.1375	>0.48
P-26	0.41±0.09	0.36±0.15	P=0.1013	>0.48

Table 3. 4. Positivity of IgM and IgG antibodies for gB (P-16) peptide in confirmed HSE, suspected HSE and control cases

Category of patients	IgM+/IgG-	IgG+/IgM-	IgM+/IgG+	Total positivity
Confirmed HSE (n=35)	09 (25.71%)	15 (42.85%)	06 (17.14%)	30 (85.71%)
Suspected HSE (n=105)	24 (22.85%)	39 (37.14%)	16 (15.23%)	79 (75.23%)
Control (n=40)	1 (2.5%)	2 (5%)	0	3 (7.5%)

Table 3. 5. Results of PCR, IgM and IgG antibodies against P-16 in follow up CSF samples obtained on day 1, 7, 14 and 21 of admission of 12 individual patients

Patient No.	Sample collection interval (days)	PCR results	IgM results	IgG results
1	1	Positive	Negative	Negative
	7	Negative	Positive	Positive
2	1	Positive	Positive	Positive
	7	Negative	Negative	Positive
3	1	Positive	Positive	Positive
	7	Negative	Positive	Positive
4	1	Positive	Negative	Negative
	7	Positive	Negative	Negative
5	1	Negative	Positive	Negative
	7	Positive	Negative	Positive
6	1	Positive	Positive	Positive
	14	Negative	Negative	Positive
7	1	Positive	Positive	Negative
	14	Negative	Negative	Positive
8	1	Positive	Negative	Negative
	14	Negative	Negative	Positive
9	1	Positive	Positive	Positive
	7	Positive	Positive	Positive
	14	Negative	Negative	Positive
10	1	Positive	Positive	Negative
	7	Positive	Positive	Positive
	14	Negative	Negative	Positive
11	1	Positive	Positive	Positive
	14	Positive	Negative	Positive
	21	Negative	Negative	Positive
12	1	Positive	Positive	Positive
	14	Positive	Negative	Positive
	21	Negative	Negative	Positive

After the identification of an immunogenic synthetic peptide of the antigenic envelope glycoprotein gB of HSV-1 and -2, the IgM and IgG antibody titer against this peptide was determined in CSF of HSE patients. The antibody titers were determined against the identified potential peptide using the method of single dilution as reported earlier.

The peptide specific antibody 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. 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 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 (Figure 3. 5).

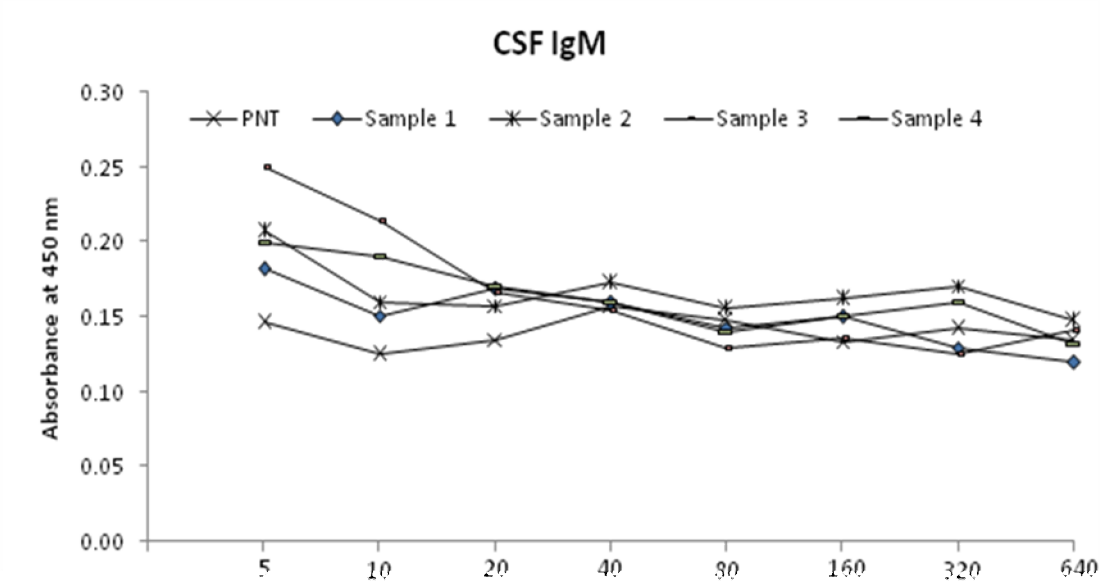
The regression analysis was carried out with $\log_{10}$ values of absorbance at each dilution of 35 PCR positive CSF with their corresponding $\log_{10}$ observed antibody titers to obtain regression equation. The regression equation with the best correlation coefficient value was henceforth used for the determination of antibody titers in CSF of HSE patients. The highest correlation was obtained at dilutions 1:10 and 1:20 for IgM and IgG analysis respectively in CSF samples (Table 3. 6). On the basis of these values, the regression equations were hence obtained (Table 3. 6). The absorbance values of samples at this

dilution were directly converted into predicted antibody titers using the regression equations.

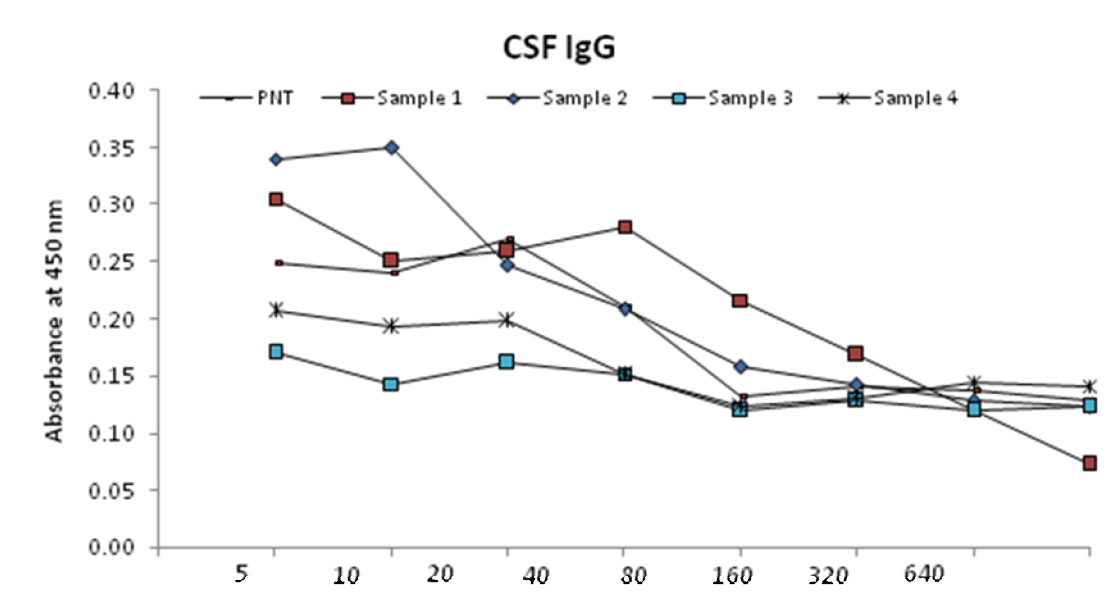
The titer values between 5-10 and 5-20 were obtained for IgM and IgG antibody analysis, respectively against P-10 in CSF of both confirmed and suspected HSE group. Geometric mean titer values of 8 for IgM; 12 and 10 for IgG were obtained in CSF of confirmed HSE and suspected HSE group respectively (Table 3. 7).

The antibody titers obtained in ELISA were correlated with HSV DNA levels. A weak negative correlation of IgM antibody titer and a positive correlation of IgG antibody titer were obtained with viral load in the CSF of HSE patients (Figure 3. 6).

Figure 3. 5. PNT baseline for determination of (a) IgM and (b) IgG antibody titer using serially diluted CSF samples



(a)



(b)

Table 3. 6. CSF and serum dilutions with their highest correlation coefficient value and the regression equation for calculation of antibody titers

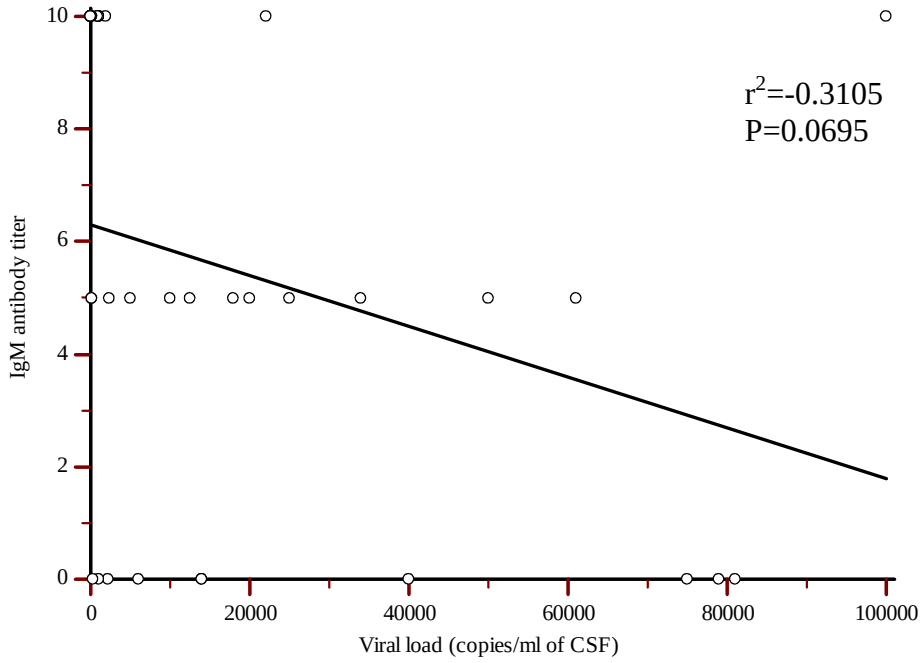
Sample	Antibody	Dilution	Correlation coefficient	Equation [y=C+mx]
CSF	IgM	1:10	0.9098	y = -0.1048 + 0.1048 x
	IgG	1:20	0.8129	y = -0.1731 + 0.2346 x

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

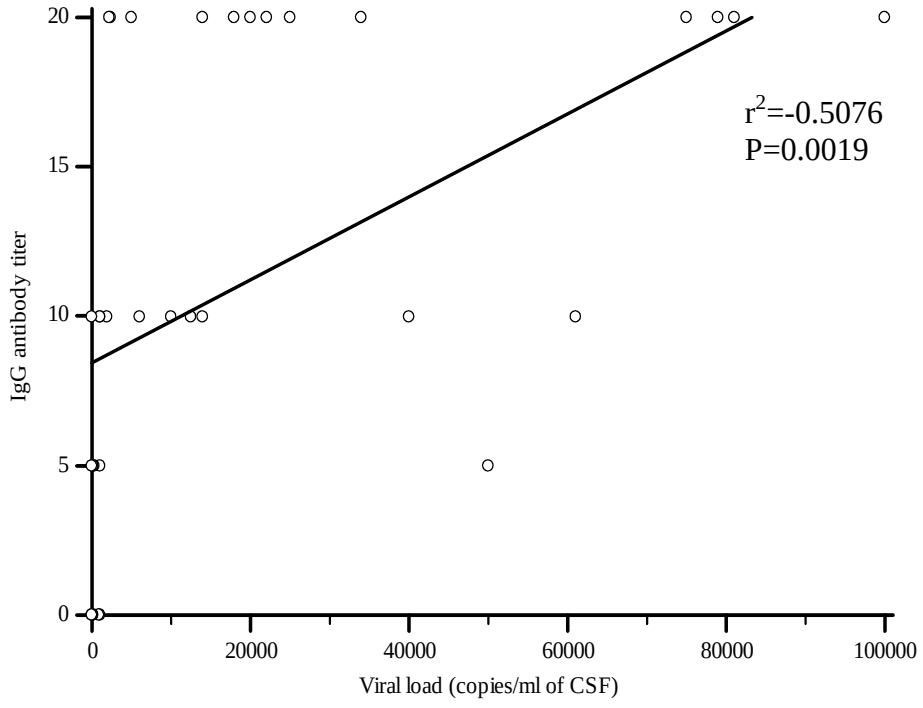
Table 3. 7. Range along with geometric mean titer of IgM and IgG antibody titer against P-16 in CSF of confirmed and suspected HSE cases

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



(a)



(b)

3. 4. DISCUSSION AND CONCLUSIONS

Detection of herpes virus-specific antibodies permits diagnosis of an infection when virologic methods such as culture, antigen detection, and PCR are impractical, too costly, or yield negative results. CSF antibody measurements may be useful in cases where CSF has been obtained at late times following onset of illness or the viral load is low in CSF. Therefore, analysis of IgM and IgG response in CSF may be a useful alternative tool for diagnosis of HSE.

The type-common antibody response against HSV is mostly attributed to glycoprotein gB while the envelope glycoprotein gG, is the only HSV antigen known to induce type-specific antibody responses ***(Ashley, 1998; Görander et al., 2003)***. These glycoprotein sequences were selected to obtain peptides with antigenic epitopes on the basis of prediction analysis by Kolaskar and Tongaonkar method.

A total of 12 peptides consisting 6 peptides each of HSV-1 and HSV-2 gG were synthesized and analyzed in cases of confirmed HSE and control groups. Studies have been reported describing the possibility of HSV serology by using synthetic peptides of gG ***(Levi et al., 1996)***. Immunodominant type-specific epitopes for human antibodies have been identified and should be useful for type-specific serodiagnosis of HSV-1 and HSV-2 ***(Marsden et al., 1998; Grabowska et al., 1999; Tunbäck et al., 2000; Kasubi et al., 2005)***. However, in this study, the selected peptides of gG region were not found to be immunodominant epitopes for the diagnosis of HSE. There are studies

supporting this finding which states the limits in reliability of gG based type-specific serologic assays for HSV-1 and 2 due to the high rates of seroreversion, defined as the change in an individual's antibody status from positive to negative over time, suggesting that their use in HSV acquisitional studies would not be useful ***(Schmid et al., 1999; Cherpes et al., 2003)***.

The ideal peptide-based seroassay should combine high sensitivity and specificity and be based on a well-conserved HSV antigen evoking a strong antibody response against antigenic determinants ***(Bergström and Trybala, 1996)***. The sequence of gB is well known to be conserved in HSV. Out of 7 peptides selected for type-common sequences of HSV-1 and HSV-2 gB, only one peptide (P-16) of gB region showed significant difference ($P<0.05$) in the absorbance between the confirmed HSE and control group for the antibody analysis in CSF. Almost 86% of the confirmed HSE cases were found to be positive for the presence IgM and/or IgG antibodies using this peptide. There is only one reported study available which determine immunity induced by peptides corresponding to the antigenic sites of gB ***(Mester et al., 1990)***. It is reported that antibodies in CSF against gB in HSV were detected earlier than antibodies to the other viral proteins, probably because of high immunogenicity of gB ***(Kahlon et al., 1987)***. One study demonstrated that gB is presented with the same kinetics as a classical early-gene product and it is the possibility that gB could be an effective target as HSV emerges from latent infection ***(Mueller et al., 2003)***. It has also been shown that the functional domains of gB involved in cell penetration and cell fusion, and the major antigenic domains are highly conserved in peripheral and CNS HSV isolates

(Sivadon et al., 1998) therefore, variation in antibody response against this protein could be least suspected.

The P-16 was further used for the determination of antibody response in cases negative by PCR but suspected for HSE on the basis of clinical and other laboratory analyses. In our study, out of 105 cases negative by PCR, approximately 75% of the cases showed IgM or IgG antibody response against P-16. Failure of the PCR in HSE patients where intrathecal antibody response is obtained has been reported ***(Puchhammer-Stöckl et al., 2001; Fomsgaard et al., 1998)***. Therefore, antibody detection using P-16 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.

In addition to that, approximately 14% of the cases positive by PCR were not found to be positive for the presence of any of the antibodies against the particular peptide. To rule out the possibilities, the IgM and IgG antibodies were 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 IgM antibody against P-16 was obtained on day 1 and 7 of admission whereas, it was not found on day 14 and 21 of admission. The IgG antibodies against P-16 were obtained in CSF on day 7, 14 and 21 of admission. All the 12 cases showed positivity by PCR in CSF collected at day 1 except one case where the IgM antibody against P-16 was detected. Thus, in cases where CSF shows negativity for HSV DNA, the IgM or IgG antibody could be detected which helps in improved diagnosis of the disease and early administration of antiviral therapy. There

were some cases in which the first CSF of follow up samples showed negativity for antibodies. The CSF samples of patients had been collected in between 4 to 12 days after the onset of illness in patients. Previous study explains why the sensitivity of this method is time dependent. One study has reported similar specificity in early and late samples, but an increased sensitivity upto 90.5% if the sample was collected more than 1 week after the onset of symptoms ***(Sauerbrei and Wutzler, 2002)***.

There are only two Indian studies that determined the serological profile of patients with HSE ***(Ratho et al., 1999; Panagariya et al., 2001)***. As far as I know, this is the first report describing the use of synthetic peptide of HSV for serological response in HSE patients. I conclude that this synthetic gB peptide (P-16) may be useful for determining IgM or IgG antibody response against HSV. The peptide based ELISA is specific and sensitive as compared to detection of the virus by PCR. The method is inexpensive and reliable.

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)***. I identified an immunodominant epitope for the determination of antibodies in the CSF of HSE patients. I aimed for development of single CSF dilution method for determination of antibody titer against this epitope.

Determination of antibody titer against peptide has rarely been adopted. However, studies have been made to determine titer of antibodies against a particular peptide. ***(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 ***(Bohl et al., 2005; Lundstig et al., 2007; Randhawa et al., 2008; 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, I 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.

As far as I 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.