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Synthetic Peptides are Better Than Native Antigens for Development of ELISA Assay for Diagnosis of Tuberculosis

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Abstract Immunodiagnosis of both pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis has remained challenging. In the present work, in-house developed synthetic peptide based antibody detection assay was assessed and validated with antigen based assay for effective diagnosis of tuberculosis (TB). The study population included both tuberculous meningitis (TBM) (n = 60) admitted to Neurology IPD wards of our Institute hospital and PTB cases (n = 57) recruited from high TB endemic zones. Peptides of five highly immunogenic *Mycobacterium tuberculosis* (MTB) proteins (Ag85B, 45 kDa, HSP-16, CFP-10 and ESAT-6) were designed and synthesized. The designed peptides were evaluated in samples of both TBM and PTB cases, respectively, using in-house developed antibody detection method. The developed tests were further compared and validated with MTB native proteins based antibody detection ELISA. Sensitivity and specificity of peptide assay were significantly higher or almost similar ($p < 0.05$) in TBM and PTB as compared to native proteins based ELISA. Among all peptides, diagnostic reliability of Ag 85B peptide A1 was higher for both forms of TB. Peptide-based antibody assay is cost effective, simple and

may be interchangeable with conventional Antigen based ELISA assays for effective diagnosis of TB in the developing world.

Keywords *Mycobacterium tuberculosis* · Tuberculous meningitis · Serodiagnosis · B-cell epitopes · Peptide ELISA

Abbreviations

TBM	Tuberculous meningitis
TB	Tuberculosis
PTB	Pulmonary tuberculosis
EPTB	Extra pulmonary tuberculosis
ELISA	Enzyme-linked immunosorbent assay
PCR	Polymerase chain reaction
Ag85B	Antigen 85 B
45 kDa	45 kilo Dalton
HSP 16	Heat Shock Protein 16
CFP-10	10 kDa culture filtrate protein
ESAT-6	6 kDa Early secretory antigenic target
CIIMS	Central India Institute of Medical Sciences
MTB	*Mycobacterium tuberculosis*
MAHAN	Meditation Addiction Health AIDS Nutrition
NTBM	Non-tuberculous meningitis
NPTB	Non pulmonary tuberculosis
WHO	World Health Organization
MIF	Molecular Immunology Foundation-Bioinformatics software
Bcepred	Prediction of linear B-cell epitopes—Bioinformatics software
ABCpred	Artificial neural network based B-cell epitope prediction server
NCBI	National Center for Biotechnology Information
BLAST	Basic local alignment search tool

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CSF   Cerebrospinal fluid
ATT   Anti tuberculosis treatment
QFT   QuantiFERON-TB Gold test
TST   Tuberculin skin test

Introduction

Tuberculous meningitis (TBM) is among a fatal form of extrapulmonary tuberculosis (EPTB) accounting for 70–80 % of all neurological meningitis cases. The estimated death rate due to TBM in India is 1.5 per 100,000 populations. Clinical diagnosis of TBM remains difficult, primarily due to non-specific clinical features which vary widely, thus creating a major obstacle in the initiation of treatment. TBM represents a highly infectious form of central nervous system (CNS) disease caused by *Mycobacterium tuberculosis* (MTB), and accounts for about 1 % of all instances of TB (Leonard and Des Prez, 1990; Cherian and Thomas, 2011). The clinical diagnosis of TBM remains challenging and is primarily based only on clinical and preliminary cerebrospinal fluid (CSF) findings without definitive microbiology test (Takahashi et al. 2012; Desai et al. 2006). Despite the development of several molecular-based methods, the role of these modified techniques was limited to a single laboratory, and they have not been widely used. A meta-analysis found that commercial polymerase chain reaction (PCR) for the diagnosis of TBM had an overall sensitivity of 56 % and a specificity of 98 % (Marx and Chan 2011; Pai et al. 2003). In 2012, Indian government of the Ministry of Health and Family Welfare banned the manufacture, import, distribution and use of serological test kits for the diagnosis of TB, However World Health Organization (WHO) still encourages further research on novel immunological methods by targeting panel of antigens and antibodies (http://www.tb-facts.org/serological-test-tb.html; Morris 2011).

Earlier in our laboratory, we have developed ELISA based antibody detection assay using panel of five MTB native proteins, namely—Ag85B (Antigen85B, Rv1886C), 45 kDa (45 kDa glycoprotein, Rv1860) HSP-16 (Heat Shock Protein 16, Rv2031c) CFP-10 (Culture filtrate Protein-10, Rv3874), ESAT-6 (Early Secretory Antigenic Target, Rv3875) for the rapid diagnosis of pulmonary tuberculosis (PTB) and EPTB (Kashyap 2014; Kashyap et al. 2004, 2009; Shekhawat et al. 2014). During the past few years, use of peptide based ELISA has been widely reported in the diagnosis of various diseases because of low production cost and good specificity (Alban et al. 2014).

There are various advantages of immunodiagnostic tests using peptides over diagnostic tests based on complex biological materials such as the whole antigen. It is very easy to standardize and validate synthetic peptides because they are chemically defined antigens and they are not derived from any biological material such as whole antigens which are often selected for immunodiagnostics because they contain multiple epitopes. Nevertheless, high sensitivity may occur at the expense of assay specificity, since these molecules may also contain cross-reactive epitopes. By screening peptides which may function as a potential B-cell epitope within an immunogenic protein, it will become easy to keep the highly specific epitopes thereby eliminating peptide epitopes that are cross-reactive or have poor specificity. Previously in our laboratory, we have developed synthetic peptides based ELISA assay using Ag85B complex protein, and achieved good sensitivity and specificity (Kashyap et al. 2013a).

In the present work, we have compared our in-house ELISA based antibody detection assay using MTB native proteins with a newly designed cost effective peptide based assay for the diagnosis of TBM and PTB in different populations, to determine better diagnostic test for tuberculosis (TB).

Materials and Methods

Ethics Statement

The study was approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences (CIIMS) and is in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) Nagpur. Written consent was obtained from all the patients after an oral explanation about the study.

Designing and Synthesis of MTB peptides

Antigenic peptides from Ag85B, 45 kDa, HSP-16, CFP-10, ESAT-6 protein sequence was determined by Kolaskar and Tongaonkar (1990) using online software (MIF) Bioinformatics software available in the protocol described elsewhere. (Morey et al. 2010; Kolaskar and Tongaonkar 1990) (Fig. 2 Supplementary information).These antigenic peptide 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 Center for Biotechnology Information) (Basic Local Alignment Search Tool). Based on the results of the blast analysis, the antigenic sequences found to be specific to Ag85B were 2 (out of 8), 45 kDa 2 (out of 12), HSP-16 1 (out of 4), CFP-10 2 (out of 4), ESAT-6 2 (out of 4). These

peptides were then checked for various physicochemical properties which included B cell epitope mapping and suitability for antibody production using Bcepred software described elsewhere. (Saha and Raghava, 2007; Goyal et al. 2014). The server is able to predict epitopes with 58.7 % accuracy, using specific methods hydrophilicity (Parker et al. 1986), flexibility (Karplus and Schulz, 1985), accessibility (Emini et al. 1985), turns (Pellequer et al. 1993), exposed surface (Janin and Wodak, 1978), polarity (Ponnuswamy et al. 1980) and antigenic propensity (Kolaskar and Tongaonkar 1990). Online Software named ABCpred was used to find out the score of the designed peptides (Mahdavi et al. 2012). Peptide sequences were then sent for synthesis at Genic Bio lab, Shanghai, China. The purity of the peptides was checked using HPLC and their quantification was done using mass spectrometry. All the designed peptides were synthesized with purity >90 % (Figure-1 Supplementary information). For comparative analysis with peptides, five native proteins of MTB strain H37Rv (C193) were obtained from Colorado State University through TB Research Materials and Vaccine Testing Contract (NO1-AI-75320). Representative amino-acid sequences for the selected peptides of MTB H37Rv antigens Ag85B, Hsp-16, 45 kDa, ESAT-6, CFP-10 are provided in (Fig. 1).

Study Population

This study was conducted in three different populations which included IPD patients from CIIMS, Nagpur and populations from two high TB endemic regions of Mominpura, Nagpur and Melghat, Amravati district. These populations were further grouped as-.

(1) A total of 120 patients ranged between 20 and 81 years 71 were males and 49 were females, admitted to the Department of Neurology and Neurosurgery at CIIMS Nagpur.
(2) A total of 64 participants ranged between 19 and 60 years, including 42 males and 22 females from the high TB endemic area of Mominpura and the malnourished tribal region of Melghat village, Amravati district.
(3) Total 50 participants ranged between 18 and 60 years, including 22 males and 28 females from a TB endemic region of Mominpura, Nagpur (Fig. 3 Supplementary information).

The patients in all categories were recruited using pre specified inclusion criteria with the help of a team of experts of TB and Chest physicians. Based on the criteria specified by them, all the patients were further categorized into the following groups:-

TBM Cases (n = 60)

Confirmed TBM Cases (n = 12)

This category included cases having CSF positive for AFB staining and/or culture and showing all signs and symptoms suggestive for TBM. PTB was ruled out in TBM cases based on negative chest X-ray and sputum culture negative for TB bacilli. These patients experienced a slight cough, stomach pain and demonstrated signs of fever and meningeal irritation.

Clinically Suspected TBM Cases (n = 48)

This group included cases having CSF negative for AFB staining but showed signs and symptoms suggestive for TBM such as sub-acute or chronic fever with features of meningeal irritation (headache, neck stiffness and vomiting, with or without other features of CNS involvement). Cases also had raised protein levels, and/or decreased glucose (CSF: blood glucose ratio <0.5), and/or pleocytosis with lymphocytic predominance and showed a good clinical response to ATT drugs.

NTBM Cases (Non-Tuberculous Meningitis) (n = 60)

NTBM Infected Control Group (n = 16)

This group included patients having CSF culture negative for TB bacilli with no signs of TBM. They had other neurological disorder such as bacterial or viral meningitis. Bacterial meningitis (n = 8) were confirmed by bacterial culture and positive by in house developed nested PCR tests for either of the organism—*Staphylococcus aureus, Klebsiella pneumonia, Enterobacteriaceae species.* Symptoms of patients in this category included high grade fever with features of meningitis. Viral meningitis (n = 8) were confirmed by PCR. Symptoms included acute onset of fever and signs and symptoms of meningeal irritation.

NTBM Non-Infected Control Group (n = 44)

This category included patients with no clinical symptoms suggestive of meningitis; normal CSF protein & sugar level with no evidence of CNS or extra-CNS tuberculosis.

PTB Cases from Melghat (n = 32) and Mominpura (n = 25)

The samples for this group were obtained with the help of camps organized from a TB-endemic region of Mominpura, Nagpur and villages of the Melghat region of Amaravati District, Maharashtra, India during 2009–2013. All
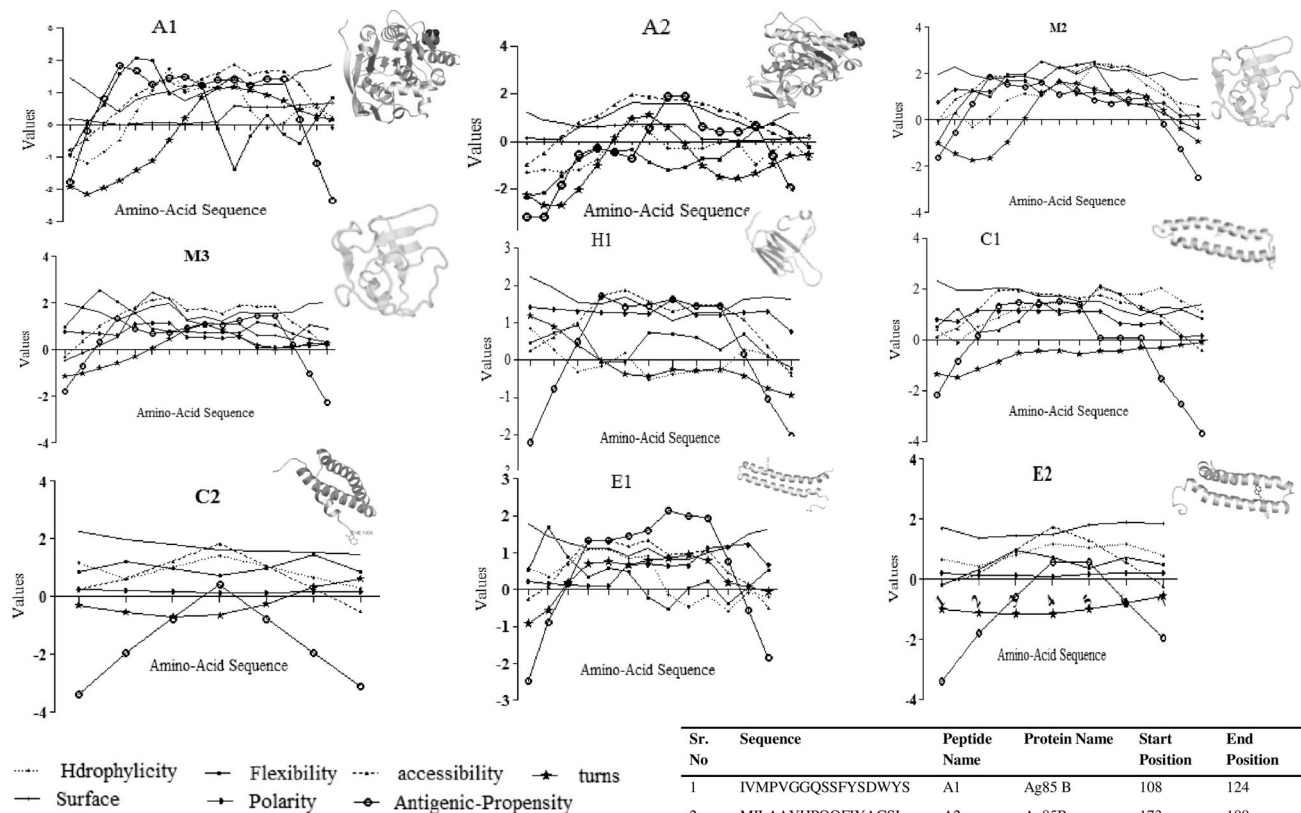
Sr. No	Sequence	Peptide Name	Protein Name	Start Position	End Position
1	IVMPVGGQSSFYSDWYS	A1	Ag85 B	108	124
2	MILAAYHPQQFIYAGSL	A2	Ag85B	173	189
3	TAASPPSTAAA	M2	45KD	50	60
4	RPLVAPPPAPAPAP	M3	45KD	284	297
5	LKTQIDQVESTAGS	C1	CFP10	25	38
6	QQQALSS	C2	CFP10	90	96
7	HPRSLFPEFSEL	H1	HSP-16	10	21
8	IQGNVTSIHSLLD	E1	ESAT-6	18	30
9	AYQGVQQ	E2	ESAT-6	50	56

Fig. 1 Shows Physico-chemical Properties (Hydrophilicity, Flexibility, Polarity, Accessibility, Antigenicity) of **a** A1 Peptide, **b** A2 peptide of Ag 85 B, **c** M2peptide, **d** M3 peptide of 45kda, **e** H1peptide of HSP 16, **f** C1 Peptide, **g** C2 peptide of CFP 10, **h** E1 Peptide and **i** E2Peptide of ESAT-6 which is obtained from the BcePred Web server along with Molecular modeling of Multiplex peptide protein predicted by the Swiss model server

participants in Melghat were re-enrolled based on the information available from the Tribal Health Research Centre, Dharni run by MAHAN trust. Participants were screened using requisite inclusion and exclusion criteria. For Mominpura region, participants were enrolled with the help of local physician and records from TB-DOTS center located in the vicinity of the study area. Participants who had all signs and symptoms which included (fever, cough, productive sputum, suggestive X-ray) and positive culture of MTB from a specimen of sputum were categorized as active PTB. In this group, the cases showed no symptoms for EPTB.

NPTB (Non-Pulmonary Tuberculosis) Group from Melghat (n = 32) and Mominpura (n = 25)

Participants with no clinical, bacteriological features of PTB, normal chest radiograph with no history of anti tuberculosis treatment (ATT), negative QFT/TST test, normal body mass index (BMI) were categorized as healthy control subjects, also with no signs of EPTB.

Blood and CSF Specimens

Blood samples from Melghat and Mominpura were collected in the plain tube. The serum was separated by centrifugation at 12,000 r.p.m. CSF was collected by lumbar puncture in patients admitted to CIIMS. Approximately 3 ml of CSF was obtained. All the samples were stored at 4 °C until further analysis.

Protocol for Antibody Detection Assay Using MTB Peptides

The protocol for in-house developed peptide based assay using MTB peptides as described by Kashyap et al. was

used to detect MTB antibodies to MTB peptides in the CSF or serum samples of selected groups. The wells of microtiter plates were coated with 100 μl of nine peptides namely—A1, A2, M2, M3, H1, C1, C2, E1 and E2 (500 μg/100 μl) diluted in phosphate buffered saline (PBS pH = 7.4) and incubated at 4 °C for overnight. Next day the wells were washed and blocked with 0.5 % BSA in PBS for 2 h at 37 °C. After blocking the plates were washed three times and 100 ul of CSF or serum samples (dilution in PBS) were added and incubated for 1 h at 37 °C. After incubation, the wells were washed and incubated with secondary antibody goat anti-human IgG-HRP conjugate (1:10,000 in PBS) for 45 min at 37 °C. The wells were washed extensively with PBS-T after the incubation followed by the addition of 100 μl of TMB/H_2O_2 substrate and incubated at room temperature for 2–3 min. The reaction was stopped by the addition of 100 μl of 2.5 N H_2SO_4 the absorbance of each well was read at 450 nm (Kashyap et al. 2013b).

Protocol for Antibody Detection Assay Using MTB Native Proteins

The protocol for in-house developed antibody detection assay using MTB native proteins described by Kashyap, et al. was used to detect MTB antibodies to MTB antigens in the CSF or serum samples of selected groups. The wells of micro-titer plates were coated with 100 ul of five MTB antigens namely—(Ag85B and Hsp16—4 μg per well. 45kD, CFP-10—1 μg per well and ESAT-6—5 μg per well) diluted in phosphate buffered saline (PBS pH = 7.4) and incubated for 3 h at 37 °C. The wells were then washed and blocked with 0.5 % BSA in PBS for 2 h at 37 °C. After blocking the plates were washed once and kept overnight at 4 °C. Next day, the plates were washed twice and 100 μl of CSF samples (1:5dilution in PBS) or Serum (1:400 dilution in PBS was added and incubated for 35 min at 37 °C. After incubation the wells were washed and incubated with secondary antibody goat anti-human IgG-HRP conjugate (1:10,000 in PBS) for 30 min at 37 °C. The wells were washed extensively with PBS-T after the incubation followed by the addition of 100 μl of TMB/H_2O_2 substrate and incubated at room temperature for 2–3 min. The reaction was stopped by the addition of 100 ul of 2.5 N H_2SO_4 the absorbance of each well was read at 450 nm.

Statistical Analysis

The statistical analyses were performed using MedCalc (version 10) statistical software. A cutoff point for optimal sensitivity and specificity for the ELISA tests was determined using the Receiver Operating Characteristic (ROC) curve analysis. The accuracy of each test was evaluated according to the area under the curve (AUC). The difference between subject categories was calculated by using a comparison of means t-test. The correlation was determined by linear regression analysis using the MedCalc software. The reactivities of individual CSF and serum samples from PTB, TBM patients and healthy controls with each individual antigen and peptide were plotted using Graph pad prism version 5.0.3(San Diego, CA, USA).

Results

Designed Peptides

Figure 1 is a diagrammatic representation of physicochemical properties of the selected peptides such as hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns with the help of Bcepred software. The software evaluated the performance of existing linear B-cell epitope prediction methods based on physicochemical properties on a non-redundant dataset. The dataset consists of 1029 B-cell epitopes obtained from Bcipep database and equal numbers of non-epitopes are obtained randomly from Swiss-Prot database. The prediction accuracy of the model on various properties varies from 52.92 and 57.53 %.

Diagnostic Potential of the Synthetic Peptides

Figure 2a–e representing scattered graphs showing the deviation of absorbance of TBM (confirmed and clinically suspected) and NTBM (infectious and non-infectious control) CSF samples from its average values for different peptides of Ag85B, 45 kDa, HSP-16, CFP-10 and ESAT-6 complex. In the plot, each dot represents an individual sample value and the horizontal bar in each group represents the average values. Based on the results, it was observed that the average value of TBM cases was significantly higher than the average value of NTBM cases. A similar trend was observed in the malnourished population of Melghat and population of highly TB endemic areas of Mominpura as shown in Fig. 3a, b.

ROC Curves

To investigate the utility of each of the nine peptides for diagnosis of TBM, ROC curves were constructed and the area under the curve (AUC) was determined. (Figure 2f–j).The AUC of the nine peptides ranged from (0.756 to 0.814). It was found that the AUC of peptide M2 and M3 of 45 kDa is >0.8, thus indicating the high discriminatory ability of the peptides for TBM and NTBM patients. Fig 2 also depicts the sensitivities and specificities of the peptide
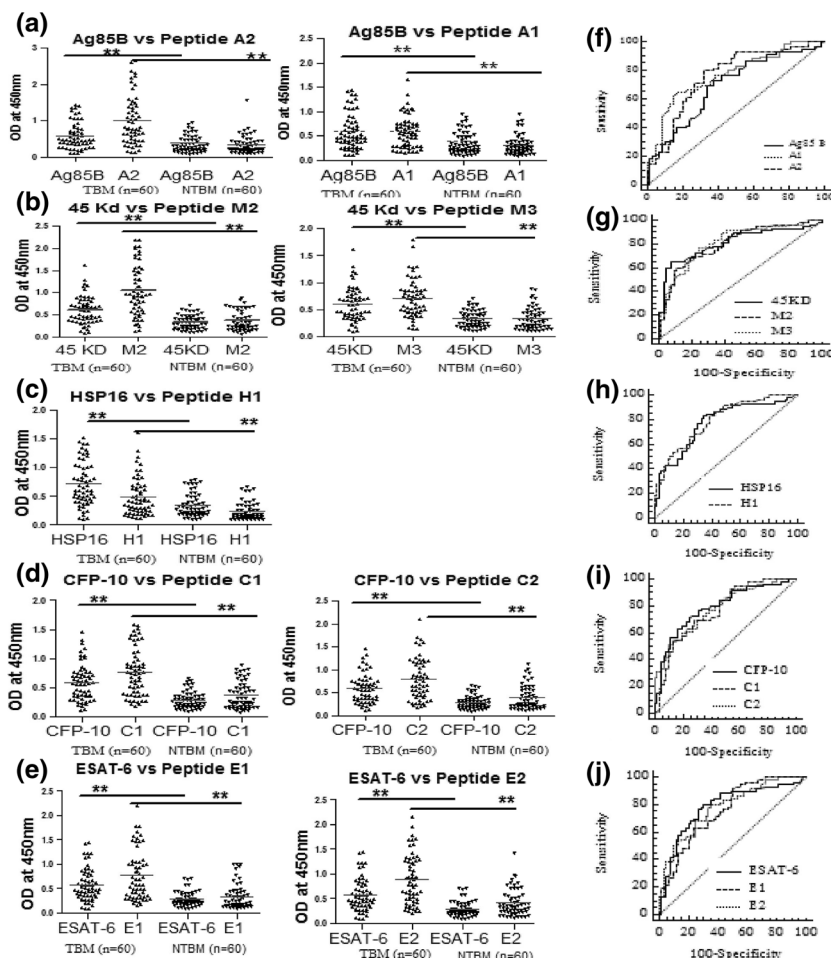
subjects	AUC (95%CL)	P-Value	Sensitivity (95% CI)	Specificity (95% CI)
Ag85 B	0.692 (0.601-0.774)	0.0001	76.66%	86.66%
A1	0.769 (0.683-0.841)	0.0001	91.66%	80%
A2	0.759 (0.673-0.833)	0.0001	93.33%	63.33%

subjects	AUC (95%CL)	P-Value	Sensitivity (95% CI)	Specificity (95% CI)
45KD	0.814 (0.732-0.879)	0.0001	88.33%	85%
M2	0.802 (0.719-0.869)	0.0001	83.33%	86.66%
M3	0.814 (0.732-0.879)	0.0001	81.66%	83.33%

subjects	AUC (95%CL)	P-Value	Sensitivity (95% CI)	Specificity (95% CI)
HSP-16	0.775 (0.689-0.846)	0.0001	78.33%	68.33%
H1	0.792 (0.708-0.861)	0.0001	71.66%	83.33%

subjects	AUC (95%CL)	P-Value	Sensitivity (95% CI)	Specificity (95% CI)
CFP-10	0.787 (0.703-0.856)	0.0001	83.33%	91.66%
C1	0.764 (0.678-0.850)	0.0001	90%	63.33%
C2	0.780 (0.695-0.850)	0.0001	88.33%	66.66%

subjects	AUC (95%CL)	P-Value	Sensitivity (95% CI)	Specificity (95% CI)
ESAT-6	0.784 (0.699-0.854)	0.0001	78.33%	98.33%
E1	0.756 (0.668-0.830)	0.0001	86.66%	65%
E2	0.778 (0.692-0.849)	0.0001	75%	88.33%

Fig. 2 *Dot Plots* of OD values of antibodies against **a** Ag85B and Peptide A1 and A2, **b** 45KD and Peptide M2 and M3, **c** HSP-16 and Peptide H1, **d** CFP-10 and Peptide C1 and C2, **e** ESAT-6 and Peptide E1 and E2 observed in the CSF samples of TBM (Tuberculous Meningitis) and Non TBM (Non-Tuberculous Meningitis) patients

(n = 60). The difference between subject categories was found to be statistically significant by Comparison of means *T*-test. **$*p < 0.001$ between different groups. **f**, **g**, **h**, **i** and **j** Shows ROC curves representing the performance of Antigens with their respective peptides for diagnosis of TBM patients

along with their native proteins in CSF samples of TBM and NTBM patients. The sensitivity ranged from 71.66 to 93.33 % and the range of specificity was found to be 63.33–98.33 %. Ag85B peptide A1 showed sensitivity and specificity higher than 90 and 80 % ($p = 0.0001$) respectively as compared to other peptides.

Correlation

We assessed the degree of correlation between whole antigens and their peptides in the CSF samples of TBM patients (Fig. 4) where the correlation between the absorbance of peptides with their native proteins was determined. It was noted that all the peptides were positively correlated with their respective antigens. Amongst them, peptide A2 of Ag85B was very significantly correlating with native proteins ($r = 0.3293$, 95 % CI, $p < 0.001$). From a linear regression analysis, $R2$ was found to be

0.1969, which indicates that the linear model fit the data quite well.

Cross-Reactivity

Figure 5 indicates the level of cross-reactivity of MTB peptides in other neurological bacterial and viral infections. No cross-reactivity was noted in other neurological disorders, indicating the better specificity of developed peptides for diagnosis of TB. The cross-reactive response against peptides of MTB in other bacterial and viral Neuro-infections, it was observed that significantly higher IgG levels were obtained in the TBM samples when compared with positive samples of bacterial meningitis cases, namely-*S. aureus, K. pneumonia, Enterobacteriaceae* and Viral meningitis cases of *Herpes simplex virus* and *Japanese Encephalitis virus*. Hence this indicates that MTB peptides do not cross react with other neurological diseases.
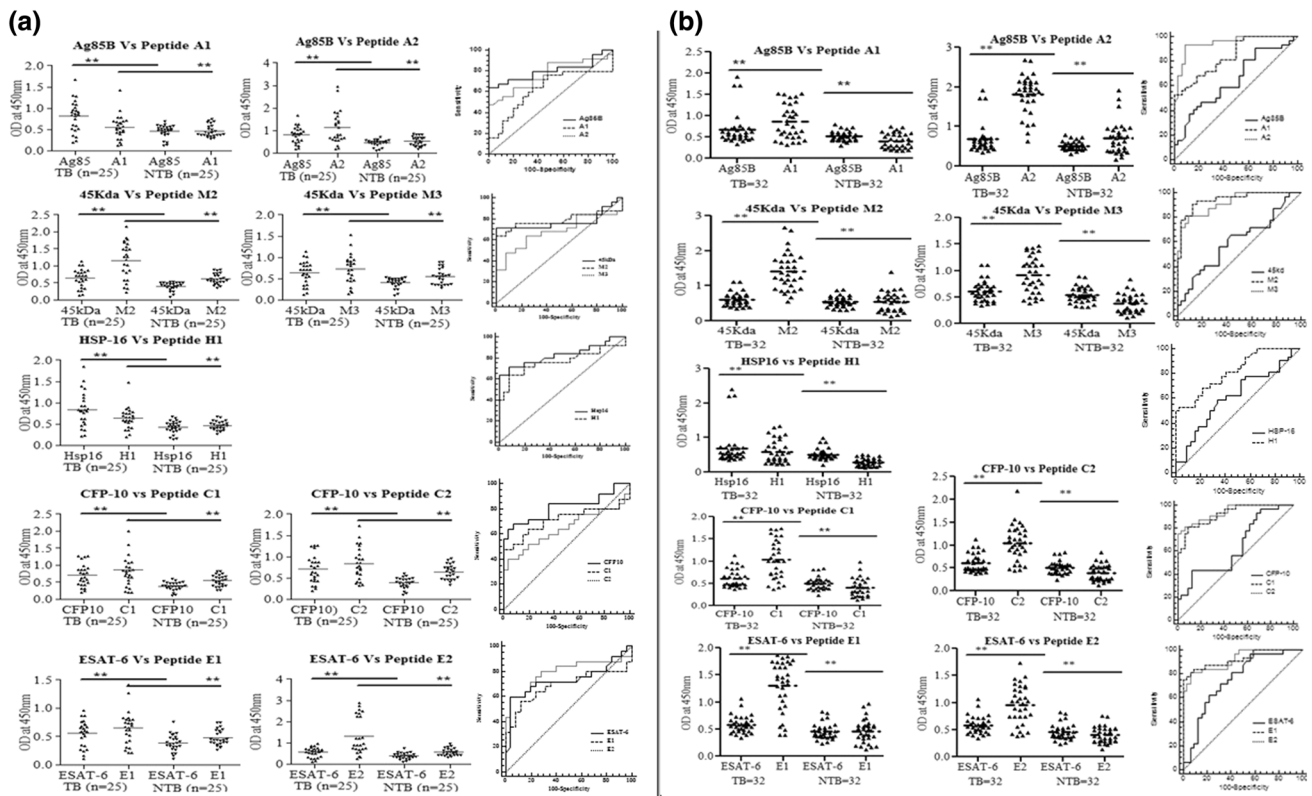
(a)

(b)

Fig. 3 **a** Dot Plots of OD values of antibodies against Ag85B and Peptide A1 and A2, 45KD and Peptide M2 and M3, **b** Dot Plots of OD values of antibodies against Ag85B and Peptide A1 and A2, 45KD and Peptide M2 and M3, HSP-16 and Peptide H1, CFP-10 and Peptide C1 and C2, ESAT-6 and Peptide E1 and E2 observed in the serum samples of PTB (Pulmonary Tuberculosis) and Non PTB (Non-Pulmonary Tuberculosis) patients (n = 32) of the malnourished population of Melghat. The difference between subject categories was found to be statistically significant by Comparison of means T-test. **p < 0.001 between different groups. **c** HSP-16 and Peptide H1, **d** CFP-10 and Peptide C1 and C2, **e** ESAT-6 and Peptide E1 and E2 observed in the serum samples of PTB (Pulmonary Tuberculosis)and Non PTB(Non-Pulmonary Tuberculosis) patients (n = 25) of a TB endemic region of Mominpura. The difference between subject categories was found to be statistically significant by Comparison of means T-test. ** p < 0.001 between different groups

Discussion

The global impact of TB is lethal to one-third of the world's population estimated to be infected with MTB. (Kashyap et al. 2013a). Increasing cases of EPTB infections have further complicated diagnostic scenario as its diagnosis is difficult. The diagnosis of both forms of TB has many complications. Therefore, there is a strong need for a simple, rapid and cost effective, accurate laboratory assay for TBM diagnosis (Haldar et al. 2009). Delayed or misdiagnosis of TBM has shown mortality and morbidity. A false-negative diagnosis exposes the patient to a severe, often fatal clinical outcome, while a patient with a false-positive diagnosis will have to undergo an unnecessarily long and expensive regimen of ATT treatment, with substantial risks of toxicity (Kim et al. 2010). In the present study, an effective synthetic peptide-based immunodiagnostic test alternative to native protein ELISA has been designed to target potential MTB antigens that are tested in CSF samples of TBM patients. We have shown that a peptide-based ELISA that identifies Ag85B, 45 kDa, HSP-16, CFP-10 and ESAT-6 peptide-specific IgG responses with >90 % sensitivity and specificity. Furthermore, the peptide-based IgG diagnostic reagents described in the current study are not only useful for TBM but may be useful for diagnosis of PTB in malnourished and TB endemic regions of the targeted population.

The use of peptides as antigens in serological diagnosis has a major benefit because peptides are cheap and easy to manufacture in a reproducible manner. Various studies have shown the efficacy of peptide-based diagnostics for identifying specific viral or bacterial infections. For a successful peptide based ELISA, the choice and design of the peptide are of major significance (Dubois et al. 2012). Although, there are several online prediction Bioinformatics tools (Bcepred, ABCpred, BepiPred, AAPPred Antigenic) that are reported to identify useful B-cell linear options based on various factors and parameters to develop immunoassays. However, in a study conducted by Juan G Costa et al. reported AAPPred and ABCpred Bioinformatics tools yield
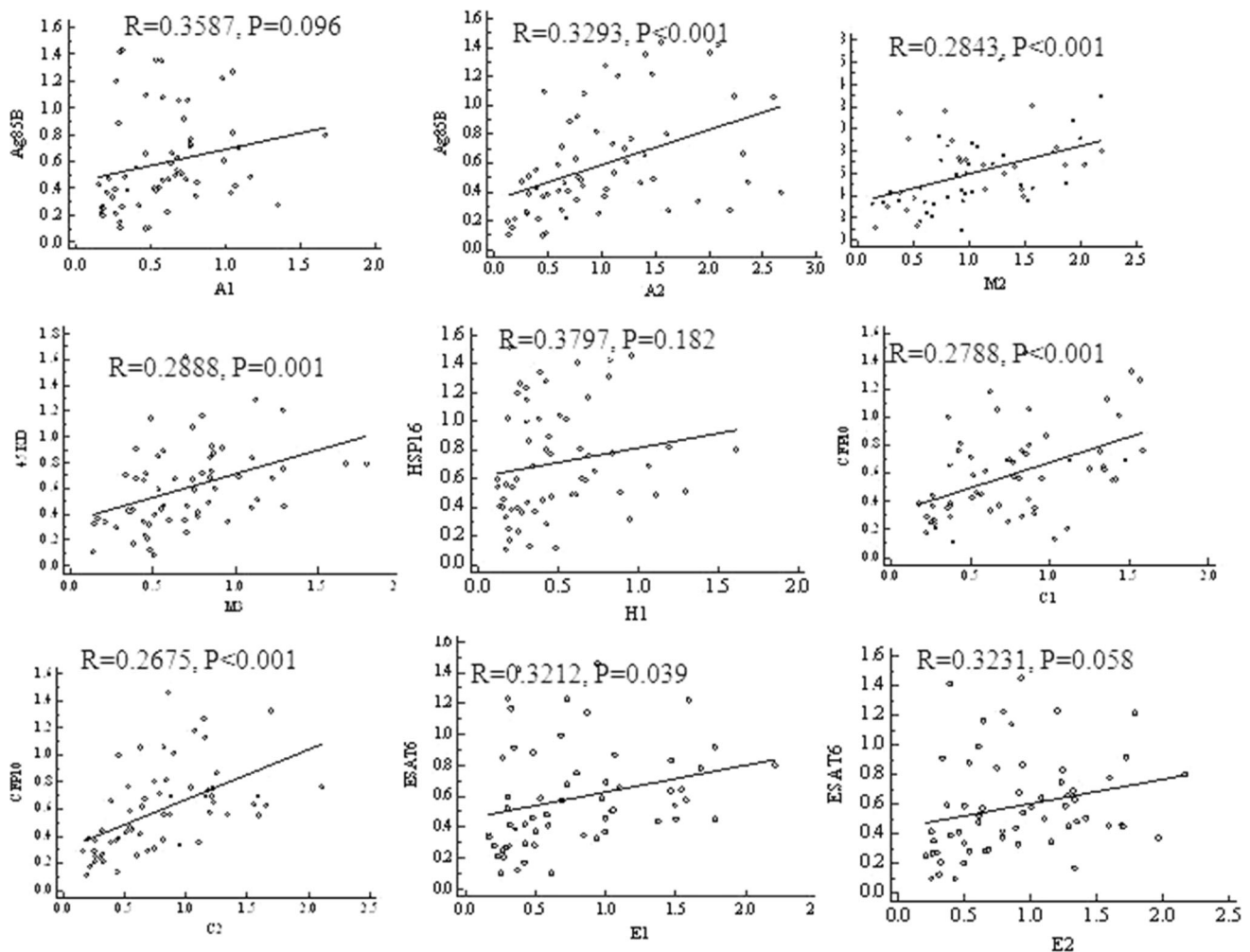
Fig. 4 Correlation between the Antigens and their Peptides **a**, **b** Ag85B and Peptide A1 and A2 **c**, **d** 45 kDa and Peptide M2 and M3, **e** HSP-16 and Peptide H1, **f**, **g** CFP-10 and Peptide C1 and C2, **h**, **i** ESAT-6 and Peptide E1 and E2 through absorbance level in the CSF samples of the study population observed in TBM (Tuberculous Meningitis)and Non TBM (Non-Tuberculous Meningitis) patients ($n = 60$)

the best results as compared with the other programs (Costa et al. 2013). There are numerous studies that have used these softwares for peptide prediction for diseases like Malaria, Leishmania, etc. (Tao et al. 2014; Assis et al. 2014).

We have used the method of Kolaskar and Tongaonkar (1990) using the MIF Bioinformatics software. Further, to be sure that the designed peptides have satisfactory physico-chemical properties, we have used Bcepred and ABCpred software. As mentioned earlier using the Bcepred software the six different parameters were evaluated for each protein to select the peptide. The software has evaluated the performance of existing linear B-cell epitope prediction methods based on physico-chemical properties on a non-redundant dataset. The dataset consists of 1029 B-cell epitopes obtained from Bcipep database and equal numbers of non-epitopes

are obtained randomly from Swiss-Prot database. The prediction accuracy of the model on various properties varies from 52.92 and 57.53 %.

We have also used software called ABCpred which aims to predict B cell epitopes in an antigen sequence, using the artificial neural network. This is the first server developed based on recurrent neural network (machine based technique) using fixed length patterns. The server is able to predict epitopes with 65.93 % accuracy, using the recurrent neural network. Prediction using ABCpred resulted in scores higher than 0.65 for all peptides. On the basis of this software, our peptides show an acceptable range of threshold and are considered to be a good B-cell epitope for antibody production. All these data suggest that selected peptides are reliable depending on the point of peptide designing.
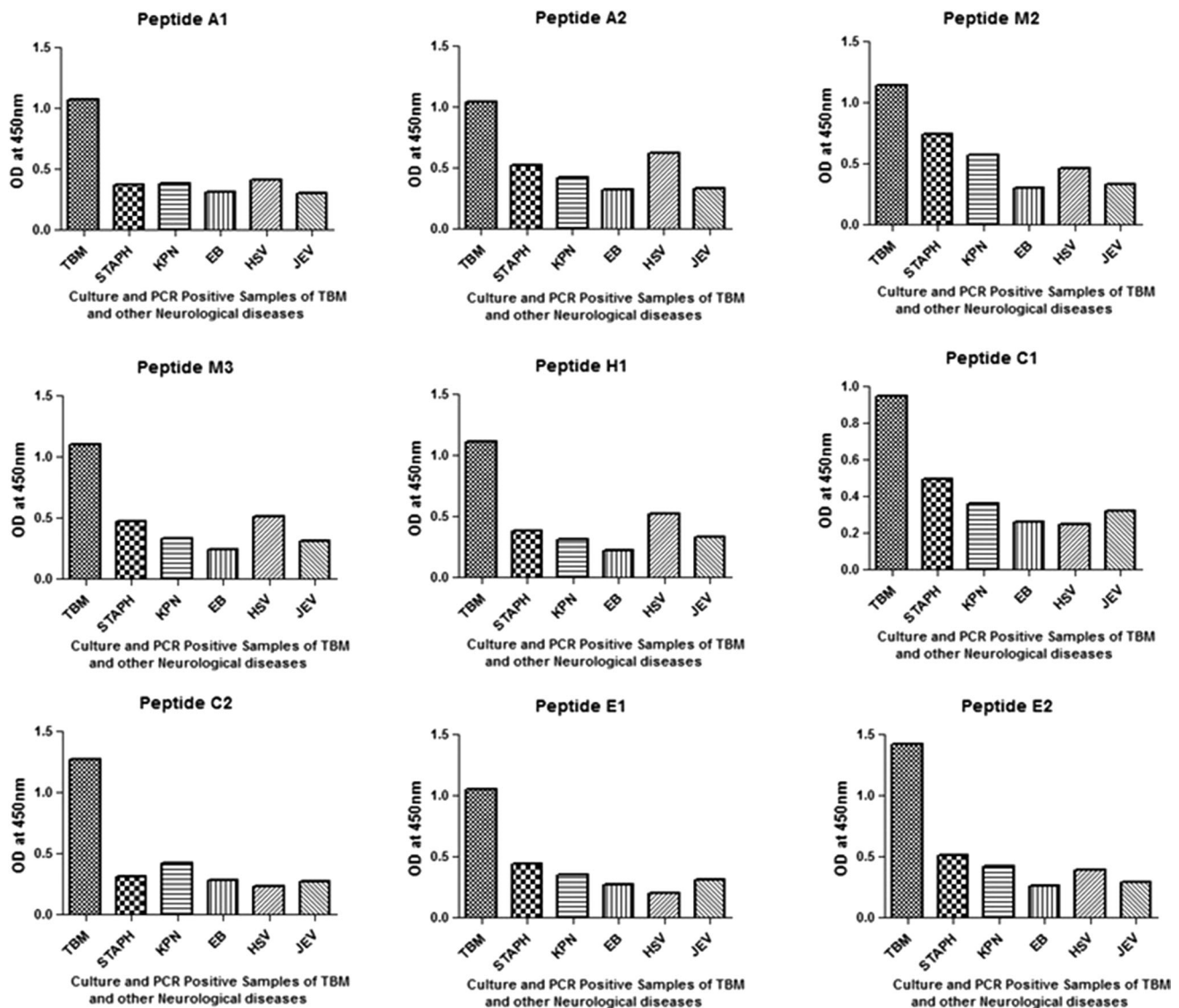
Fig. 5 Levels of IgG Antibodies detected in CSF Samples of Bacterial and Viral Meningitis patients against the Peptides from *Mycobacterium tuberculosis* A1 and A2 Peptide of Ag85B, M2 and M3 Peptide of 45KD, H1 Peptide of HSP-16, and C1 and C2 Peptide of CFP-10, E1 and E2 Peptide of ESAT-6

Altogether, our results indicate that these types of multiple strategies are useful for selecting diagnostic antigens. There are studies which have been conducted for detecting different forms of TB. But in our study, we are targeting panel of synthetic peptides, which is also an extension of the previous studies with the panel of synthetic peptides that have reported utilization of ELISA for detection of antibodies that are reactive against MTB synthetic peptides. A study by Araujo et al. suggested the use of Ag85A and ESAT-6 peptides which is in accordance with our study. Hence we have included the peptides of ESAT-6 in our panel. A similar study conducted by Goyal et al. suggested the use of peptides of antigens of RD1 and RD2 namely CFP-10 and ESAT-6. Based on the conclusion of these studies, we have selected a panel of MTB peptides that has the advantage over other studies, which depicts/shows the use of antibody detection in the diagnosis of TB patients. A study by Salman et al. suggested that ESAT-6 peptide mixtures have a good sensitivity for the diagnosis of PTB infection, which adds one more reason for selecting ESAT-6 in the panel of our peptide. In conclusion, these carefully selected peptides and the developed tests with these multiplex peptides can be used as promising tool for the diagnosis of PTB and EPTB (Araujo et al. 2013; Goyal et al. 2014; Salman et al. 2012).

Immunodiagnostic tests are especially attractive for use in the resource limited settings where TB is endemic and

settings where a sophisticated laboratory infrastructure and highly trained personnel are not available (Shen et al. 2008). An ideal TB diagnostic test must not only provide a high sensitivity and specificity, but must also be susceptible to bulk production. Although native whole protein antigens are easier to obtain and have been used for the detection of specific antibodies in several diagnostic tests, they are costly and may lead to some cross-reactivity. In contrast peptide designing and synthesis is a costly affair, but if the peptides are prepared in bulk it will reduce the cost of synthesis. Also, the large-scale synthesis of peptides allows the easy production of compatible antigenic moieties, most peptides are relatively stable, and the use of peptides eliminates the inclusion of cross-reactive regions of the protein antigens, thus increasing the specificity which can be achieved with full-length native proteins.

Antibodies are highly specific markers for diagnosis of various diseases. Identification of peptide epitopes that may detect different subgroups of TB patients might have diagnostic and prognostic significance. We have investigated a panel of nine peptides derived from MTB proteins and compared it with our in-house serological assay based on immunodominant antigens. Out of 12 in 7 confirmed cases of TBM we found that only one of the native protein antibody was positive, but in the case of peptide ELISA, it gave positivity in all the peptides. Hence it can be concluded that peptides detect various sub-groups of the disease.

We have extended our study and determined the role of identified potential peptide based ELISA test in serum samples of PTB patients from malnourished populations of Melghat and high TB endemic region of Mominpura, Nagpur. Earlier in our laboratory, we have demonstrated the use of in-house antibody detection ELISA assay using MTB native proteins in the same population (Kashyap et al. 2013a; Panchbhai et al. 2011). We have compared our developed peptide based assay with our in-house antibody detection ELISA assay using MTB native proteins for the evaluation of predictive values of the test in the serum samples of PTB patients. It was observed that the sensitivity and specificity of the cost effective peptide based assay using MTB peptides are more or less similar with in-house antibody detection ELISA assay using MTB native proteins in this population.

Conclusion

This study is a cohort study and needs to be extended using more number of samples. The current study provides the proof of concept that peptides (epitopes) derived from carefully selected immunogenic antigens having the potential for the development of an immunodiagnostic test

for TB and can be used interchangeably with full length antigen based ELISA.

Compliance with Ethical Standards

Conflicts of Interest The authors affirm that this article content has no conflict of interest.

Informed Consents The study was approved by the Institutional Ethics Committee. Written consents were obtained from each participant and oral explanation about the research study was presented to all participants.

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