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The Rv3874-Rv3875 chimeric protein shows a promiscuous serodiagnostic potential for tuberculosis

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ABSTRACT

Tuberculosis (TB) stays a major cause of death globally after COVID-19 and HIV. An early diagnosis to control TB effectively, needs a fast reliable diagnostic method with high sensitivity. Serodiagnosis involving polyclonal antibodies detection against an antigen of *Mycobacterium tuberculosis* (*Mtb*) in serum samples can be instrumental. In our study, Rv3874 and Rv3875 antigens were cloned, expressed, and purified individually and as a chimeric construct in *Escherichia coli BL21*. Enzyme-Linked Immunosorbent Assay (ELISA) based findings revealed that the Rv3874-Rv3875 chimeric construct was two-fold more sensitive (59.7%) than the individual sensitivities of Rv3874 (28.4%) and Rv3875 (24.9%) for 201 serum TB positive samples. Furthermore, the fusion construct was a little more sensitive (60.4%) for male subjects than that for females (58.8%). Lastly, our preliminary findings, molecular insights of secondary structure, and statistical and *in silico* analysis of each construct also advocate that CEP can be considered a better immunodiagnostic tool in addition to previously reported EC skin test.

1. Introduction

Tuberculosis (TB) remained the second major infectious disease after COVID-19 with elevated death rates owing to disruption of services during the peak COVID-19 pandemic in 2021. A considerable decline in TB monitoring of new and existing cases resulted in an enhanced death toll and the primary targets of the WHO End TB Strategy 2015–2020 could not be achieved. About 20.2 million people of all age groups remained untreated worldwide, which accounts for a 50% reduction in treatment and 1.5 million deaths due to TB [1]. Pakistan is still on the list of eight countries that account for two third of thirty high TB burden countries in 2020. For effective control of the disease, a more sensitive, less expensive, and optimally scaled-up diagnostic test is highly needed. Early diagnosis in high TB incidence countries including Pakistan, can be possible with the help of an overly sensitive serodiagnostic test. Given differential antibody detection for the same antigen in various patients, a combination of different antigenic proteins is needed to check the antibody response leading to a more sensitive diagnostic test.

Antigenic proteins commonly used in serodiagnosis of tuberculosis include pstS1, HSP, LprG, MPT63, MPT64, HSPX, TB31.7, MPT70, fbpA, fbpB, fbpC, fbpD, esxA (ESAT-6) and esxB (CFP10) [2]. The use of single native antigens in TB serodiagnosis has not been successful to get reproducible sensitivity and specificity [3]. The development of more sensitive fusion antigens with enhanced epitope availability can result in practical and economical TB serodiagnosis. For effective antibody-based diagnostics, different unique combinations of TB antigens (full length and truncated) were designed in our labs, and their diagnostic potential was evaluated against a wide range of individuals [4–9]. A truncated variant of PstS1 antigen (tnPstS1) had improved diagnostic sensitivity (43%) as compared to its full form (36%). Similarly, tn2FbpC1-tnPstS1 fusion was comparatively more sensitive (72.2%) than its individual truncated antigens [4,9]. Rv2031c-Tn1Rv1984c-Rv1352, a combination of three antigens had also shown enhanced sensitivity of 71.4% [5]. Another recombinant trimeric fusion protein, Rv1793-Rv2628-tnRv2608 had 66% diagnostic sensitivity and 100% [6]. Likewise, a tetrameric HSPX-EspC-CFP7-PPE57 fusion showed

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improved sensitivity of 69% [7].

Regions of difference-1 (RD1) handle host interactions and virulence. It has gotten significant attention due to its high immune response. It is not present in all BCG vaccine strains and its deletion completely removes seven genes (Rv3872 to Rv3878) which function as a "virulence-associated type VII secretion system" (1°, 12). This system includes the two important secretory antigenic proteins Rv3874 (CFP10) and Rv3875 (ESAT-6). There is ample evidence that they engage in virulence in pathogenic mycobacteria [10,11]. That is why both these antigens are targets for a vaccination with and without combination with BCG in addition to their use in diagnosis. The only available live attenuated vaccine MTBVAC which is developed from *Mycobacterium tuberculosis*, also holds the RD1 region. Recently, it has also been reported that Rv3874 and Rv3875 were expressed differentially in MTBVAC, and so CFP10 and ESAT6 proteins were only found in the intracellular fraction of MTBVAC in comparison to BCG. So, Rv3874 and Rv3875 presence in MTBVAC can make it a strong candidate to replace BCG in infants at least. It has also entered first in human adult clinical trials [12,13].

Rv3874 and Rv3875 fusion had also been tried with Heat shock protein (HSPX) and Ag85B, and other different adjuvants to vaccinate TB-infected mice and guinea pigs. Although this cocktail induced both B and T cell immune responses in mice, it had a minor effect in guinea pigs infected with *Mtb*. So, this mixture could serve as a post-exposure prophylactic subunit vaccine by controlling disease progress after more successful animal trials [14].

On the other hand, recombinant fusion Rv3875-Rv3874 is being considered as an alternative to a tuberculin skin test for the diagnosis of latent TB. Both antigens are also a vital part of a cocktail of peptides used for differential diagnosis of TB infected and uninfected vaccinated animals in the "DIVA" test [15]. So, fusions of full or truncated Rv3874 and Rv3875 antigens in different combinations and antigens are still areas of interest for TB researchers. Recently, the high specificity of Rv3874-Rv3875 chimeric protein has also been justified by comparing it with conventional skin test with the Enzyme-Linked Immunosorbent Spot (ELISpot) test and the Interferon-Gamma Release Assay (IGRA). Even more this chimeric fusion was equally effective for the diagnosis of latent TB infection, so there is a possibility of such neo-epitopes' formation at the junction of these two proteins which are absent in native proteins [16].

In another study, it has been reported that the fusion elicited an immune response in intradermal and ELISpot assays with diagnostic accuracy superior to that of TB-PPD for diagnosis of latent TB. The area under the receiver operating characteristic curve (AUC) of the fusion was 0.870 (95% CI, 0.796–0.944) while the AUC in the TB PPD test remained 0.686 (95% CI, 0.585–0.786). When all participants were tested, also the ELISPOT assay had an AUC value of 0.908 (95% CI, 0.852–0.965) comparable to the fusion Rv3875-Rv3874 with 0.849 (95% CI, 0.835–0.952) with no serious side effects on participating subjects. So, after further determining the safety and efficacy of the fusion, it may be used as a promising tool in identifying TB disease [17].

Diagnostic accuracy of Rv3875- Rv3874 in terms of safety, specificity, and sensitivity for skin test was determined for TB infection in adult Chinese individuals and was named as EC skin test (after ESAT-6 and cfp10 native antigens) as contrary to Tuberculin Skin Test (TST). Using the EC test, a bacillus Calmette Guerin (BCG) model was set up for the diagnostic assessment of TB infection. The participants including healthy individuals and TB patients were first assessed with the T-SPOT TB Test which is a modified form of ELISPOT, followed by EC Skin Test and TST. The EC skin test showed sensitivity (87.5%) like that of the T-SPOT TB test (86.5%). It was concluded that the EC test had high consistency with the T-SPOT TB test in BCG-vaccinated adults with no serious adverse effects. So, the BCG vaccination had no impact on the accuracy of the EC skin test [18].

In our study, we have designed fusion constructs from full-length native Rv3874 and Rv3875 to produce the Rv3874-Rv3875 (dubbed as CEP after the names of CFP10 or Rv3874 and ESAT-6 or RV3875) by using reverse order of the antigens as contrary to previously reported [17]. The B cell immune response and diagnostic parameters such as specificity and sensitivity of our engineered constructs were also evaluated using sera of TB affected and normal individuals from the Pakistani population.

2. Materials and Methodology

2.1. Epitope prediction

In this study, Rv3874 and Rv3875 antigens were included as full-length native antigens. One B-cell epitope for Rv3874 was obtained from the immune epitope database and analysis source (IEDB) [19]. As Rv3875 has no reported B cell epitopes, the Bepipred-1.0 Linear Epitope Prediction tool (http://tools.iedb.org/bcell) was used to predict putative B cell Epitopes. This prediction tool uses an antigenic propensity scale for polypeptides for the location of continuous epitopes [20]. A threshold of 0.35 was set as in the graph with residues positions versus residue outcomes (Fig. 1a). Total of seven epitopes with a threshold length of ten, were selected for the fusion including those retrieved from IEDB. The results for the position of the active residues and sequence length were consistent with Bepipred 1.0 linear epitope prediction results. Bepipered-2 software was also used to assess any increase in the number or span of active epitopes in the fusion (Fig. 1 b-d).

2.2. Cloning, expression, and purification

Full-length *Rv3874* (303-bp) and *Rv3875* (288-bp) genes were amplified using genomic DNA of *Mtb strain H37Rv* [21]. The primers used for *Rv3874* were pF1 and pR1 while pF2 and pR2 were used for *Rv3875* (Table 1). Rv3874 and Rv3875 both were amplified using the same PCR program conditions as shown in Table 2. The PCR products were cloned into pJET1.2 followed by the transformation in *E. coli* DH10β cells. Sub-cloning was performed using pET28a (+) as described previously [22] followed by double digestion of the amplified Rv3874 and the recombinant pET28a (+) with *Nde*I and *Bam*HI, while the same was executed for Rv3875 with *Bam*HI and *Hin*dIII. Digested products were ligated and transformed into DH10β cells.

Amplified *Rv3874* without stop codon and pET28a (+)-*Rv3875* were digested with *Nde*I and *Bam*HI followed by mutual ligation forming pET28a (+)-Rv3874-*Rv3875*. Ligation of the inserts was confirmed through restriction digestion and commercial sequencing (*Apical Scientific Selangor, Malaysia*).

The sub-clones were expressed in *E. coli* BL21 CodonPlus (DE3) RIPL as described previously [21]. The IPTG (0.1 mM) induced cells with expression of the histidine-tagged recombinant protein (25 kDa), were pelleted, and re-suspended in the buffer having 20 mM Tris-Cl (pH 8.0), 0.2 M NaCl, and 1 mM PMSF followed by cells 'disruption by sonication (Sonics Vibra-cell 53 Churchill Hill Rd New Town CT USA). The total cell proteins were spun at 5000 rpm for 12 min. The supernatant having soluble fractions and re-suspended pellet with insoluble proteins were resolved on SDS-PAGE and the densitometric method was used for the determination of protein expression level using a gel documentation system (SynGene UK). RV3875 showed partial solubility while others were completely soluble. RV3875 was obtained in both pellets as well as supernatant. RV3875 was solubilized using a gradient of 8 M, 6 M, 4 M, 2 M, and Zero molar Urea in 50 mM Tris-Cl. All proteins were purified through Ni column chromatography by previously reported protocols [22]. The purity of soluble protein fractions was assessed by SDS-PAGE analysis. The salts in eluted fractions were removed by dialysis using 20 mM Tris-Cl (pH 8.0).

2.3. Collection of plasma samples

Blood plasma samples from 201 cultures confirmed TB patients of all

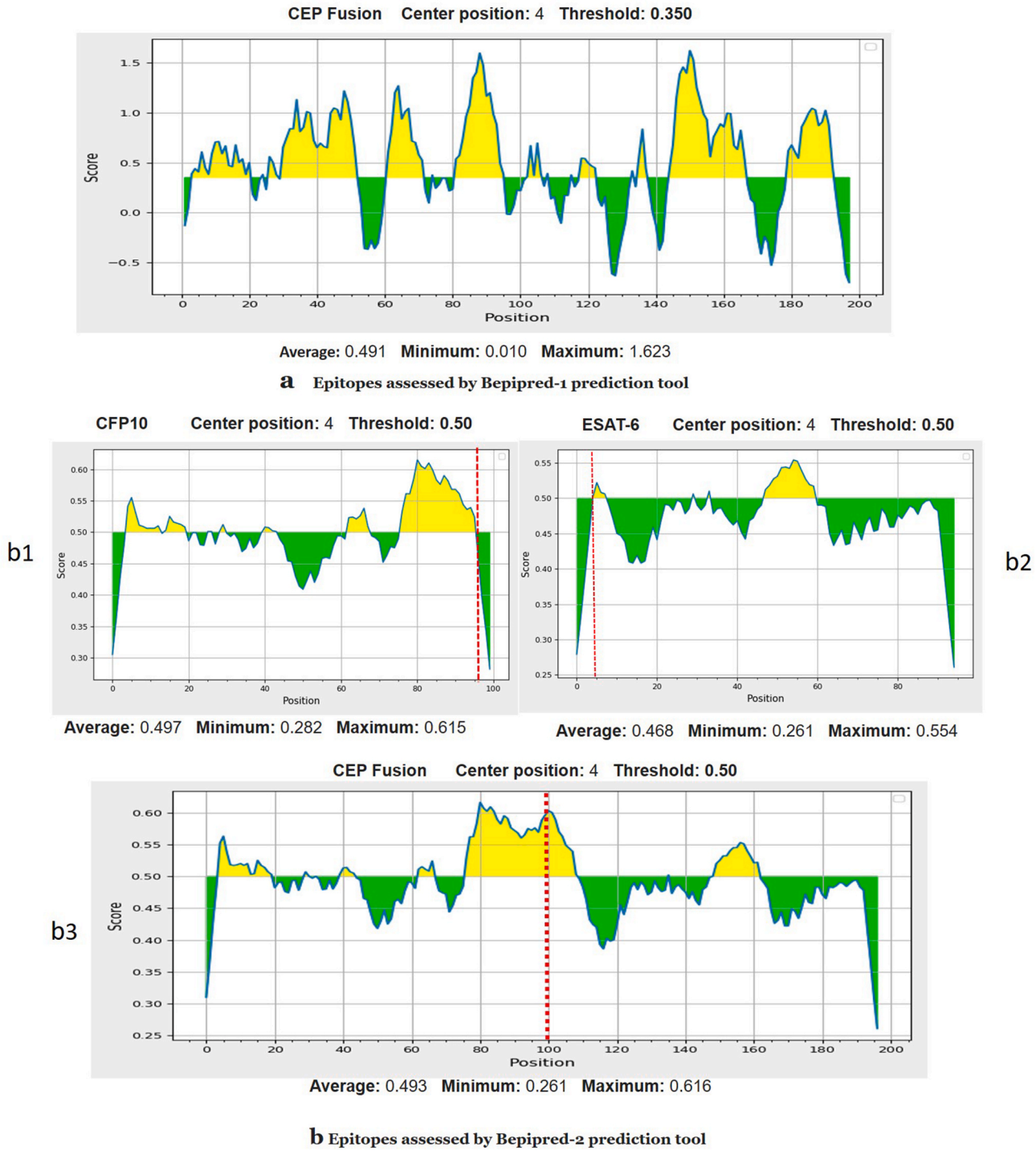

Fig. 1. (a) B-cell epitope prediction for CEP fusion based on protein linear sequence using Bepipred-1 (b) CFP10, ESAT-6, and CEP fusion epitopes predicted using Bepipred-2. Note the presence of two neo epitopes in **(b3).** The first one is at position 5–20 while the other is at the junction of the two genes spanning 77–109 amino acids with a 77–96 region common to native CFP10. The yellow-colored epitopes above the threshold show the feasible B-cell epitopes while regions with green color show inactive residues. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
PCR Primer specifications for antigenic proteins (highlighted regions show the restriction sites).

Antigen with gene size	Primer Name	Sequence 5′-3′	Restriction sites	Genomic complement Location
Rv3874/cfp10 (303bp)	**pF-1**	AGAGCATATGGCAGAGATGAAGACCGA	***Nde*I**	**4352274–293**
	pR-1	ATTAGGATCCGAAGCCCATTTGCGAGG	***Bam*HI**	**4352560–576**
Rv3875/ESAT-6 (288bp)	**PF-2**	ATATGGATCCATGACAGAGCAGCAGTG	***Bam*HI**	**4352609–625**
	PR-2	ATATAAGCTTCTATGCGAACATCCCAGTGA	***Hind*III**	**4352877–896**

Table 2
PCR Conditions for native antigens. (Both antigens amplified using same PCR program).

Antigens	Initial Denaturation	Repeating cycles = 30			Final Extension
		Initial Denaturation	Annealing	Extension	
Rv3874 & Rv3875	95 °C for 5 min	95 °C for 45sec	65 °C for 45sec	72 °C for 1 min	72 °C for 10 min

age groups and both sexes, were gathered from Gulab Devi Chest Hospital (GDCH), Lahore. Sample collection was done from mid of December 2017 to the last of February 2019. Acid Fast Bacillus (AFB) culture microscopy was used for further screening and samples were classified as culture positive and culture negative by a previously described protocol [23]. All the sera samples were collected from post-exposure but untreated patients. Clinical findings based on symptoms were also recorded and these are reported in Table 3. Fifty healthy individuals volunteered their plasma samples and were designated as healthy controls. These controls were reported to have no TB history, smoking habit, or any other medical complications. All subjects in the study group were from urban areas and the outskirts of Lahore, Pakistan. Informed choice for sampling was given in written on a prescribed consent form. Standard operating procedures about healthcare conditions and dealing with TB patients were followed [24]. All these practices also met the criteria given in the Declaration of Geneva for patient care.

2.4. Evaluation of rabbit antisera

Antisera for Rv3874 and Rv3875 were produced in Rabbits according to the previously described protocol [25]. In short, native antigens at 600 μg/500μl in 1X Phosphate buffer saline were mixed with 300 μl of Freund's Complete Adjuvant (FCA). Resultant emulsions were mixed in 300 μl of Freund's Incomplete Adjuvant (FIA) and slowly injected intramuscularly at equal intervals of one month after the first dose. Six rabbits, three male, and three female, *Strain Oryctolagus cuniculus*, used in the research were obtained from an animal house for experimental animals in Lahore. The rabbits were acclimatized for 14 days in a suitable cage in the animal house of the department under the supervision of the veterinarian. The medical condition was vigilantly checked during the whole experiment. Diet in pellets was supplied regularly in addition to *Trifolium alexandrinum* (common name, berseem). The drinking water supply was kept on a needed basis. Rabbits were kept at a consistent moderate temperature of 25 °C. Before drawing blood, the rabbits were anesthetized. The immune reactivity of all proteins was checked against the *anti*-Rv3874 and *anti*-Rv3875 antibodies by ELISA [26].

2.5. Immune reactivity of the antigenic proteins

ELISA protocol with slight modifications was used as already reported from our lab [26]. The readings were recorded on an ELISA reader (HUMAREADER plus, human GMBH) by a protocol reported earlier [26].

Table 3
Clinical findings based on patient history.

Clinical history	Conditions	TB Patients (n = 201)
Diagnosis category	culture positive	120
	culture negative	81
Cough	Yes/No	170/31
	< Two Weeks	36
	>Two weeks	134
Fever	present/Not present	155/46
Loss of appetite	present/Not present	178/23
Weight loss	present/Not present	189/12
Night sweats	present/Not present	138/63

2.6. Statistical analysis

Calculations based on ELISA results were made by defining a cutoff value. Cutoff values were calculated by adding a factor to the mean of $OD_{450/630nm}$ of all the samples from healthy controls and this factor was calculated by multiplying the standard deviation in the OD values of the samples from healthy controls by 3. All the blank subtracted OD values were normalized by dividing them by their relevant cutoff values. All the samples having a cutoff value ≥ 1, were considered positive. Sensitivity was calculated by dividing the number of positive samples by the total number of patients understudy and multiplied by a hundred to get percentage sensitivity. Specificity was calculated by dividing the number of healthy samples having normalized OD of healthy samples greater than the cut-off value by a total number of healthy samples and multiplied by a hundred. Then this value was subtracted from a hundred to get specificity.

Receiver operating characteristic (ROC) analysis for all the OD values was used to get the AUC. Non-parametric Mann-Whitney algorithm was used to compare outcomes in normal individuals and patients. All the calculations were performed in Graphpad Prism 9 software. (Graph-Pad Software Inc., San Diego, CA). A diagnostic test evaluation calculator from *MedCalc* online software was used for further statistical analysis (Table 4). The quality of the antibody detection was also calculated statistically by Matthews Correlation Coefficient (MCC) (https://www.medcalc.org/calc/).

2.7. In silico structural analysis

Raptor-X Server was used for molecular modeling of proteins [27]. It predicts protein secondary and tertiary structures, distance map with improved alignment accuracy, solvent accessibility as well as functional annotation and binding sites. It is a template-based modeling tool. Different tools and servers of bioinformatics were used to design chimeric models followed by validation, solvent accessibility of amino acid residues, and their visualization as mentioned previously [25]. Further, the Ramachandran plot statistics analysis was performed for finding favorable residues in the fusion.

2.8. Assessment of vaccine potential

The online prediction tool Vaxijen was also used to assess the vaccine potential of all the recombinant antigens with favorable outcomes.

3. Results

3.1. Epitopes of Rv3874, Rv3875, and CEP fusion

One epitope with IEDB ID 120421 and sequence AVVRFQEAANKQK (54–66) for the antigenic protein Rv3874 was found by using a qualitative binding assay [28] while four epitopes were retrieved from Bepipred-1.0 which are EMKTDAATLAQEAGNFER (3–20) [29], DQVESTAGSLQGQWRGAAGTAA (30–51), AANKQKQELDE (61–71), and VQYSRADEEQQQAL (81–94). As no epitope for Rv3875 has been reported so far, its two predicted epitopes are AWGGSGSEAYQGVQQKWDATATE (42–64) and SEAGQAMASTEGNV (77–90). To get a chimeric protein consisting of all the said epitopes, full-length Rv3874 and Rv3875 were fused (Figs. 1 and 2). The position of the epitopes in the two individual antigens and their fusion was also

Table 4
Sero-reactivity outcomes of CEP Fusion compared with individual proteins.

Antigens	Total = 201				Culture Positive = 120		Culture Negative = 81		Specificity
	Detected sera	%age Immune response	MCC Co-efficient	Diagnostic accuracy	Detected sera	%age Immune response	Detected sera	%age Immune response	
Rv3874	57	28.4	0.27	42.6%	25	21%	32	39.5%	100%
Rv3875	50	24.9	0.20	39.04%	35	29.1%	16	19.7%	96%
CEP	120	59.7	0.46	67.3%	63	52.5%	55	68%	98%

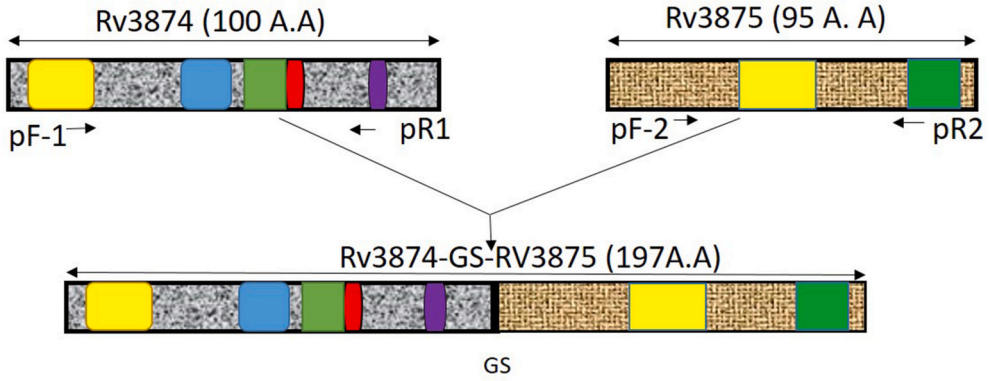

Fig. 2. Schematic illustration for the construction of fusion CEP from single proteins where GS (shown as a black bar) stands for two extra amino acids added because of *Bam*HI restriction site insertion at the point of fusion and resultant fusion is showing the additive effect of individual epitopes.

analyzed using the software Bepipred-2. (Fig. 1b–d). According to this software, the epitope at the N terminal residues of CFP10 (5-20) [29] became more pronounced when the two antigens were fused. Also, the N terminal residues of Rv3875 (residues1-7) in association with C terminal residues of CFP10 (97–100) in the fusion molecule became a part of the active epitopic region (Fig. 1d).

3.2. Cloning, expression, and purification

The expressed native and fusion proteins were analyzed by SDS-PAGE and protein bands thus resolved were at expected sizes. The expression level of CEP was 90%. Rv3875 was partially soluble while the rest gave soluble expression. Purification by nickel column yielded 98% pure fusion protein (Fig. 3). The recovery of CEP was 47% after purification.

3.3. Evaluation of rabbit antisera

The antibodies raised cross-reacted with the antigens against which they were raised. Also, the fusion molecule Rv3874-Rv3875 cross-reacted with the antibodies raised against each of the constituent antigens. Rv3874 and Rv3875 ESAT-6 cross-reacted with the two antibodies. The polyclonal rabbit antisera were used to check reactivity with native and fusion proteins through ELISA. The antigenic concentrations were optimized. The CEP showed more reactivity as compared to native Rv3874 and Rv3875. It means antibodies showed improved response to concentrations of the fusion protein as shown in Fig. 4.

3.4. Immune reactivity of the antigenic proteins

All antigenic proteins exhibited considerable results on ELISA with a comparison of OD values of plasma samples from TB patients with healthy controls. Signal to noise ratios (ratio of average OD for the TB sera to the average OD of sera from healthy controls) of Rv3874, Rv3875, and CEP were 4.5, 2.8, and 5.58 respectively, which showed greater immune identification in the case of CEP as compared to individual Rv3874 or Rv3875.

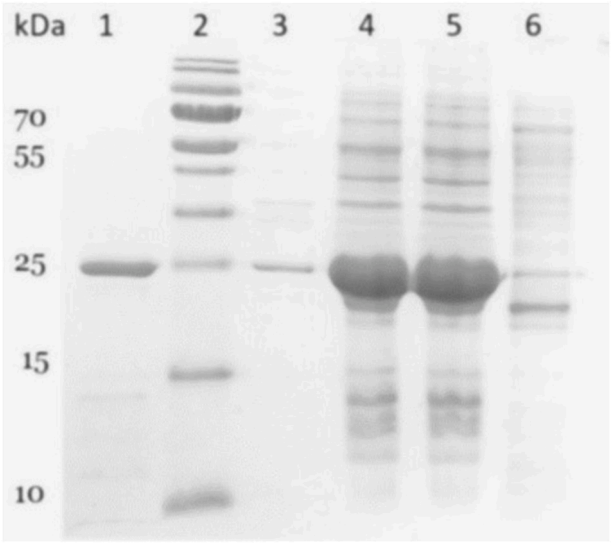

Fig. 3. SDS PAGE showing expression of recombinant fusion protein: **1**-purified fusion protein Rv3874-Rv3875 (CEP) at 25 kDa position, **2**- PAGE Ruler Pre-Stained Protein Ladder, range, 10–180 kDa, **3** shows pellet **4** shows Supernatant, **5** induced (or Total cell lysate having over-expressed fusion protein) at 0.1 mM IPTG and **6** shows an un-induced sample.

The antigen Rv3874 detected 57 (28.4%) samples out of 201 TB samples. Similarly, Rv3875 diagnosed 50 (24.9%) samples. The CEP fusion antigen detected 120 TB sera with increased sensitivity of 59.7% (Table 4) while the combined sensitivity of both proteins was 53.3%. Specificities of Rv3874, Rv3875, and CEP were 100%, 96%, and 98% respectively. (Fig. 5a).

The sensitivities of Rv3874, Rv3875, and CEP in the male group were 27.9%, 27%, and 60.4% respectively and these were 28.8%, 22.2%, and 58.8% in females, respectively. The sensitivities of Rv3874, Rv3875, and CEP in the subjects of age group (1-15 years) [29] were 25%, 38%, and 62.5%. The sensitivities in the age range (16–35 years) were 24.6%,

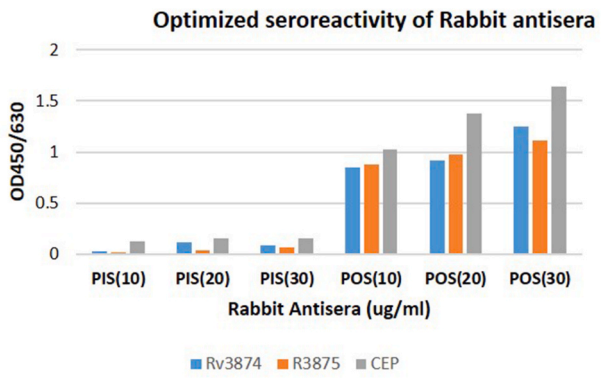

Fig. 4. Rabbit antisera response against corresponding antigens where PIS refers to pre-immune sera and POS stands for post-immune sera.

23%, and 55.5% while the sensitivities for the 36–55 years age range were 38.5%, 25%, and 67.3%. The sensitivities above the age of 55 were 26.6%, 33.3%, and 66.6%, respectively (Fig. 6c). Graphpad Prism 9 software was used to record the ROC curve for the diagnostic potential of all the recombinant antigens (Fig. 5b). Values for the AUC for Rv3874, Rv3875 and CEP were 0.91, 0.90 and 0.97, respectively. Hence CEP diagnostic potential was most efficient.

3.5. *In silico structural analysis*

Raptor-X server was used to develop 3D molecular models of recombinant proteins. All the *in silico*-derived protein models (Fig. 7 a-f) showed good authentication scores with high solvent accessibility using the tools and software described previously [30]. In the CEP fusion, a combined effect of B-Cell epitope region residues with active participation in antigen-antibody interaction was observed. Ramachandran plot data also supported the same. It showed 96% amino acids (170 residues) in the most favorable region as well as 3.4% amino acids (6 residues) in additional allowed regions and zero% disallowed regions (Fig. 8). The plot has 2 Å resolution with an R factor >20 making the fusion a strong antigen in terms of maximum exposure of amino acids.

3.6. *Assessment of vaccine potential*

The vaccine potential of all the antigenic proteins was also predicted by Vaxijen, an online prediction tool used to foresee the protective efficacy of immunogenic proteins for use as a subunit vaccine. CEP showed a vaccine potential of 0.67 at a threshold of 0.4, which further consolidated the idea of residue arrangement in a favorable orientation to mount an immune response.

4. Discussion

Fusion protein-based serodiagnosis of TB can be economical and handy in a high TB burden and economically poor countries like Pakistan, yet it is subject to the significantly increased sensitivity of fusion chimeras. Using multistage antigens to reach this goal may result in increased costs. So, the formation of fusion constructs from different antigens to enhance the diagnostic efficiency of ELISA will be cheaper. However, a fusion of multiple full-length genes may result in an unstable size range. Therefore, in this study, pairwise antigens were used to make the fusion molecule. When Rv3875, was fused to the C terminal of Rv3874, a soluble overexpression of Rv3874-Rv3875 chimeric protein was seen. Linking Rv3875 at *N*-terminal also resulted in the enhanced recovery of the Rv3874-Rv3875 to 300 mg/L/OD_{600}.

Irrespective of the exact stage of infection, the symptoms in patient history are indicative of the invasive infection stage. The serum samples were collected before the start of any drug intervention. It means under these circumstances the patient sera will yield enough antibodies due to ongoing immune response.

Solvent accessibility of epitopic residues means their better exposure and interaction with antibodies [31]. The CPORT server uses molecular models for solvent accessibility analysis thereby supporting the possibility that the fusion of two antigenic proteins aligns the epitopes more effectively in an additive manner. This will allow more efficient antigen-antibody interaction thus enhancing its sensitivity. A sensitivity of 59.7% for the fusion CEP is higher than the combined sensitivities of the two contributing antigens (53.3%). It is because of conformational re-orientation of the antigens in the fusion state. It is further supported by the appearance of a stronger epitope at the N terminal of Rv3874 (residues 5-20) and its C terminal residues (97–100) which became epitopically more active in association with N terminal (residues 1-7) of Rv3875 (Fig. 1b–d).

The diagnostic evaluation outcomes for native and fusion proteins are consolidated by statistical analysis in the form of a ROC curve. CEP showed AUC value of 0.973 which is greater than any of the individual antigens Rv3874 or Rv3875. Further, this AUC value of CEP is greater than that obtained in the EC test which means changing the position of ESAT-6 from the N terminal to the C terminal of the fusion results in the enhanced epitopic potential of the fusion protein as contrary to reported earlier in reverse order [17,18].

Our fusion Rv3874-Rv3875 also shows greater sensitivity (59.7%) as compared to single native antigens with high and soluble expression

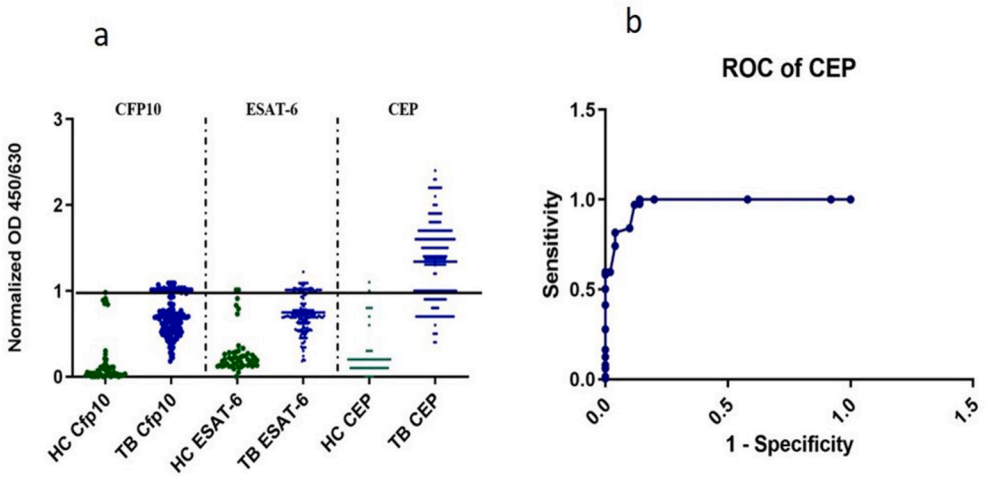

Fig. 5. Depiction of ELISA outcomes using Graph pad Prism 9. (a) Scatter dot plot (b) ROC curve showing the diagnostic evaluation of CEP fusion.

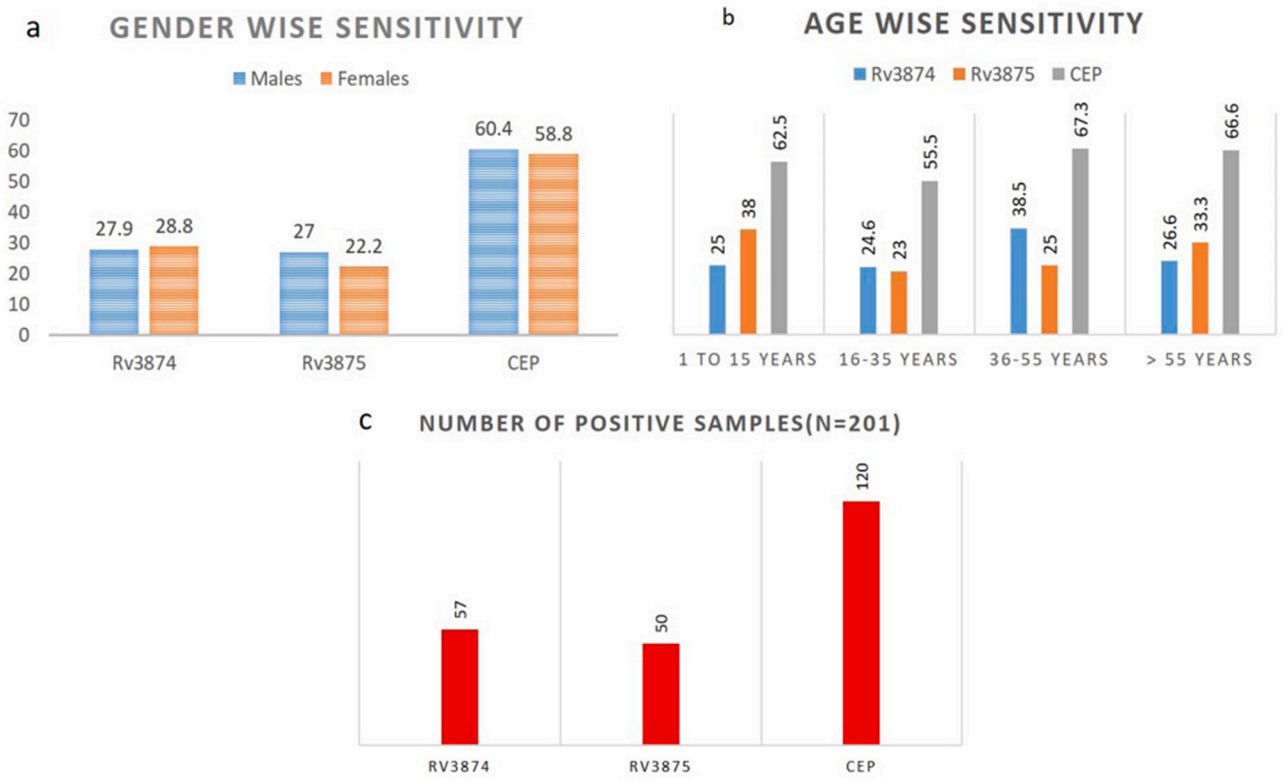

Fig. 6. Sero-detection efficiency of native proteins and CEP fusion in categories based on: (a) number of positive plasma samples (b) Gender (c) age groups.

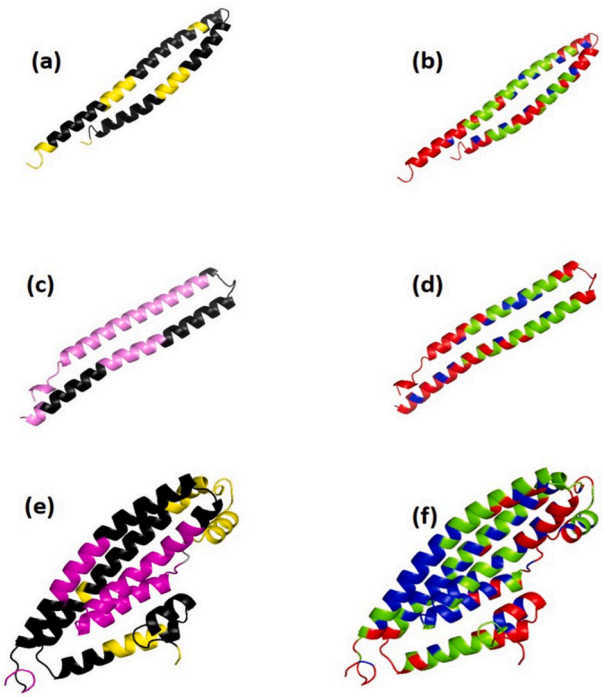

Fig. 7. (a, c, and e) Ribbon models of Rv3874, Rv3875 and their fusion CEP generated through RaptorX (Epitopes are shown in black color). Fig. 7 (b, d, and f) Ribbon models in 3D as predicted by CPORT showing solvent accessibility of native and fusion molecules. Here the responsive amino acids are labeled with red color along with green color showing amino acids with enhancing effect for interaction with antibodies. The blue color shows nonsporting residues. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

thereby making the chromatographic purification easier. This sensitivity is comparable to that of the EC test [17,18] as well as to the tri-protein fusionTriFu64 recently reported from our lab which showed a sensitivity of 66% [6]. Therefore, CEP has a good potential in making a cheap, dependable, and rapid serodiagnostic tool for TB.

5. Conclusions

The production of chimeric antigenic proteins is an economical and more applicable way compared to bulk peptide synthesis. Our Rv3874-Rv3875 chimeric protein may serve as a milestone for developing more chimeric molecules with greater serodiagnostic potential. The chimeric Rv3874-Rv3875 protein has shown almost a two-fold enhancement in sensitivity due to the cumulative effect of epitopic proteins and the formation of new epitopes by possible neo-peptides at the junction of adjacent proteins. Attachment of RV3875 to the C terminal of Rv3874 as compared to the reverse order used in the EC test resulted in the favorable epitopic arrangement of each antigen with enhanced sensitivity (59.7%) and AUC value (0.97). The sensitivity of CEP fusion is comparable to the recently reported trimeric TriFu64 fusion from our lab with a sensitivity of 66%. Yet, these are the preliminary findings, and further studies are still needed to confirm practical applications of CEP fusion.

Ethical approval

Ethical guidelines regarding patient care, as well as the use of experimental animals, were approved by the Ethics Committee, School of Biological Sciences, University of the Punjab, Lahore, Pakistan vide letter no. SBS/765/17 and followed accordingly.

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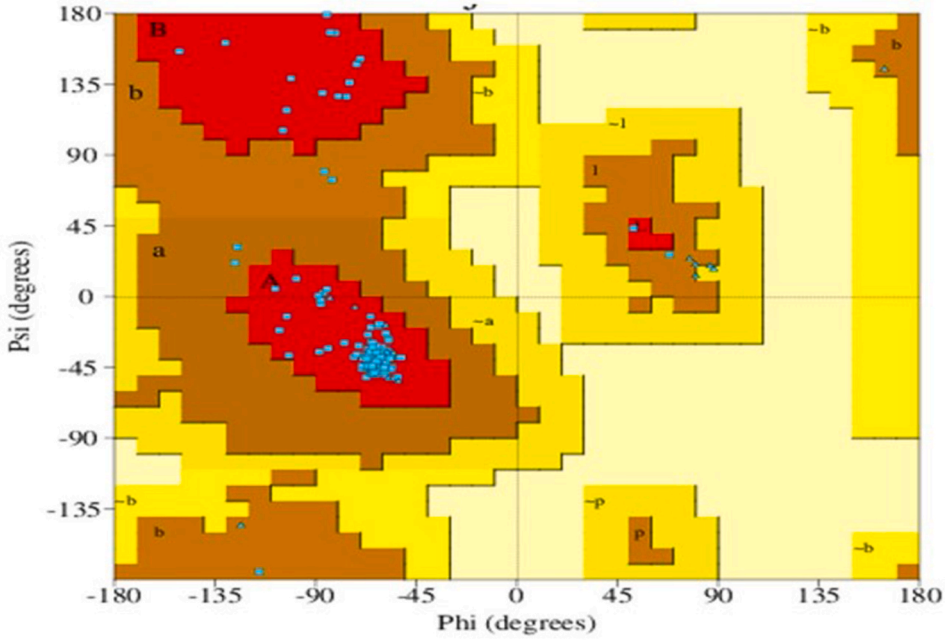

Fig. 8. Analysis of CEP Using Ramachandran plot where red colored regions in the plot show residues in the most favored position, the yellow color shows additional allowed regions for residues, and the light-yellow color depicts generously allowed regions for residues. The white color is for the disallowed region. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Declaration of competing interest

None.

Author contribution

Conceptualization, resource contribution, and overall supervision were by MWA. NM performed major experiments and data analysis. MA also executed data analysis. Demographical parameters and plasma samples of volunteers were recorded by ZUN and IHK. Critical reading and draft writing were by NM, NH, MS and MWA.

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References

[1] Organization WH. Global tuberculosis report 2021. Geneva: WHO; 2021. p. 2021.
[2] Zhou F, Xu X, Wu S, Cui X, Fan L, Pan W. Protein array identification of protein markers for serodiagnosis of *Mycobacterium tuberculosis* infection. Sci Rep 2015 Oct 20;5:15349. https://doi.org/10.1038/srep15349. PMID: 26481294; PMCID: PMC4642701.
[3] Yan Z, Yi L, Wei P, Jia H, Wang J, Wang X, Yang B, Gao X, Zhao Y, Zhang H. Evaluation of panels of *Mycobacterium tuberculosis* antigens for serodiagnosis of tuberculosis. Int J Tubercul Lung Dis 2018;22:959–65.
[4] Afzal M, Khurshid S, Khalid R, Paracha RZ, Khan IH, Akhtar MW. Fusion of selected regions of mycobacterial antigens for enhancing sensitivity in serodiagnosis of tuberculosis. J Microbiol Methods 2015;115:104–11.
[5] Akhter M, Arif S, Khaliq A, un-Nisa Z, Khan IH, Akhtar MW. Designing fusion molecules from antigens of *Mycobacterium tuberculosis* for detection of multiple antibodies in the plasma of TB patients. Tuberculosis 2020:101981.
[6] Sulman S, Shahid S, Khaliq A, Ambreen A, Khan IH, Cooper AM, Akhtar MW. Enhanced serodiagnostic potential of a fusion molecule consisting of Rv1793, Rv2628, and a truncated Rv2608 of *Mycobacterium tuberculosis*. PLoS One 2021 Nov 12;16(11):e0258389. https://doi.org/10.1371/journal.pone.0258389. PMID: 34767571; PMCID: PMC8589213.
[7] Arif S, Akhter M, Khaliq A, un-Nisa Z, Khan IH, Akhtar MW. Serodiagnostic evaluation of fusion proteins from multiple antigens of *Mycobacterium tuberculosis* for active TB. Tuberculosis 2021;127:102053.
[8] Khurshid S, Afzal M, Khalid R, Khan IH, Akhtar MW. Improving sensitivity for serodiagnosis of tuberculosis using TB16. 3-echA1 fusion protein. Tuberculosis 2014;94:519–24.
[9] Khurshid S, Khalid R, Afzal M, Akhtar MW. Truncation of PstS1 antigen of *Mycobacterium tuberculosis* improves diagnostic efficiency. Tuberculosis 2013;93:654–9.
[10] Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, et al. Type VII secretion–mycobacteria show the way. Nat Rev Microbiol 2007;5:883–91. https://doi.org/10.1038/nrmicro1773.
[11] Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999;284:1520–3.
[12] Aguilo N, Uranga S, Marinova D, Monzon M, Badiola J, Martin C. MTBVAC vaccine is safe, immunogenic and confers protective efficacy against *Mycobacterium tuberculosis* in newborn mice. Tuberculosis 2016 Jan;96:71–4. https://doi.org/10.1016/j.tube.2015.10.010. Epub 2015 Nov 30. PMID: 26786657; PMCID: PMC4727503.
[13] Aguilo N, Gonzalo-Asensio J, Alvarez-Arguedas S, Marinova D, Gomez AB, Uranga S, Martin C. Reactogenicity to major tuberculosis antigens absent in BCG is linked to improved protection against Mycobacterium tuberculosis. Nat Commun 2017;8(1):1–11.
[14] Chen L, Xu M, Wang ZY, Chen BW, Du WX, Su C, Shen XB, Zhao AH, Dong N, Wang YJ, Wang GZ. The development and preliminary evaluation of a new *Mycobacterium tuberculosis* vaccine comprising Ag85b, HSPX, and CFP-10: ESAT-6 fusion protein with CpG DNA and aluminum hydroxide adjuvants. FEMS Immunol Med Microbiol 2010 Jun 1;59(1):42–52. https://doi.org/10.1111/j.1574-695X.2010.00660.x. Epub 2010 Feb 17. PMID: 20298499.
[15] Jones GJ, Whelan A, Clifford D, Coad M, Vordermeier HM. Improved skin test for differential diagnosis of bovine tuberculosis by the addition of Rv3020c-derived peptides. Clin Vaccine Immunol 2012 Apr;19(4):620–2. https://doi.org/10.1128/CVI.00024-12. Epub 2012 Feb 1. PMID: 22301696; PMCID: PMC3318272.
[16] Hill PC, Jackson-Sillah D, Fox A, Franken KL, Lugos MD, Jeffries DJ, Donkor SA, Hammond AS, Adegbola RA, Ottenhoff TH, Klein MR, Brookes RH. ESAT-6/CFP-10 fusion protein and peptides for optimal diagnosis of *Mycobacterium tuberculosis* infection by ex vivo enzyme-linked immunospot assay in the Gambia. J Clin Microbiol 2005 May;43(5):2070–4. https://doi.org/10.1128/JCM.43.5.2070-2074.2005. PMID: 15872224; PMCID: PMC1153808.
[17] Li F, Xu M, Qin C, Xia L, Xiong Y, Xi X, Fan X, Gu J, Pu J, Wu Q, Lu S, Wang G. Recombinant fusion ESAT6-CFP10 immunogen as a skin test reagent for tuberculosis diagnosis: an open-label, randomized, two-center phase 2a clinical trial. Clin Microbiol Infect 2016 Oct;22(10). https://doi.org/10.1016/j.cmi.2016.07.015. 889.e9-889.e16, Epub 2016 Jul 21. PMID: 27451942.
[18] Xu M, Lu W, Li T, Li J, Du W, Wu Q, Liu Q, Yuan B, Lu J, Ding X, Li F, Liu M, Chen B, Pu J, Zhang R, Xi X, Zhou R, Mei Z, Du R, Tao L, Martinez L, Lu S, Wang G,

Zhu F. Sensitivity, specificity, and safety of a novel ESAT6-CFP10 skin test for tuberculosis infection in China: 2 randomized, self-controlled, parallel-group phase 2b trials. Clin Infect Dis 2022;74(4):668–77. https://doi.org/10.1093/cid/ciab472. PMID: 34021314; PMCID: PMC8886919.

[19] Vita R, Zarebski L, Greenbaum JA, Emami H, Hoof I, Salimi N, et al. The immune epitope database 2.0. Nucleic Acids Res 2009;38:D854–62. https://doi.org/10.1093/nar/gkp1004. PMID: 19906713.

[20] Larsen JEP, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. Immunome Res 2006;2:2. https://doi.org/10.1186/1745-7580-2-2. PMID: 16635264.

[21] Khalid R, Afzal M, Khurshid S, Paracha RZ, Khan IH, Akhtar MW. Fusion molecules of heat shock protein HSPX with other antigens of *Mycobacterium tuberculosis* show high potential in the serodiagnosis of tuberculosis. PLoS One 2016;11:e0163349. https://doi.org/10.1371/journal.pone.0163349. PMID: 27654048.

[22] Sambrook J, Russell DW, Russell DW. Molecular cloning: a laboratory manual (3-volume set). New York: Cold spring harbor laboratory press; 2001.

[23] Khurshid S, Khalid R, Afzal M, Akhtar MW. Truncation of PstS1 antigen of *Mycobacterium tuberculosis* improves diagnostic efficiency. Tuberculosis 2013;93:654–9. https://doi.org/10.1016/j.tube.2013.07.005. PMID: 23978525.

[24] Khaliq A, Ravindran R, Hussainy SF, Krishnan VV, Ambreen A, Yusuf NW, et al. Field evaluation of a blood-based test for active tuberculosis in endemic settings. PLoS One 2017;12:e0173359. https://doi.org/10.1371/journal.pone.0173359. PMID: 28380055.

[25] Hopewell PC, Pai M, Maher D, Uplekar M, Raviglione MC. International standards for tuberculosis care. Lancet Infect Dis 2006;6:710–25. https://doi.org/10.1016/S1473-3099(06)70628-4. PMID: 17067920.

[26] Afzal M, Khurshid S, Khalid R, Paracha RZ, Khan IH, Akhtar MW. Fusion of selected regions of mycobacterial antigens for enhancing sensitivity in serodiagnosis of tuberculosis. J Microbiol Methods 2015;115:104–11. https://doi.org/10.1016/j.mimet.2015.06.003. PMID: 26068786.

[27] Kallberg M, Wang H, Wang S, Peng J, Wang Z, Lu H, et al. Template-based protein structure modeling using the RaptorX web server. Nat Protoc 2012;7:1511–22. https://doi.org/10.1038/nprot.2012.085. PMID: 22814390.

[28] Baassi L, Sadki K, Seghrouchni F, Contini S, Cherki W, Nagelkerke N, Benjouad A, Saltini C, Colizzi V, El Aouad R, Amicosante M. Evaluation of a multi-antigen test based on B-cell epitope peptides for the serodiagnosis of pulmonary tuberculosis. Int J Tubercul Lung Dis 2009 Jul;13(7):848–54. PMID: 19555534.

[29] Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, et al. The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc Natl Acad Sci USA 2003;100:12420–5. https://doi.org/10.1073/pnas.1635213100.

[30] Scarabelli G, Morra G, Colombo G. Predicting interaction sites from the energetics of isolated proteins: a new approach to epitope mapping. Biophys J 2010;98:1966–75. https://doi.org/10.1016/j.bpj.2010.01.014. PMID: 20441761.

[31] de Vries SJ, Bonvin AM. CPORT: a consensus interface predictor and its performance in prediction-driven docking with HADDOCK. PLoS One 2011;6:e17695. https://doi.org/10.1371/journal.pone.0017695. PMID: 21464987.