Original Article

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Microbiology Section

Novel Synthetic Peptide-based Enzyme Linked Immunosorbent Assay for Antibody Detection in Viral Infections of the Central Nervous System

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ABSTRACT

Introduction: Viral infections of Central Nervous System (CNS) are associated with severe neurological sequelae and can lead to significant morbidity, if not adequately diagnosed and treated. An immunological assay detecting antibodies in the Cerebrospinal Fluid (CSF) of the patients is widely used for diagnosis of viral infections of the CNS. In the present study, diagnostic efficacy of in-house designed synthetic peptide based Enzyme Linked Immunosorbent Assay (ELISA) was evaluated for detection of antibodies against neurotropic viruses panel such as Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Varicella Zoster Virus (VZV), Japanese Encephalitis Virus (JEV), Dengue (DENV), West Nile Virus (WNV) and Chandipura Virus (CHPV) in CSF of suspected cases.

Aim: To determine the diagnostic efficacy of in-house developed synthetic peptide-based ELISA in viral infections of the CNS.

Materials and Methods: A retrospective study was conducted in Central India Institute of Medical Science, Nagpur, Maharashtra, India. The suspected case of CNS viral infections of the patients were enrolled in this study from January 2013 to December 2015. Total 150 suspected cases of viral CNS infections and 135 control cases were recruited. Total 32 synthetic peptides of highly immunogenic proteins of respective seven neurotropic viruses were designed and synthesised. The designed peptides were evaluated in CSF samples of both the viral CNS infections and control cases for detection of Imunoglobulin G (IgG) and IgM antibodies using in-house developed antibody detection method. The developed tests were further compared with commercially available antibody detection ELISA kit. The sensitivity and specificity of peptide-ELISA were determined by Receiver Operating Curve (ROC) analysis and p-value was calculated by comparison of means by t-test.

Results: Out of total 150 cases of CNS viral infections, a total of 31 CSF samples including 15 for CMV and 16 of JEV were positive by in-house peptide ELISA. The synthetic peptides STGDVVDISP, KQKSLVEL, RTLEVFKE, RSSNVED of CMV and ITYECPK, RRSVSVQT, GESSLVN of JEV showed a significant difference in mean absorbance value for IgM and IgG antibodies in CSF of suspected cases of viral CNS infection.

Conclusion: The results demonstrated that the synthetic peptide-based ELISA is rapid, cost-effective, simple and efficient immunodiagnostic assay for initial screening of viral CNS infection.

Keywords: Bioinformatics, Central nervous system viral infection, Cerebrospinal fluid, Commercial enzyme linked immunosorbent assay, Neurotropic viruses

INTRODUCTION

Viral infections of the CNS occur sporadically and have been extensively studied because of potential for permanent neurological damage or death [1]. The neurotropic viruses are the main aetiological agent for infections of the CNS. The common neurotropic viruses causing CNS infection belongs to herpes viruses, flavi viruses, coxsackie virus and adeno virus families along with several others like mumps, measles, polio, echo and rabies viruses [2]. Among all neurotropic viruses, major outbreaks of JEV, DENV, WNV and CHPV have been reported in India with significant mortality and morbidity [3-6]. Herpes virus family members including VZV, CMV and EBV have been known for long causing infections of the CNS [7-9].

Clinical diagnosis of viral CNS infections remains difficult due to non specific clinical presentations, thus posing a major challenge to clinicians in confirmatory diagnosis. The present clinical and laboratory diagnosis of CNS viral infection is based on medical history and examination of CSF for protein and glucose content. Neuroimaging such as Computed Tomography (CT), Magnetic Resonance Imaging (MRI) and Electroencephalography are also performed for diagnosis [10]. However, these methods lack specificity for the identification of the infecting organism. Molecular diagnostic assays using Polymerase Chain Reaction (PCR) are widely used for diagnosis of viral infections of the CNS, due to high sensitivity. However, such techniques although sensitive but require elaborate infrastructure facilities and are beyond most diagnostic capacities in underdeveloped regions [11]. An immunological assay detecting antibodies against viruses provides a less expensive method for early diagnosis [12]. In the current serological assay for the detection of viral antibodies, the viral lysate is mostly used [13]. However, the standardisation of viral lysate composition is difficult as it contains many viral antigens which may cause cross-reactivity and give a false positive result.

Earlier study have reported the diagnostic utility of synthetic peptide-based ELISA methods for detection of antibodies in some neurological viral infection like herpes simplex encephalitis and chikungunya [14,15]. Some immunodiagnostic methods based on synthetic peptides derived from antigenic proteins of viruses and bacteria has been reported in human infections in recent years [16, 17]. Synthetic peptide-based assay helps to differentiate subtle changes in monoclonal antibody specificities and has the major advantage of being cost-effective, than conventional antigen based tests. In the present study, authors report a utility of novel and cost-effective in-house developed synthetic peptide-based ELISA assay for the detection of viral CNS infections in patients with suspected viral infections admitted to the tertiary care hospital.

MATERIALS AND METHODS

The retrospective study was conducted in patients admitted to the Inpatient Department (IPD) wards of Central India Institute of Medical Sciences, Nagpur, Maharashtra, India with suspected viral CNS infections were enrolled in the present study from January 2013 to December 2015 and the study was approved by the Ethical Committee of Central India Institute of Medical Sciences (CIIMS), Nagpur (CIIMS/IEC/04/2008). Written consents were obtained from all the patients or their relatives those enrolled in the study.

Sample size: The sample size was calculated by using Raosoft online sample size calculator.

Study Procedure

The patients admitted to the IPD wards of Central India Institute of Medical Sciences, Nagpur, India with suspected viral CNS infections were enrolled in the present study from January 2013 to December 2015 and were analysed in the same duration. Neurological diagnostic investigations were performed during the first week of hospitalisation; these investigations included the acid fast staining, India ink and Gram stain, microbial culture, Human Immunodeficiency Virus (HIV) status, estimation of protein and sugar level 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. The HIV and Herpes Simplex Virus (HSV) positive samples were excluded from the study.

Approximately 3-5 mL of CSF was collected under all aseptic precautions by standard lumbar puncture technique from all the patients. The collected samples were divided into two fractions, one fraction was subjected for IgG/IgM/PCR/antigen analysis and the other fraction was used for microbial analysis along with cell count, protein and sugar content. All the samples were stored at -20°C until further analysis. The initial samples were collected from patients before treatment and from some of the patients, whenever possible, during treatment. The clinical data of patients was collected from case record forms. Clinically, all the patients (n=285) were divided as discussed below:

a) Suspected viral infections of the CNS cases (n=150)

The patient's inclusion criteria involved the presence of headache, altered mental status (low level of consciousness, behaviour or personality changes) with acute onset of fever, clinical features consistent with viral encephalitis and other clinical manifestations (e.g. focal neurological deficits, seizures) and CSF findings showing mild increase in protein, glucose often normal and mild pleocytosis.

b) Non viral infections of the CNS cases control

1. Non infectious neurological disorders (n=105)

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

2. Other infectious cases (n=30)

Patients included in this group had Tuberculous Meningitis (TBM), pyogenic meningitis or 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 and patients showed good clinical response to antituberculous drugs.

Non tuberculous infectious meningitis: This group included patients having pyogenic or fungal meningitis.

Peptide Designing and Synthesis

For designing of antigenic peptide, immunogenic antigens of viruses namely glycoprotein B (gB) of CMV, glycoprotein E (gE) of VZV, glycoprotein E (gE) of WNV and glycoprotein G (gG) of CHPV, membrane protein Early antigen- diffuse (EA-D) of EBV, premembrane protein (prM) of JEV and non structural proteins (NS) of DENV were targeted. Sequences of respective viral proteins were obtained by using EXPASY proteomic server- UniProtKB/Swiss-Prot. The antigenic peptides were determined on the basis of Kolaskar AS and Tongaonkar PC methods by using online software "Molecular Immunology Foundation-Bioinformatics" [18]. The designed peptides sequences were then subjected to multiple sequence alignment to check homology with other organisms and to obtain the sequence similarities with other non redundant protein database sequences of different species using NCBI BLAST (National Centre for Biotechnology Information, Basic Local Alignment Search Tool). A total of 32 peptides were designed having varying antigenicity.

Peptides synthesis: Peptides were custom synthesised by Hong Kong GenicBio BioTech Co., limited with 95% purity and quantity of 10 mg each with no modification. The peptides were finally dissolved in a concentration of 1 mg/mL of Phosphate Buffer Saline (PBS), pH 7.4.

Standardisation of Peptide ELISA

Prior to the development of synthetic peptide-based ELISA for antibody detection, peptide concentration was initially standardised using 100 ng to 800 ng of peptide per well [14,15]. About 100 µL of different concentration of peptides were diluted in PBS and coated in microtitre wells and incubated overnight at 4°C. Next day after giving one wash with Phosphate Buffered Saline with Tween-20 (PBST), wells was blocked with 0.5% Bovine Serum Albumin (BSA) and incubated for 2 hrs at 37°C. The plate was washed three times with PBS and 100 µL of 1:5, 1:10; 1:20 and 1:40 diluted CSF samples were added to the wells and incubated for 1 hr at 37°C. After washing three times with PBST, 100 µL of secondary antibody 1:5000, 1:10000, 1:20000 and 1:40000 (Goat anti-human IgG/IgM Horse Radish Peroxidase (HRP) conjugate Bangalore, Genei) were separately added and incubated for 45 min at 37°C. The plate was washed again with PBST and 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB)/H_2O_2 substrate solution was added and the plate was incubated for 3 min. The reaction was stopped by adding 100 µL of 2.5N H_2SO_4 in each well. The absorbance of the colour developed in each well was read at 450 nm.

Antibody Detection by Indirect ELISA

Indirect ELISA protocol was followed as previously standardised for Herpes simplex virus and Chikungunya viral CNS infections [14,15]. Peptides (500 ng/100 µL) for individual viruses were separately diluted in PBS and coated in the clear microtiter well plate and incubated at 4°C for overnight. Next day after giving one wash with PBST, wells was blocked with 0.5% BSA and incubated for 2 hrs at 37°C. The plate was washed three times with PBS and 100 µL of diluted CSF samples (1:10) of suspected viral CNS infections cases along with known positive and negative control of respective viruses, and blank were added to the wells and incubated for 1 hr at 37°C. After washing three times with PBS 100 µL of secondary antibody (Goat anti-human HRP conjugated antibody, 1:5000 for IgM and 1:10,000 for IgG) were separately added and incubated for 45 min at 37°C. The plate was washed again with PBS and 100 µL of TMB/H_2O_2 substrate solution was added and the plate was incubated for 3 min. The reaction was stopped by adding 100 µL of 2.5 N H_2SO_4 in each well. The absorbance of the colour developed in each well was read at 450 nm.

Positive and Negative Reference Control in ELISA

Negative reference control was selected from the pooled CSF of non infectious controls that had no prior history of viral infections. For positive reference control, commercial antibodies were taken are as follows- EBV and DENGUE (Biorbyt, USA), CMV, VZV and JEV

(Abnova, USA) and for CHPV and WNV (Invitrogen USA). Antibodies were used as per the manufacturer's instructions. ELISA was run with the positive control, negative control and sample blank (PBS).

JEV antibody detection by using commercial kit: JEV ELISA kit (InBios, Seattle, WA, USA) was used for the detection of IgG/IgM antibodies. This assay employs a recombinant antigen named as JERA which acts as a rapid serological marker for the detection of JEV infections. The protocol for JEV IgG/IgM detection was followed as per kit instructions.

CMV antibody detection by using commercial kit: The LUCIO CMV ELISA kit (Germany) was used for the qualitative detection of IgG/IgM antibodies for CMV in CSF of suspected viral encephalitis patients. The protocol for CMV IgG/IgM detection was followed as per kit instructions.

STATISTICAL ANALYSIS

The statistical analyses were performed using Medcalc (version 10) statistical software. A cut-off point for optimal sensitivity and specificity for the ELISA tests was determined using the ROC curve analysis. A p-value was calculated by comparison of means by paired t-test.

RESULTS

The CSF samples were collected from the patients from the IPD wards of Central India Institute of Medical Sciences, Nagpur and categorised into suspected CNS viral infection cases and control groups based on the clinical history, biochemical, microbiological and pathological analysis of CSF samples and neuroimaging findings. The peptides selected for antibody detection in patient's samples showed less or no homology among them using NCBI BLAST tool. [Table/Fig-1] shows the list of viral antigens and their synthetic peptide sequences for antibody detection. [Table/Fig-2,3] shows the Mean±SD value of absorbance of all synthetic peptides for binding of IgG and IgM antibodies in CSF from suspected and control groups. Among all 32 peptides, significant difference in mean absorbance value for IgG and IgM antibodies were obtained in peptides namely A2, A3, A4, A5 of CMV and E1, E2, E3 of JEV in suspected group as compared to control groups. The cut-off values were found to be >0.331, >0.332, >0.387, >0.389 for IgG and >0.304, >0.352, >0.372, >0.391 for IgM antibodies of CMV Similarly, in case of JEV the cut-off values are >0.333, >0.328, >0.316 for IgG and >0.357, >0.391, >0.322 for IgM antibodies. The cut-off value for absorbance of these peptides as determined by ROC analysis and on the basis of cut-off values, the positivity of IgG and IgM antibodies in CSF samples of suspected cases were determined for peptides showing a significant difference between suspected and control groups. No significant difference was obtained in Mean±SD value of EBV, VZV, DENV, WNV and CHPV viral peptides.

[Table/Fig-4] shows total % positivity of IgG and IgM antibodies for CMV and JEV detected by peptide ELISA in suspected viral CNS infections cases.

S. No.	Virus	Proteins	Peptide designation	Peptide sequence	Amino acid position
1	Cytomegalovirus	Glycoprotein B (907 AA)	A1	STRYVTVKD	221-229
			A2	STGDVVDISP	266-274
			A3	KQKSLVEL	404-411
			A4	RTLEVFKE	480-487
			A5	RSSNVFD	564-570
2	Epstein-Barr virus	Early antigen D (404 AA)	B1	YGRTCVLGK	91-99
			B2	RPEFVKL	122-128
			B3	TASSLQK	172-178
			B4	SPRVQPLGT	315-323
			B5	HPKKVKQA	391-398
3	Varicella zoster virus	Glycoprotein E (623 AA)	C1	ITNPVRASVLRYDD	24-37
			C2	SVYEPYYHSD	49-58
			C3	RHKIVNVDQ	143-151
			C4	QGQRLIEVSVEE	167-178
			C5	TLKFVDT	462-468
4	Dengue serotype 1	Non structural proteins (352 AA)	D1	RELKCGSGIFVTN	11-23
			D2	EEGVCGI	51-57
	Dengue serotype 2		D3	ITPELNH	71-77
			D4	EKQDVFCDS	173-181
	Dengue serotype3		D5	SPKRLATAI	37-45
			D6	HLGKLELDFNY	270-280
	Dengue serotype 4		D7	ALTPPVSDLKY	45-55
			D8	SSEVCDH	175-181
5	Japanese encephalitis virus	Pre-membrane protein (167 AA)	E1	ITYECPK	49-50
			E2	RRSVSVQT	86-93
			E3	GESSLVN	95-101
6	West Nile virus	Glycoprotein E (501 AA)	F1	GEVTVDC	183-189
			F2	GTKTFLVHR	206-214
7	Chandipura virus	Glycoprotein G (521 AA)	G1	RGHWVDPLFVGG	152-163
			G2	QEKPLLPLFKECPAG	257-271
			G3	IDGPVLK	350-356
			G4	PLDHPQLPHAQS	420-431

[Table/Fig-1]: List of viral proteins and selected peptide with their amino acid sequences.

Viruses	Peptides	Test (n=150) Mean±SD	Control (n=135) Mean±SD	p-value (t-test)	Cut-off value	95% Confidence interval
CMV	A1	0.19±0.10	0.18±0.07	0.3339	>0.208	0.589 to 0.711
	A2	0.28±0.10	0.17±0.06	<0.0001	>0.331	0.670 to 0.784
	A3	0.31±0.11	0.16±0.06	<0.0001	>0.332	0.687 to 0.799
	A4	0.33±0.15	0.17±0.07	<0.0001	>0.387	0.669 to 0.783
	A5	0.32±0.15	0.18±0.08	<0.0001	>0.389	0.672 to 0.789
EBV	B1	0.19±0.09	0.16±0.06	0.5457	>0.215	0.439 to 0.567
	B2	0.18±0.09	0.16±0.08	0.7613	>0.211	0.442 to 0.570
	B3	0.21±0.10	0.18±0.08	0.6491	>0.215	0.510 to 0.637
	B4	0.20±0.09	0.18±0.09	0.0621	>0.212	0.509 to 0.635
	B5	0.19±0.08	0.18±0.07	0.2647	>0.201	0.552 to 0.676
VZV	C1	0.17±0.10	0.15±0.08	0.0652	>0.221	0.542 to 0.667
	C2	0.2±0.11	0.20±0.09	1.0000	>0.271	0.541 to 0.668
	C3	0.19±0.09	0.18±0.07	0.2998	>0.204	0.487 to 0.614
	C4	0.2±0.10	0.18±0.09	0.0783	>0.228	0.565 to 0.689
	C5	0.18±0.10	0.16±0.07	0.0539	>0.243	0.485 to 0.613
DENV 1	D1	0.20±0.10	0.18±0.08	0.0652	>0.211	0.606 to 0.727
	D2	0.19±0.10	0.17±0.08	0.0652	>0.204	0.576 to 0.699
DENV 2	D3	0.20±0.11	0.18±0.07	0.0716	>0.205	0.583 to 0.706
	D4	0.20±0.09	0.18±0.09	0.0621	>0.206	0.589 to 0.711
DENV 3	D5	0.21±0.18	0.19±0.12	0.2762	>0.21	0.601 to 0.722
	D6	0.17±0.12	0.15±0.06	0.0813	>0.199	0.548 to 0.673
DENV 4	D7	0.20±0.10	0.18±0.09	0.0783	>0.222	0.598 to 0.719
	D8	0.2±0.09	0.19±0.09	0.3498	>0.205	0.573 to 0.697
JEV	E1	0.32±0.14	0.17±0.10	<0.0001	>0.333	0.689 to 0.801
	E2	0.31±0.15	0.18±0.10	<0.0001	>0.328	0.757 to 0.858
	E3	0.30±0.14	0.18±0.07	<0.0001	>0.316	0.765 to 0.865
WNV	F1	0.2±0.12	0.18±0.09	0.1157	>0.206	0.601 to 0.722
	F2	0.18±0.08	0.17±0.05	0.2126	>0.234	0.575 to 0.698
CHPV	G1	0.16±0.09	0.15±0.06	0.2762	>0.211	0.519 to 0.645
	G2	0.16±0.08	0.15±0.06	0.2377	>0.203	0.542 to 0.667
	G3	0.18±0.09	0.17±0.08	0.3245	>0.225	0.629 to 0.748
	G4	0.17±0.09	0.16±0.05	0.2544	>0.214	0.590 to 0.712

[Table/Fig-2]: Mean±SD, absorbance value and p-value along with cut-off value determined by ROC analysis of peptides for IgG detection.
CMV: Cytomegalovirus; EBV: Epstein-barr virus; VAZ: Varicella zoster virus; DENV: Dengue serotypes; JEV: Japanese encephalitis virus; WNV: West nile virus; CHPV: Chandipura virus

Virus	Peptides	Test (n=150) Mean±SD	Control (n=135) Mean±SD	p-value (t-test)	Cut-off value	95% Confidence interval
CMV	A1	0.16±0.12	0.14±0.10	0.1299	>0.286	0.541 to 0.680
	A2	0.22±0.16	0.14±0.11	<0.0001	>0.304	0.608 to 0.741
	A3	0.25±0.16	0.13±0.08	<0.0001	>0.352	0.639 to 0.769
	A4	0.29±0.15	0.13±0.09	<0.0001	>0.372	0.652 to 0.780
	A5	0.3±0.18	0.15±0.12	<0.0001	>0.391	0.644 to 0.773
EBV	B1	0.19±0.13	0.13±0.07	0.2030	>0.214	0.531 to 0.671
	B2	0.14±0.12	0.12±0.07	0.0913	>0.202	0.553 to 0.692
	B3	0.16±0.15	0.13±0.10	0.0505	>0.223	0.551 to 0.689
	B4	0.17±0.15	0.14±0.12	0.0652	>0.213	0.563 to 0.701
	B5	0.16±0.11	0.14±0.10	0.1108	>0.192	0.521 to 0.661
VZV	C1	0.14±0.12	0.12±0.08	0.1028	>0.236	0.541 to 0.680
	C2	0.19±0.16	0.13±0.09	0.0556	>0.248	0.550 to 0.689
	C3	0.14±0.13	0.13±0.08	0.4409	>0.231	0.535 to 0.674
	C4	0.16±0.14	0.14±0.10	0.1705	>0.292	0.549 to 0.687
	C5	0.15±0.12	0.13±0.09	0.1157	>0.216	0.555 to 0.693
DENV 1	D1	0.17±0.15	0.13±0.10	0.0605	>0.209	0.559 to 0.697
	D2	0.16±0.13	0.13±0.10	0.1499	>0.221	0.570 to 0.707
DENV 2	D3	0.16±0.13	0.14±0.10	0.1499	>0.232	0.582 to 0.718
	D4	0.16±0.12	0.13±0.09	0.0258	>0.199	0.581 to 0.717

DENV 3	D5	0.16±0.14	0.14±0.11	0.1843	>0.221	0.568 to 0.706
	D6	0.16±0.13	0.14±0.10	0.1499	>0.245	0.584 to 0.720
DENV 4	D7	0.15±0.11	0.13±0.08	0.0832	>0.235	0.587 to 0.722
	D8	0.17±0.16	0.14±0.09	0.1576	>0.252	0.591 to 0.726
JEV	E1	0.23±0.16	0.14±0.09	<0.0001	>0.357	0.654 to 0.782
	E2	0.25±0.16	0.15±0.11	<0.0001	>0.391	0.642 to 0.772
	E3	0.24±0.17	0.16±0.12	<0.0001	>0.322	0.614 to 0.747
WNV	F1	0.16±0.14	0.14±0.11	0.1843	>0.204	0.539 to 0.678
	F2	0.15±0.12	0.13±0.08	0.1028	>0.193	0.516 to 0.657
CHPV	G1	0.14±0.12	0.12±0.09	0.1157	>0.197	0.556 to 0.694
	G2	0.14±0.11	0.12±0.08	0.0832	>0.211	0.530 to 0.670
	G3	0.15±0.12	0.13±0.07	0.0913	>0.206	0.527 to 0.667
	G4	0.15±0.12	0.13±0.08	0.1028	>0.196	0.543 to 0.682

[Table/Fig-3]: Mean±SD, absorbance value and p-value along with cut-off value determined by ROC analysis of peptides for IgM detection.

S. No.	Viruses	IgG positive	IgM positive	Total positivity
1	CMV	7.3% (11)	1.3% (2)	10% (15)
2	JEV	8.7% (13)	4% (6)	10.7% (16)

[Table/Fig-4]: Total percent positivity of IgG and IgM antibodies for CMV and JEV in CSF samples of suspected viral CNS infection group.

To ascertain diagnostic efficacy of in-house ELISA, ELISA positive cases of JEV and CMV were also compared with the commercial kit [Table/Fig-5]. Out of total 150 suspected cases of CNS viral infections, a total of 31 samples were positive by in-house ELISA. Out of these 31 positive cases, 25 cases were positive by the commercial kit.

S. No.	Viruses	Positive by in-house ELISA	Positive by commercial ELISA
1	CMV	15 (10%)	13 (8.7%)
2	JEV	16 (10.7%)	12 (8%)
3	Total positive cases	31 (20.7%)	25 (16.7%)

[Table/Fig-5]: Comparison of positivity in CSF samples by in-house peptide ELISA and commercial ELISA.

[Table/Fig-6] shows the clinical characteristics of viral suspected cases were also compared in two groups namely; peptide ELISA positive and peptide ELISA negative cases. Out of total 150 viral suspected cases, 119 were peptide ELISA negative and 31 were positive by peptide ELISA. Out of these, 15 cases were positive for CMV and 16 cases for JEV. Neck stiffness, lesion in brain, reduced level of consciousness and behaviour disturbance was significantly (p=0.019, p=0.044, p=0.003, p=0.026) more in peptide ELISA positive cases as compared to negative cases. The clinical outcome of positive and negative cases showed significant variation (p=0.006). It was observed that more number of cases showed severe clinical outcome in peptide ELISA positive cases as compared to negative cases. No significant variation was observed in other clinical characteristics of patients between both the groups.

Patients characteristics	Peptide ELISA positive (n=31)	Peptide ELISA negative (n=119)	p-value (t-test)
Gender			
Male	19 (61%)	70 (59%)	0.840
Female	12 (39%)	49 (41%)	
Seizures			
Present	18 (58%)	39 (33%)	0.358
Absent	13 (42%)	80 (67%)	
Vomiting			
Present	10 (32%)	35 (29%)	0.826
Absent	21 (68%)	84 (71%)	
Headache			
Present	18 (58%)	46 (39%)	0.066
Absent	13 (42%)	73 (61%)	
Fever			
Present	19 (61%)	88 (74%)	0.184
Absent	12 (39%)	31(26%)	
Magnetic resonance imaging			
Lesions	14 (45%)	30 (25%)	0.044*
Normal	17 (55%)	89 (75%)	
Neck stiffness			
Present	9 (29%)	13 (11%)	0.019*
Absent	22 (71%)	106 (89%)	
Reduced level of consciousness			
Present	21 (68%)	44 (37%)	0.003*
Absent	10 (32%)	75 (63%)	
Behaviour disturbance (Slurred speech, drowsy, disoriented)			
Present	22 (71%)	57 (48%)	0.026*
Absent	9 (29%)	62 (52%)	
Limb weakness			
Present	11 (35%)	22 (18%)	0.0931
Absent	20 (65%)	97 (82%)	
Acyclovir treatment given	17 (55%)	65 (54%)	1.00
Clinical outcome			
Expired	5 (16%)	6 (5%)	0.006*
Improved with neurodeficit	5 (16%)	4 (3%)	
Full recovery	17 (56%)	96 (81%)	
Static	2 (6%)	10 (8%)	
Deteriorate	2 (6%)	3 (3%)	
CSF parameter (range)			
Total leukocyte count	2-150	10-250	-
Sugar, mg/dL	26-249.67	37-415	
Protein, mg/dL	26-140.2	31.33-296	

[Table/Fig-6]: Comparative baseline clinical characteristics between peptide ELISA negative and positive cases of viral suspected CNS infections.
*p<0.05 Significant

[Table/Fig-7] shows concordance in CNS viral infections cases between peptide ELISA and commercial ELISA. Twenty five samples were positive while 119 were negative by both the tests. Thus, the concordance existed between the results of the peptide-based ELISA and commercial ELISA was found to be 96%. The confirmed viral CNS infections cases of CMV and JEV were also compared with controls cases as determined by the cut-off values of antigenic peptides of respective viruses and significant variation was observed between confirmed case and controls groups [Table/Fig-8].

Test	Commercial ELISA positive (n=25)	Commercial ELISA negative (n=125)	Concordance
Peptide ELISA positive (n=31)	25	6	96%
Peptide ELISA Negative (n=119)	0	119	

[Table/Fig-7]: Concordance between peptide ELISA and commercial ELISA.

CNS. A total of 32 peptides of viruses consisting of five peptides each for CMV, EBV, and VZV, eight peptides of DENV serotypes (1, 2, 3, 4), three for JEV, two for WNV and four for CHPV were synthesised and analysed in cases of suspected viral infections and controls groups. Present study results indicated that out of 32 peptide sequences, peptides of only CMV and JEV showed a significant difference for antibody analysis in CSF samples of suspected cases.

Viruses	Peptides	Test (n=25)	Control (n=135)	p-value (t-test)	Test (n=25)	Control (n=135)	p-value (t-test)
		Mean±SD (IgG)			Mean±SD (IgM)		
CMV	A1	0.19±0.07	0.18±0.07	0.5127	0.18±0.13	0.14±0.10	0.0824
	A2	0.40±0.09	0.17±0.06	<0.0001	0.42±0.24	0.14±0.11	<0.0001
	A3	0.38±0.12	0.16±0.06	<0.0001	0.43±0.23	0.13±0.08	<0.0001
	A4	0.41±0.15	0.17±0.07	<0.0001	0.44±0.23	0.13±0.09	<0.0001
	A5	0.39±0.18	0.18±0.08	<0.0001	0.41±0.23	0.15±0.12	<0.0001
JEV	E1	0.45±0.13	0.17±0.10	<0.0001	0.38±0.23	0.14±0.09	<0.0001
	E2	0.53±0.20	0.18±0.10	<0.0001	0.34±0.18	0.15±0.11	<0.0001
	E3	0.52±0.17	0.18±0.07	<0.0001	0.34±0.18	0.16±0.12	<0.0007

[Table/Fig-8]: Comparison of confirmed viral CNS infections cases with controls samples for IgG and IgM antibodies.

DISCUSSION

In the present study, authors have described a utility of synthetic peptide-based ELISA for rapid diagnosis of viral CNS infection. As viruses are largely responsible for a majority of infectious diseases of the CNS [1]. The identification of the infectious agent is challenging because laboratory findings and clinical symptoms may mimic each other. In recent years, antibody detection in the CSF has become one of the widely used tests for the diagnosis of CNS viral infections when other methods such as cultivation and antigen detection are unsuitable, costly or give negative results. Although PCR is recognised as the standard laboratory techniques for diagnosis of viral CNS infections but has some limitations; it remains negative in cases where samples have been obtained at a later period or when viral load in samples is less and it required sophisticated technology, well-trained personnel and still not available in many centers of developing countries [15]. In a case of some medically important flavi viruses IgM capture, ELISA is most commonly used as a diagnostic assay in many laboratories [19].

Earlier studies have reported the use of antigenic viral proteins for selection of peptides used for the detection of antibodies [20-22]. For ideal peptide-based seroassay, peptides should be designed on a well conserved and specific region of the viral proteins that evoke a strong antibody response. The major antigenic determinants used in serological assays are the glycoproteins, membrane proteins and non structural proteins of the viruses. In herpes viruses such as VZV, CMV, and EBV; gE, gB, and EA-D respectively, have been shown to be the major immunogenic antigens for neutralising antibodies [23-25]. In flavi viruses, glycoprotein such as gE (WNV) membrane proteins such as prM (JEV), non structural proteins such as NS (DENV) and gG (CHPV) elicits strong antibody response thus acting as major antigenic determinants [26-29]. These sequences of proteins in viruses were selected for peptides designing on the basis of antigenic epitope prediction analysis by Kolaskar AS and Tongaonkar PC method [18].

Several studies have reported the use of the peptide as a marker for diagnosing of the CNS viral infections. In a study done by Jun Xu et al., they designed a synthetic peptide of JEV envelope protein and used it for detection of antibodies in JEV CNS infections [30]. A study performed by Giessauf A et al., has reported the role of synthetic peptides in screening of rubella virus infection [31]. Similarly, Smith RS et al., used this peptide-based approach for Epstein-Barr virus diagnosis by using a peptide designed specifically against the Epstein-Barr virus nuclear antigen (EBNA) [32]. In the similar pattern, the present study was intended to design, synthesise and evaluate the synthetic peptides of respective viral proteins instead of whole viral protein for detection of antibodies in CSF of patients with suspected viral infections of the

The JEV infection is most prevalent in eastern and southern Asia and causes an estimated 50,000 cases and 15,000 deaths annually. In India, approximately 1,500 to 4,000 cases are reported every year and major epidemics are reported from northern India [33]. The CMC infection of the CNS occurs most commonly in immunosuppressed patients, newborn infants and some studies also reported that CMV reactivation and sever infections in immunocompetent patients [34]. It is estimated that about 40,000 children are born with CMV, resulting in about 400 fatal cases each year and up to 10-15% of the infection, develop one or more long-term neurological sequelae [35].

The results obtained from the present study suggested that the peptides STGDVVDISP, KQKSLVEL, RTLEVFKE, RSSNVED of CMV and ITYECPK, RRSVSVQT, GESSLVN of JEV are highly conserved and antigenic, therefore, the synthetic peptides of glycoprotein gB of CMV and membrane protein prM of JEV can consider as a potential biomarker for diagnosis of CMV and JEV CNS infection. On comparing the peptide ELISA results with the commercial kit, it was found that approximately 81% of peptide ELISA positive cases (approximately, 73% of CMV and 62% of JEV) were also positive in commercial ELISA kit. Martinez Viedma MDP et al., used the synthetic peptide-based ELISA for the diagnosis of Zika virus infections and reported the diagnostic efficacy of peptide-ELISA as compared to commercial kit [36]. Similarly, Saxena V used the peptide-based ELISA and PCR as diagnostic tools for diagnosis of JEV infection and reported 61% positivity by ELISA, however all samples were negative for PCR [37]. However, the negativity for some of the samples by the commercial kit depicts the enhanced sensitivity of the in-house peptide ELISA in detecting cases with lower baseline levels of antibodies against specific epitopes of the viral antigen in their CSF. None of the cases were found to be positive for VZV, EBV, DENV, WNV, and CHPV by peptide ELISA for these viruses in present study cohort. After tracking the clinical history of this peptide ELISA negative cases the patients responded well on antiviral and mix therapies. So, there would be the possibility that these cases have unknown viral aetiology or may have non infectious aetiology. The peptide-based ELISA is being developed for small infrastructure laboratories and it requires simple to handle technology which can be beneficial for initial screening of CNS viral infections. The most important part for the development of successful peptide ELISA is the choice and design of the peptides. Specificity of peptide-based ELISA can be enhanced by averting the selection of cross-reactive sequence from the antigenic proteins.

Limitation(s)

The study has certain limitations, as the cases positive by peptide ELISA was confirmed by only one of the commercial kit and more

number of samples is required to justify the difference in positivity index (20% in present study) with the two reported assays under the study and the sample size was less because it was focused on only those patients referred to our tertiary care centre over a limited time period.

CONCLUSION(S)

The results obtained from the study demonstrated that the synthetic peptides derived from the immunogenic antigen of JEV and CMV having the potential to detect the serological response of these viruses in CNS infection cases. The in-house developed synthetic peptide-based ELISA is a reliable, rapid, cost-effective and less cumbersome procedure which can be adopted in any diagnostic laboratory with limited resources, especially in developing countries for the initial screening of selected neuroviruses CNS infections.

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