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Exploration of some new secretory proteins to be employed for companion diagnosis of *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis (TB) is a highly infectious disease and its early and precise diagnosis is essential to reduce morbidity and mortality of patients. Since the routine diagnostic tests (like Monteux, AFB smear microscopy, chest X-Ray) do not give infallible results, additional tests are always recommended. Therefore to address the concerns about non-specificity of the present battery of diagnostic tests, we have attempted to analyze some unique secretory antigens which could be able to identify the stage specific infection of MTB. In this study, we have used recombinant proteins CFP-10, ESAT-6, Ag85 A, Ag85B, Ag85C, PE3, PE4 and Mycp1 to eliminate heterogeneity and cross reactivity in clinical diagnosis. Amplified genes were cloned and over-expressed in *Escherichia coli* BL21 (DE3). The recombinantly purified proteins were used as antigens against 158 sera samples of TB patients. Secretory proteins showed better response than the PPD control. Among all the used antigens PE3 and PE4 proteins showed better reactivity levels among all the groups of TB patients. The secretions of CFP-10 and ESAT-6 were also higher as compared to other secretory proteins like Ag85 A, Ag85B, Ag85C and MycP1.The clinical use of these newly identified secretory antigens could be of significant value for the confirmatory, rapid, simple and low-cost diagnosis of TB patients.

1. Introduction

Tuberculosis (TB), one of the most common diseases all over the world, is highly infectious and caused by *M. tuberculosis (Mtb)*. The emergence of MDR-TB has been an area of growing concern to human health worldwide and is posing a threat to the control of TB [1,2]. The organism most commonly affects lungs in case of pulmonary TB (PTB), and other extra-pulmonary tuberculosis (EPTB) when it affects other organs [3].

Early diagnosis of TB is very essential to reduce mortality in patients affected [4–6]. Diagnosis is often difficult to establish using the standard methods, because of the difficulties in obtaining specimens [3,6].

Arloing described the first serodiagnostic test for TB based on haemagglutination, but since then the progress in serodiagnosis has been slow [7]. In the last decade, studies on new assays that use various antigens for measurement of serum antibodies against *Mtb* in patients with TB have been reported [4,6]. ELISA-based serological tests to detect antibodies against *Mtb* are simple and inexpensive and are a potential practical tool for the diagnosis of active PTB and EPTB. However, almost all the assays are limited by sensitivity, especially in smear-negative TB patients. The reported specificity of 90% is acceptable for a serodiagnostic test [4,6].The completion of *Mtb* genome sequence has led to the identification of several antigens that can be utilized for accurate diagnosis and control of TB. According to a recent data, approximately 205 cultures filtrate proteins are secreted by *Mtb* and immunodiagnostics seems to be an ideally suited diagnostics modality for PTB and EPTB [6,8]. The recombinantly purified proteins are able to eliminate heterogeneity and cross reactivity. Detection of *Mtb* components in clinical specimen using cell-mediated immune (CMI) responses, the Tuberculin sensitivity test (TST) with purified protein derivative (PPD) as one of the CMI-based tests has low specificity [3,8,9]. The availability of the completely sequenced genome of *Mtb* has provided tools for the identification of bacterial antigens that are useful in the development of new reagents to diagnose and control TB [9,10].

More recently, evidence has accumulated on the accuracy of Xpert *MTB*/RIF against various EPTB forms. Thus, Xpert *MTB*/RIF should now be considered a central test in the work-up of EPTB and should be used

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along with existing tools such as microscopy, liquid cultures, and histopathology to arrive at the final diagnosis [5,8,11].

Secreted virulence factors are regarded as the key mediators for mycobacterial pathogenicity [12,13]. Pathogenic strains of *Mtb* can have up to five different T7S systems, designated as ESX-1 to ESX-5 [14]. Phylogenetic and comparative genomic analyses have shown that these different ESX systems have probably evolved by gene duplication, in the order ESX-4, ESX-1, ESX-3, ESX-2, and the most recently evolved ESX-5 [15].

The well-studied system is ESX-1, which *Mtb* pathogens use to secrete the T-cell antigens ESAT-6/CFP-10 in addition to other ESX secreted proteins (Esp) which are required for progression of disease [16,17]. Mycosin-1 protease (MycP1) is essential for ESX- 1 secretion in pathogenic Mtb and for efficient DNA conjugation in the avirulent saprophyte *M. smegmatis* [12]. ESAT-6 and CFP-10 are prominent T-cell antigens, the genes for which are encoded on a segment termed as Region of differentiation (RD1) [12,15]. MycP1 is one of six conserved components that is found in all five T7SS, and is thus designated MycP1-5 in accordance to their associated systems [12]. The other T7SS of known function are involved in iron acquisition (ESX-3) [14] or export of the virulence-related PE/PPE family of proteins (ESX-5) [14,16,17]. Proteins exported by the T7SS have been detected in culture filtrates and in cell wall fractions as lower molecular weight/proteolytically processed species when compared to those which are not exported. *Mtb* secretes several highly immunogenic proteins across the cell wall using the ESX-1 transport system [18–20], and these virulence factors cause lung tissue inflammation and necrosis [17,21].

A significant portion (10%) of the *Mtb* genome encodes two unique protein families, themultigene proline-glutamic acid/ proline- proline-glutamic acid (PE/PPE) families. The PE/PPE groups of proteins are potential source of antigenic variation and have critical roles in the pathogenesis of *Mtb* as well [20,22,23]. The PE/PPE genes are expressed upon various environmental cues during infection, and many of the PPE proteins have been found to be strongly immunogenic [9,22–24].

In the fight against TB, secretory proteins have been potent weapons with their utilization in several different types of diagnostic kits based on serodiagnosis, interferon-γ release assays (IGRA) and PCR [25]. They are also components of prominent subunit booster vaccines under clinical trials [26]. However, the development of effective tuberculosis control strategy is a continuing process and it requires extensive identification and characterization of all potent virulence factors of *Mtb*.

Among the major antigens of RD1 locus, early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are encoded by the genes *esxA* and *esxB* respectively and form a 1:1 heterodimeric complex *in vitro*. ESAT-6 is the best characterized protein within the RD1 region. It has been recognized as an important stimulator of T-cells both *in vitro* and *in vivo* [4,11]. This gives the significance of ESAT-6 and CFP-10 secretion in mycobacterial virulence and the importance of these antigens as diagnostic tools for the detection of TB [15,27]. For determining the usefulness of CFP-10, ESAT-6, Ag85A, Ag85B, Ag85C, PE3, PE4 and Mycp1 in TB diagnosis, we have recombinantly expressed these proteins and purified to homogeneity, to use as antigens against the sera obtained from TB patients, and from healthy controls.

2. Materials and methods

2.1. Materials

Restriction enzymes and cloning vector *pTZ57R/T* (Fermentas, USA) and ligation kit (Roche, Germany) were used. Expression vectors*pTriEx4* and *pET28a*, E. coli strains DH5α and BL21 (DE3) competent cells (Invitrogen Life Technologies, USA) were used. The nickel–nitrilotriacetic acid (Ni–NTA), agarose, Gel extraction Kit (Qiagen, USA), QIA prep Spin Miniprep Kit (GE health care), Dialysis tubing cellulose membrane (MW 10,000) (Sigma, USA) were also used.

Secondary antibodies linked with horseradish peroxidase (HRP), were purchased through Santa Cruz Biotech Inc., isopropyl-D-thiogalactopyranoside (IPTG) (Merck, USA), Ultra-pure Milli- Q water was used in this study. Polymerase Chain reaction (PCR) mix was obtained from Life technologies. Plasmid DNA purification, Gel extraction kits were obtained from Qiagen and GE healthcare. Luria Bertani (LB) broth, Luria Bertani agar (LBA), nutrient broth (NB), nutrient broth agar (NBA) agar powder, Middle brook 7H9 (MB7H9) broth, Middle brook 7H10 (MB7H10) agar medium, albumin-dextrose-catalase (ADC) and oleic acid-albumin-dextrose-catalase (OADC) enrichments, Lowenstein-Jensen (LJ) medium powder, MGIT 7 ml medium tube, PANTA with supplement OADC, SIRE lyophilized antibiotics were procured from BD Biosciences and Difco.

2.2. Study population

One hundred and seventy two active TB patients were recruited from Department of Respiratory Medicine, King George's Medical University (KGMU), UP, Lucknow, India. Only 158 patients were selected for the study. The selected 158 patients were diagnosed as PTB and EPTB patients, registered under Revised National tuberculosis Control Programme (RNTCP), attending OPD or admitted to the hospital from July 2015 to January 2016 were enrolled for this study. For the study, only those patients (1) with detectable acid-fast bacilli in the sputum bacilloscopy or (2) culture confirmed TB, and who had undergone clinical and chest X-ray examinations, i.e., patients with active pulmonary TB, were included. The age of the patients varied between 19 and 62 years (average age 40.5 years). HIV infection, diabetes, hepatitis, hypertension, pregnancy, malnutrition, malignancies, and alcoholism were in the exclusion criteria. Forty four healthy individuals, without prior history of mycobacterial disease, were included in the healthy/control group. Healthy individuals were identified, based on extensive questioning and assessment by medical professionals specialized in respiratory diseases (Tables 1 and 2). Based on this assessment, none of the individuals identified as a healthy control or had a prior history of TB, clinical symptoms of TB, or direct contact with TB patients. The age of the controls varied between 19 and 44 years. Average age was 31.5 years. All the patients and controls gave informed consent

Table 1
Demographic Profile of Selected Patients.

S.No	Variables	No. of samples (n = 158)	Percentage%
1	Year July 2015- January 2016	158	————
2	**Residence**		
	Urban	98	62.02
	Rural	60	37.97
3	**Gender**		
	Male	96	60.75
	Female	62	39.24
4	**Age**		
	19– 30	22	13.92
	31- 40	95	60.12
	41- 50	31	19.62
	51- 62	10	06.32
5	**Family History of TB**		
	• Yes	102	64.55
	• No	48	30.37
	• Not Known	08	05.05
6	**Past History of Pulmonary TB**		
	• Yes	100	63.29
	• No	58	36.70
7.	HIV Status	All negative	100%
8.	Diabetes Mellitus	All negative	100%
9	**Patients category**		
	• Primary TB	51	32.27
	• Drug Resistant Cases	30	18.98
	• Relapse cases	48	30.37
	• EPTB	29	18.35

Table 2
Results of Culture of AFB positive sputum specimen (n = 172).

Results of Culture	Number	Percentage %
Growth of Mycobacteria	158	91.86
Contamination	O4	02.32
No growth of Mycobacteria	04	02.32
Mycobacteria other than TB (MOTT)	06	03.48
Total	172	100

for blood sampling after the written information was provided.

2.3. Drug susceptibility test (DST)

Drug susceptibility Test (DST) was performed every 2–3 months with MDR patients (Table 3). History relevant to TB such as time and duration, demographic factors, symptoms of TB, AFB load, and outcome of patients was recorded in predesigned data sheet. The sensitivity and resistance pattern of clinical isolates of *Mtb* to first line 4 anti-tuberculosis treatment (ATT) drugs were carried out with the standard procedures [28].

2.4. Confirmation test of TB patients

The diagnosis of TB and EPTB was based on typical clinical, laboratory, radiological, histopathological and microbiological findings (Table 4). *IS6110* due to its high numerical and positional polymorphism has become a widely used marker in the epidemiological studies. All TB positive cases were reconfirmed by PCR with primer of *IS6110,* PCR product was checked on 1% agarose gel in which 158 cases were found positive for *Mtb.*The healthy controls were TB negative.

2.5. Ethical approval

Ethical approval for the study was granted by Research cell King George's Medical University, UP, Lucknow Institutional Ethics Committee (IEC) vide approval no. *3178/Ethics/R.cell-15 dated 07/1/2015 (Ref Code: 70th ECM II-B/PI).*

2.6. Collection of sera samples

The sera samples from selected patients and healthy controls were collected and were divided into five groups: (i) Primary/ New cases of PTB group contained 51 patients (ii) MDR-TB (n = 30) (iii) Relapse TB cases (n = 48); (iv) EPTB (n = 29). All sera were stored at −20 °C until use.

2.7. Bacterial strains and genomic DNA isolation

Mtb H_{37}Rv was obtained from ATCC and sub cultured on LJ medium at 37 °C for 4 weeks. Fully developed colonies were stained by ZN stain for confirmation of AFB. Their genomic DNA was isolated by using Phenol: Chloroform method described earlier [29]. The complete sequences of all selected genes were obtained from Tuberculist database. The genes were amplified by using specific primers (Table S1).

2.8. Cloning and sub cloning

The genes were amplified by PCR, cloned in *pTZ57R/T* vector (Fermentas, USA) and sub cloned in expression vectors (*pTriEx4 or pET28a*), positive clones were confirmed by PCR amplification and restriction digestion method. The restriction sites for specific genes were mentioned in Table S2.

2.9. Expression and purification of proteins

The expression vector containing specific genes mentioned in supplementary data Table S2. The *E. coli* BL23 (DE3) containing specific recombinant vectors were induced at particular condition, mentioned in Table S2. *E. coli* (Bl21) with *pTriEx4*and *pET28a*, PE3, PE4 and CFP-10 cells were harvested by centrifugation and disrupted by sonication as described earlier. Ni–NTA column was used to purify these proteins. The supernatant was applied, to Ni^2+-charged His-Tagged columns pre-equilibrated with 50 mM Tris–HCl, 10 mM EDTA, 100 mM NaCl. After sample loading leave the column for 4 h at 4 °C, the column was washed with lysis buffer (50 mM Tris–HCl, 10 m MEDTA, 100 mM NaCl, 10 mM imidazole). PE3, PE4 and CFP-10 were eluted using a linear gradient with imidazole (100–500 mM).

Other proteins like Ag85 A, Ag85B, Ag85C, MycP1 and ESAT-6 were not present in soluble fraction; hence urea buffer was used for their purification. The purified proteins were analyzed by sodium Dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by Coomassie Brilliant Blue R250 staining. Refolding was done by dialysis with gradient and purity of the proteins was analyzed by SDS-PAGE (Figure S1). All the antigens of *Mtb*; PE3, PE4 Ag85a, Ag85b, Ag85c, Mycp1, CFP-10, ESAT-6 and PPD (Beacon diagnostics) (as standard antigens) were used in the assay.

3. Detection of seroreactivity

Detection of antibodies against eight recombinant proteins from of *Mtb* $H_{37}Rv$, i.e. PE3, PE4, Ag85 A, Ag85B, Ag85C MycP1 CFP-10 and ESAT-6 was carried out in active TB patients sera by ELISA. ELISA plates (Nunc, Denmark) were coated with 1.0 µg of protein in 100 µl / well of selected antigens in duplicate, and incubated overnight at 4 °C and subsequently the unbound antigens were removed by washing 3–4 times with PBS–T buffer (PBS buffer containing 0.05% Tween-20). Each antigen has been normalized and has been used in equal amount (1.0 µg for each protein) for ELISA to maintain uniformity. Free binding sites were blocked with PBS–T buffer supplemented with 1% BSA. Sera samples from different groups of TB patients and healthy individuals were diluted in PBS-T (1:200, 1:400, 1: 600: 1:800, 1:1000 and 1:2000) for the standardization of optimum concentration. Finally 1:1000 dilutions of sera were used for the presented results. The PPD as a positive control and 1% BSA with PBS-T as a negative control were used. Plates were washed 3–4 times with PBS-T and secondary antibody rabbit anti-human IgG conjugated to HRP (Santa Cruz 1:10,000 dilution) was added for 1 h at room temperature. After washing, o-phenylenediamine

Table 3
Drug resistant and Susceptibility profile of patients.

S. No.	Name of drugs	No. of patients with sensitive strains (%)	No. of patients with resistant strains (%) n = 158
1	Isoniazid(INH)	148; (93.67%)	10; (06.32%)
2	Rifampicin(Rif)	146; (92.40%)	12; (07.59%)
3	Streptomycin(SM)	153; (96.20%)	05; (03.16%)
4	Ethambutol(EMB)	155; (98.10%)	03; (01.89%)
	Total	————	30; (18.98%)

Table 4
Categories of TB patients & their confirmation by different methods.

Groups of TB patients	Number of patients	Case history	PPD test	Radiology	AFB of sputum/other samples	PCR with *IS6110*	Culture of samples	MGIT-960 of samples	Total positive cases
Primary TB	60	60 +ve	60 +ve	40+ve	45+ve	51+ve	51+ve	51+ve	51+ve
MDR- TB	30	30+ve	30+ve	30+ve	30+ve	30+ve	30+ve	30+ve	30+ve
EPTB	34	34+ve	31+ve	20+ve	10+ve	29+ve	29+ve	29+ve	29+ve
Relapse TB	48	48+ve	48+ve	48+ve	48+ve	48+ve	48+ve	48+ve	48+ve
Healthy Cases	44	44-ve	44-ve	———	———	———	———	———	44-ve

Total cases = 172 ; (Positive cases = 158); Total Healthy cases = 44.

dihydrochloride (OPD) (Sigma) with 0.05% hydrogen peroxide in phosphate-citrate buffer, (pH 5.0), was added for colorimetric analyses. After 30 min, the reaction was stopped with 2 N H_2SO_4 (50 µl/well), and plates were read on a micro plate ELISA reader at 490 nm.

The results were expressed by ELISA index (EI), calculated by the formula: EI = S/(B + 3SD), where, S is the average optical density value of the duplicate test samples and B corresponds to the average optical density value of the duplicate negative controls and the SD is the standard deviation of duplicate negative controls. Sensitivity (i.e. the conditional probability of correctly identifying the diseased subjects by test) and specificity (i.e. the conditional probability of correctly identifying non-diseased subjects by test) for these antigens were determined using ROC analysis. The cut off was selected at the point located nearest to the left upper corner of the ROC curve Cartesian space, and it represented the best accuracy, sensitivity, and specificity by ROC analysis. A positive likelihood ratio (LR+) is the ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease. The negative likelihood ratio (LR−) is the ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease. The comparison between the groups was carried out through the non-parametric using MedCalc Statistical (Version 16.2.0). Statistical significance was set at 5% ($P < 0.05$).

4. Results

4.1. Patients

The study was carried out on selected 158 cases along with 44 healthy controls (Table 4). Their demographic profiles and other details are summarised in Tables 1 and 2. Among 158 cases 96 (60.75%) were male and remaining 62 (37.97%) were female. Majority of the patients came from urban area. None of the patients had HIV and Diabetes mellitus (Table 1). Most patients' excreted large numbers of bacilli in sputum (median score, 2.0). Out of 172 collected samples, 158 (91.86%) clinical isolates were cultures positive for mycobacteria. Contamination was found in 4 (02.32%), no growth was in 4 (02.32%) and growth of mycobacteria other than TB (MOTT) were found in 06 (03.48%) (Table 2).

DST for *Mtb* isolates was done with first line anti tuberculosis treatment (ATT) drugs in accordance with the standard procedures [28]. Thirty clinical isolates displayed phenotypic resistance to Rifampicin (RIF), Isoniazid (INH), Ethambutol (ETM) and Streptomycin (SM). Highest mono drug resistance (07.59%) was found in RIF followed by INH (06.32%) (Table 3).

4.2. Over-expression and purification of the proteins

The details of over expressed and purified recombinant proteins are summarized in Table S2 and selected recombinant proteins purified by Ni-NTA affinity chromatography on SDS gel are shown in Fig. 1 & figure S1.

4.3. Serological reaction

4.3.1. Antibody response of various groups of TB patients and control group to the various antigens

ELISA was carried out on 96-well flat-bottomed polystyrene microplates by slight modification of methods reported earlier. In our study, we identified the serodiagnostic potential of PPD, PE3, PE4, ESAT-6 andCFP-10, Ag85 A, Ag85 Band MycP1of *Mtb* by using ELISA technique. Recombinant purified proteins obtained were subjected to ELISA (Figs. 2, 3). PPD was used as positive control and blank wells served as negative controls (Figs. 2, 3). The antibody response to eight antigens was analyzed for well-characterized PTB and EPTB patients and healthy controls (Fig. 2). Significant levels of IgG antibodies against CFP-10, PE3 and PE4 antigens were found in sera of active TB patients when compared with the healthy control group (Fig. 2). Sera samples from different groups of TB patients and healthy individuals were diluted in PBS-T and its 100 µl /well were added in duplicate. The variations in Optical Density (OD) values were observed with Ag85 complex. Antibody binding values against Ag85 A was higher when compared to Ag85B & Ag85C in primary TB patients' population. The levels of MycP1 and Ag85C antigens were found to be very low and did not differ significantly between the studied groups (Fig. 2). All the selected antigens demonstrated high accuracy in discriminating between diseased verses non-diseased condition and showed better response in all groups of TB patients than in PPD (Figs. 2, 3).

Members of ESAT-6 family such as ESAT-6 and CFP-10 were found to have higher OD values than other antigens. ESAT-6 and CFP-10 have been the good markers of TB infection and are also the components of important vaccines (Fig. 2).

4.3.2. Comparative antigens level in TB patients

The OD values of studied antigens are summarized in Fig. 4a & b. PPD level was found to be very low in comparison to all recombinant secretory antigens. Samples from healthy control had the lowest reactivity with PPD. The results of ELISA with recombinant proteins showed that PE3 secretion level was much higher among all the eight secretory proteins. PE4 was found to be second highest to PE3 among all group of TB patients (Fig. 4a & b). It was followed byAg85B, ESAT-6, CFP-10 and Mycp1 respectively. Among Ag85 complex, the OD value of Ag85 A was found to be slightly higher than Ag85C. In new cases of TB group, the OD of Ag85 A was found much higher and almost equal to PE3 antigen; whereas level of Ag85C was very high and almost equal to ESAT-6, CFP-10, Ag85B and MycP1. The sequence from higher to lower level of variations in OD values in all groups of TB patients (PE3 > PE4 > Ag85B > ESAT-6 > CFP-10 > MycP1 > Ag85 A > Ag85C > PPD > healthy case) was established (Fig. 4a).

The group-wise observation of antigenic variation in TB patient from higher to lower for new cases of TB group was (PE3* > PE4 > Ag85A > Ag85B > ESAT-6 > Ag85C > CFP-10 > MycP1 > PPD): for MDR-TB group (PE3* > PE4 > Ag85B > ESAT-6 > MycP1 > Ag85C > CFP-10 > Ag85 A > PPD): for EPTB group (PE3* > PE4 > Ag85B > ESAT-6 > CFP-10 > MycP1 > Ag85 A > Ag85

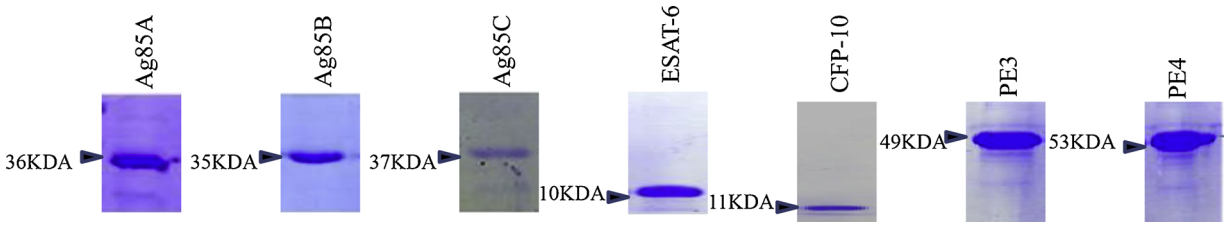

Fig. 1. Selected recombinant proteins purified by Ni-NTA affinity chromatography and analyzed on SDS-PAGE.

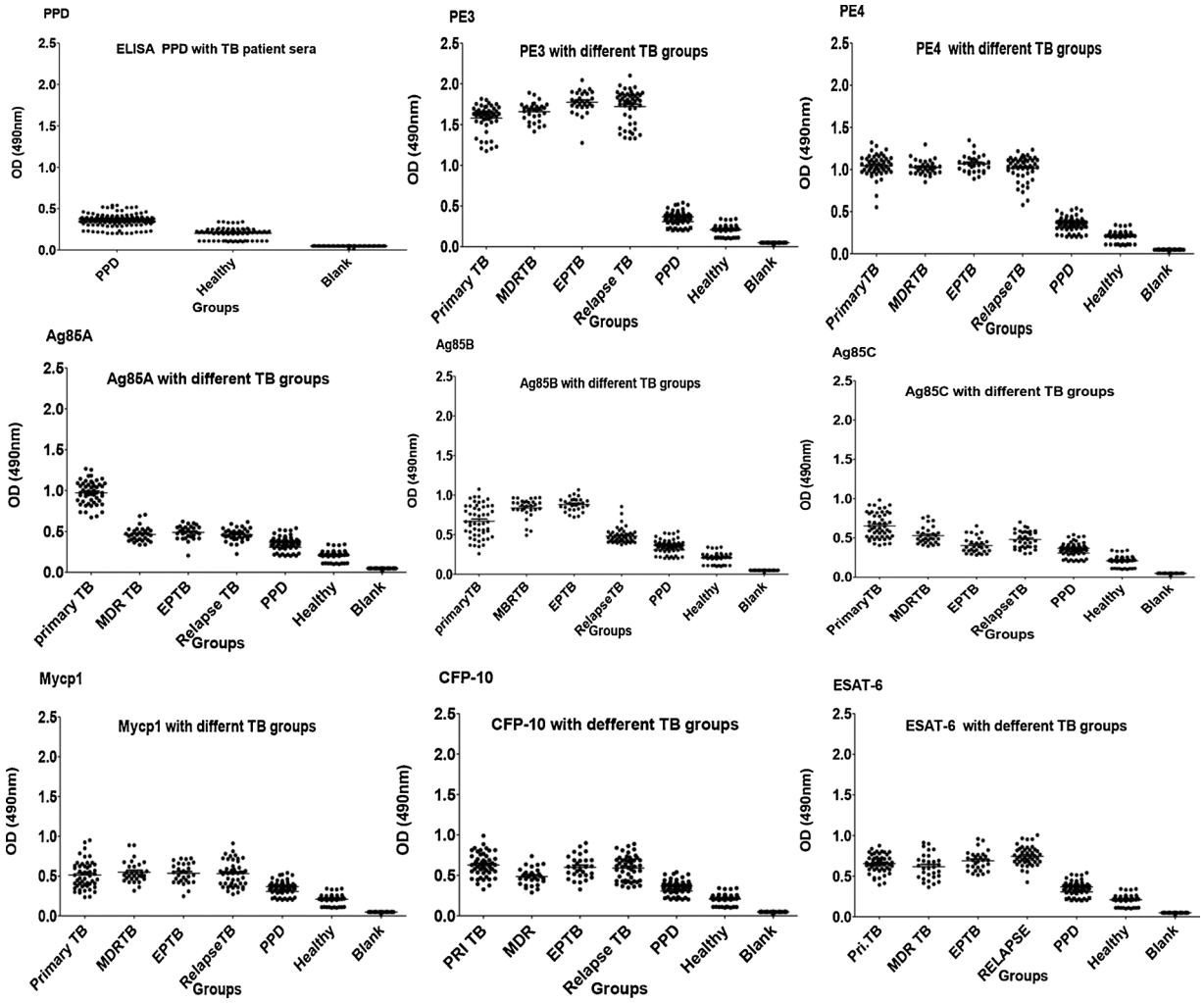

Fig. 2. Scatter Plot showing Sero-reactivity of the antibody IgG response to the recombinant antigens in various groups of TB patients and healthy individuals: PPD (As Standard); PE3; PE4; Ag85 A; Ag85B; Ag85C; Mycp1; CFP-10; ESAT-6. Each well was coated with 100 µg of antigens; 1:1000 sera dilution in PBST was used. Results are represented as ELISA index (EI); Statistical significance based on the differences between the healthy and patient groups is indicated as *$p < 0.05$. The bars indicate the medians.

C > PPD) and for Relapse TB cases group (PE3* > PE4 > ESAT-6 > CFP-10 > MycP1 > Ag85B > Ag85C > Ag85 A > PPD) (Fig. 4b). A very low serological response in some TB patients has been shown and the responses have been associated with the time of clinical onset of symptoms.

5. Discussion

TB is one of the oldest recorded highly prevalent microbial human infections. Its late diagnosis and treatment increase the risk of disease dissemination and further decreases the chances of survival of patients [2,10]. Serodiagnosis by ELISA has been widely explored over the years, in the diagnosis of TB. Despite rapid advances in molecular genetics for detection of mycobacteria, it is clear that interest in serodiagnosis remains high, especially for those situations in which a specimen may not contain the infecting agent in particular in EPTB cases. Immune response to excretory-secretory proteins of *Mtb* has been of diagnostic interest in TB [3,4,6,11,12].

Mycobacterium uses specialized type VII (ESX) secretion systems to export proteins across their complex cell walls. *Mtb* encodes five non-redundant ESX secretion systems, with ESX-1 being particularly important to disease progression. This ESX-5 system has been found to be important for granuloma formation, cell-to-cell spread of mycobacteria, and its substrates strongly modulate the human macrophage response [14,19]. All *esx* loci encode extracellular membrane bound proteases called mycosins (MycP) that are essential for secretion and have been shown to be involved in the processing of type VII exported proteins

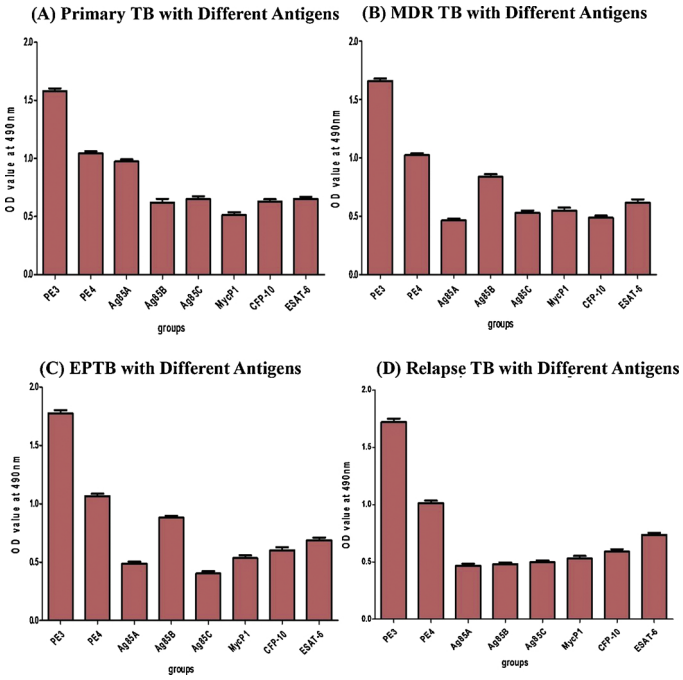

Fig. 3. Histogram showing antibody responses with selected purified antigens in various groups of TB patients; (A) Primary TB cases; (B) MDR- TB cases (C) EPTB cases (D) Relapsed cases; OD values at 490 nm; 10–15 OD = Maximum (+ + +); 05–10 OD = Moderate (+ +); 0- 5= Mild (+).

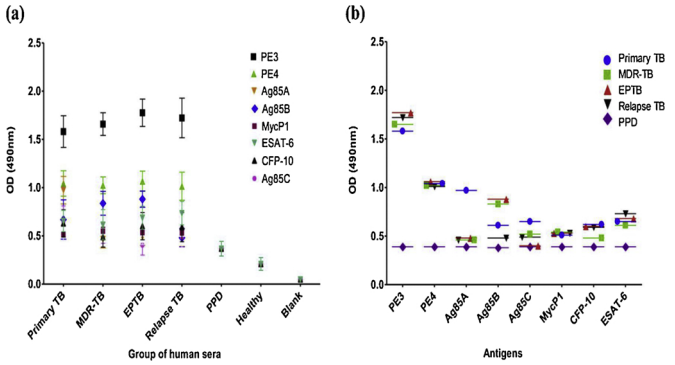

Fig. 4. Comparative Sero- reactivity against recombinant proteins from of *Mtb* $H_{37}Rv$, in various groups of active TB patients. Fig. 4a; Variations in OD values of antigens in various groups of TB patients; Fig. 4b; Level of selected recombinant proteins in various grades of TB category.

[14]. MycP1 is essential for ESX-1 secretion in pathogenic *Mtb* and DNA conjugation in the avirulent saprophyte *M. smegmatis*. The well-studied system is the ESX-1, which *Mtb* uses to secrete the T-cell antigens (ESAT-6/ CFP-10) in addition to other ESX secreted proteins (Esp) that are required for progression of disease [19]. A T7S system that has attracted a lot of attention in recent time is ESX-5, which is restricted to pathogenic mycobacterial species such as *Mtb, M. leprae, and M. marinum* [15].

The other T7SS of known function are involved in (ESX-3) export of the virulence-related PE/PPE family of proteins (ESX-5) [15,30,31]. Proteins exported by the T7SS have been detected in culture filtrates and in cell wall fractions as lower molecular weight/proteolytically processed species when compared to those which are not exported. *Mtb* secretes several highly immunogenic proteins across the cell wall using the ESX-1 transport system [14,19,28]. In the fight against TB, secretory proteins could be the potent weapons with their utilization in several different types of kits based on serodiagnosis, interferon-γ release assays (IGRA) and PCR [25,28,32–34].

Due to the increasing burden of MDR-TB and XDR-TB, the serological methods relying on the detection of circulating antibodies against *Mtb*-specific antigens are an effective diagnostic tool *in vitro* and would be an attractive progress, as immunoassays are simple, rapid, inexpensive and may offer the possibility to detect cases missed by standard sputum smear microscopy [32,33].

ESAT-6 and CFP-10, the secreted proteins from internalized mycobacteria, have been implicated in a range of functions including virulence, potent T-cell antigenicity and cytolysis. Among the major antigens of RD1 locus, ESAT-6 and CFP-10 are encoded by the genes *esxA* and *esxB* respectively. Both are the members of the ESX-5 system of *Mtb*, which is the most recent ESX system evolutionarily and is restricted to pathogenic mycobacteria like *Mtb.* ESAT-6 is the best characterized protein within the RD1 region [8,10,28,34]. This signifies ESAT-6 and CFP-10 secretion in mycobacterial virulence and the importance of these antigens as diagnostic tools for the detection of TB [24,26,30]. ESAT-6 and CFP-10 are also components of QuantiFERON-TB Gold-In Tube and T-SPOT TB, the IGRA based diagnostic kits, highlighting the importance of this family in vaccine development as well as in TB diagnosis [35–37]. Several other members of the ESAT-family also elicit strong humoral and cellular immune responses. Overlapping peptides of ESAT-6 and CFP-10 offer increased specificity over the PPD skin test when they were used in ELISA assay for the diagnosis [38,39].

Since the 1880s, laboratory diagnosis of TB has relied primarily on microscopy and culture. *Mtb* PE_PGRS family has been widely proposed as molecular mantra against deflect host immunity. PE/PPE proteins are present in both pathogenic and saprophytic mycobacteria. There is a hypothesis that PE/PPE proteins are a source of antigenic variation which causes the bacteria to escape host immune response [13,22,24]. There is some information about the biological role of PPE and PE proteins in host–bacteria interactions such as efficiency of phagocytosis, mycobacterial growth in macrophages, inhibition of phagosome maturation, and virulence. They can also induce T and B cell immune response because of potentially rich source of B and T cell epitopes. Some evidences show that these family members are cell wall-associated and they may have a role in modifying, the host immune response [22–24,36,37].

This has led to the development of highly specific immune responses that prime the infected cells to become efficient mycobacterial killers.

Various studies have been undertaken to develop serodiagnostic tests using proteins of *Mtb* which are known to be immunodominant and early markers for TB [10,37–40]. The data reveal that the sera from the new TB patients mounted comparatively higher antibody responses against PE3 than those of healthy controls (Fig. 3a & b) [16]. The antigens selected for our study have been earlier reported to be the strong targets for human B- and T-cell responses [23,33–36].

ESAT-6, CFP-10 are members of the ESX-5 system of *Mtb*, which is the most recent ESX system evolutionarily and is restricted to pathogenic mycobacteria like *Mtb*. Overlapping peptides of ESAT-6 and CFP-10 offer increased specificity over the PPD skin test when they were used in ELISA assay for the diagnosis [16,31,35]. These recombinant proteins offer a realistic and cost effective alternative to PPD and due to higher sensitivity and specificity recombinant ESAT-6, CFP-10 and Ag85 A can be better candidates for TB detection when compared with conventional diagnostic methods. Immune responses to CFP-10, ESAT-6, Ag85 A, Ag85B, Ag85C, PE3, PE4 and Mycp1 have been shown to be significantly higher in active pulmonary and extrapulmonary TB than PPD (Figs. 2, 3, 4a & b; Figure S2 a & b) [16,18,32,36,38]. Several studies have detected antibodies in sera of patients with active TB against a variety of *Mtb* antigens [16,33,38,39]. Since Ag85 A and Ag85B share around 77% of amino acids identity, one could expect them to have common immunodominant epitopes Ag85 A and Ag85B also show strong humoral and cellular responses, based on which they have been selected for subunit vaccines [38–40]. Furthermore, the Ag85 A and Ag85B proteins were also recognized in PBMC of PTB patients and individuals undergoing treatment; measured by elevated proliferation and IFN-γ production [41–46]. A significantly higher response was shown with smear and culture positive PTB than culture negative patients. It was in accordance with Broger et al [44]. Patients with active disease presented high levels of IgG against CFP-10, ESAT-6 and PE3 (Fig. 4 a; Figure S2 a & b). Many studies have detected antibodies in sera of patients with active TB against a variety of *Mtb* antigens reporting varying degrees of sensitivity and specificity [47–50].

6. Conclusion

In the present study, antibody responses to recombinant CFP-10, ESAT-6, Ag85 A, Ag85B, Ag85C and Mycp1were investigated in Indian patients with active PTB & EPTB and were compared to healthy individuals. These results indicate that *Mtb* actively produces and secretes various antigens during the active stage of TB. The TB infection continues to be transmitted at unacceptably higher rates partly due to delayed diagnosis which is also associated with a range of various factors. Several studies have reported prominent levels of PE3, PE4, and have also found that PE3 is highly expressed during active human lung infection. It can further be incorporated with other potent *Mtb* antigens to improve their accuracy for serodiagnosis. Overall, our study suggests that PE3 and PE4 are immune-dominant and stage specific antigens that could be used for diagnosis of *Mtb*. The outcomes resulting from the investigation of these unique proteins are certain to improve the understanding of pathogenesis of *Mtb* and also for development of new vaccines and new therapeutic agents for controlling and treating mycobacterial infections.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.03.010.

References

[1] World Health Organization, Global Tuberculosis Report, Available from: World Health Organization, Geneva, 2016http://www.who.int/tb/publications/globalreport/en/2016.
[2] R. Prasad, N. Gupta, A. Banka, Multi drug resistant tuberculosis/rifampicin-resistant tuberculosis: principles of management, Lung India 35 (2018) 78–81.
[3] K.R. Steingart, L.L. Flores, N. Dendukuri, I. Schiller, et al., Commercial serological tests for the diagnosis of active pulmonary and extrapulmonary tuberculosis: An updated systematic review and Meta analysis, PLoS Med. 8 (2011) e1001062.
[4] P. Waghmare, G. Wankhade, A. Mazumdar, K. Wandile, et al., SEVA TB ELISA Multi antigen and antibody assays for serodiagnosis of suspected cases of pulmonary and extra pulmonary tuberculosis in tertiary care hospital – A retrospective study, Asian Pac. J. Trop. Dis. 2 (2012) S827 32.
[5] World Health Organization, 2013 Policy Update: Xpert MTB/RIF Assays for the Diagnosis of Pulmonary and Extrapulmonary TB in Adults and Children. WHO/HTM/TB/2013.6, WHO, Geneva, Switzerland, 2013.
[6] P. Waghmare, G. Wankhade, L. Jena, B.C. Harinath, Excretory secretory proteins released during growth of *Mycobacterium tuberculosis* (H37Ra), with diagnostic potential in pulmonary and extra pulmonary tuberculosis, Mycobact. Dis. 6 (2016) 215–221.
[7] S. Arloing, Agglutination de bacille de la tuberculosevrate, C. R. Acad. Sci. 126 (1898) 1398–1400.
[8] D. Lawn, P. Mwaba, M. Bates, A. Piatek, et al., Advances in tuberculosis diagnostics: the Xpert MTB/RIF assay and future prospects for a point-of-care test, Lancet Infec Dis. 1 (2013) 349–361.
[9] H. Pandey, F. Fatma, S.M. Yabaji, M. Kumari, et al., Biophysical and immunological characterization of the ESX-4 system ESAT-6 family proteins Rv3444c and Rv3445c from *Mycobacterium tuberculosis* H37Rv, Tuberculosis 109 (2018) 85–96.
[10] B.C. Harinath, Tuberculosis serodiagnostics: Ban and after, Int. J. Mycobacteriol. 6 (2017) 323–325.
[11] A. Singh, A.K. Gupta, K. Gopinath, P. Sharma, et al., Evaluation of 5 Novel protein biomarkers for the rapid diagnosis of pulmonary and extra-pulmonary tuberculosis: preliminary results, Sci. Rep. 7 (2017) 44121.
[12] P. Brodin, M.I. de Jonge, L. Majlessi, C. Leclerc, M. Nilges, S.T. Cole, Functional analysis of early secreted antigenic target-6, the dominant T-cell antigen of *Mycobacterium tuberculosis*, reveals key residues involved in secretion, complex formation, virulence, and immunogenicity, J. Biol. Chem. 280 (2005) 33953–33959.
[13] P. Brodin, L. Majlessi, L. Marsollier, M.I. de Jonge, D. Bottai, Demangel, et al., Dissection of ESAT-6 system 1 of Mycobacterium tuberculosis and impact on immunogenicity and virulence, Infect. Immun. 74 (2006) 88–98.
[14] A.M. Abdallah, J. Bestebroer, N.D. Savage, P.K. de, et al., Mycobacterial secretion systems ESX-1 and ESX-5 play distinct roles in host cell death and inflammasome activation, J. Immunol. 187 (2011) 4744–4753.
[15] VanPittiusN.C. Gey, S.L. Sampson, H. Lee, Y. Kim, et al., Evolution and expansion of the Mycobacterium tuberculosis PE and PPE multigene families and their association with the duplication of the ESAT-6 (esx) gene cluster regions, BMC Evol. Biol. 6 (2006) 95–98 15.
[16] B. Lechartier, J. Rybniker, A. Zumla, S.T. Cole, Tuberculosis drug discovery in the post post-genomic era, EMBO Mol. Med. 6 (2014) 158–168.
[17] M. Solomonson, F.H. Pitter, A.W. Gregory, W. Nobuhiko, J.G. Robert, et al., 2013. Structure of the mycosin-1 protease from the mycobacterial ESX-1 protein type VII secretion system, J. Biol. Chem. 288 (2013) 17782–17790.
[18] R.L. Skjot, T. Oettinger, I. Rosenkrands, P. Ravn, et al., Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens, Infect. Immun. 68 (2000) 214–220.
[19] Y. Bai, Y. Xue, H. Gao, L. Wang, T. Ding, et al., Expression and purification of *Mycobacterium tuberculosis* ESAT-6 and MPT64 fusion protein and its immunoprophylactic potential in mouse model, Protein Expr. Purif. 59 (2008) 189–196.
[20] A.M. Abdallah, N.D. Savage, Z.M. van, L. Wilson, et al., The ESX-5 secretion system of *Mycobacterium marinum* modulates the macrophage response, J. Immunol. 181 (2008) 7166–7175.
[21] M.E. Feltcher, J.T. Sullivan, M. Braunstein, Protein export systems of *Mycobacterium tuberculosis*: novel targets for drug development? Future Microbiol. 5 (2010) 1581–1597.
[22] J. Campuzano, D. Aguilar, K. Arriaga, J.C. Leon, et al., The PGRS domain of *Mycobacterium tuberculosis* PE-PGRS Rv1759c antigen is an efficient subunit vaccine to prevent reactivation in a murine model of chronic tuberculosis, Vaccine. 25 (2007) 3722–3729.
[23] S.K. Singh, R. Kumari, D.K. Singh, S. Tiwari, et al., Putative roles of a proline-glutamic acid-rich protein (PE3) in intracellular survival and as a candidate for subunit vaccine against Mycobacterium tuberculosis, Med. Microbiol. Immunol. 202 (2013) 365–377.
[24] N. Khan, K. Alam, S. Nair, V.L. Valluri, et al., Association of strong immune responses to PPE protein Rv1168c with active tuberculosis, Clin. Vaccine Immunol. 15 (2008) 974–980.

[25] T. Matsumoto, T. Yamazaki, Kekkaku, The evaluation of the utility of Quanti FERON TB-Gold In-Tube, QFT-GIT 89 (2014) 743–755.
[26] A.K. Luabeya, B.M. Kagina, M.D. Tameris, H. Geldenhuys, et al., First-in-human trial of the post-exposure tuberculosis vaccine H56:IC31 in *Mycobacterium tuberculosis* infected and non-infected healthy adults, Vaccine 33 (2015) 4130–4140.
[27] S.M. Yabaji, A.K. Mishra, A. Chatterjee, R.K. Dubey, et al., Peroxiredoxin-1 of macrophage is critical for mycobacterial infection and is controlled by early secretory antigenic target protein through the activation of p38 MAPK, Biochem. Biophys. Res. Commun. 494 (2017) 433–439.
[28] P.T. Kent, G.P. Kubica, Public Health Mycobacteriology: a Guide for the Level III Laboratory, Centers for Disease Control and Prevention, Atlanta, GA, 1985, pp. 125–129. Tuberculosis. Ind J Tuberc. 54.
[29] E.B. Hill, L.G. Wayne, M. Gross, Purification of mycobacterial deoxyribonucleic acid, J. Bacteriol. 112 (1972) 1033–1039.
[30] H. Pandey, S. Tripathi, K. Srivastava, D.K. Tripathi, S. Kant, K.K. Srivastava, A. Arora, Characterization of Rv3444c and Rv3445c: The ESAT-6 Family Members from the Most Primitive ESX-4 System of*Mycobacterium tuberculosis* H37Rv, BBA – Gen. Subj. 1861 (2) (2017) 396–408.
[31] A.F. Carey, J.M. Rock, I.V. Krieger, M.R. Chase, M. Fernandez-Suarez, J.C. Gagneux S Sacchettini, T.R. Ioerger, S.M. Fortune, et al., TnSeq of *Mycobacterium tuberculosis* clinical isolates reveals strain-specific antibiotic liabilities, PLoS Pathog. 14 (2018) e1006939.
[32] N.N. Chegou, K.G. Hoek, M. Kriel, R.M. Warren, Tuberculosis assays: past, present and future, Expert Rev. Anti. Ther. 9 (2011) 457–46941.
[33] A. Majumdar, P.D. Kamble, B.C. Harinath, Detection of circulating free and immune-complexed antigen in pulmonary tuberculosis using cocktail of antibodies to *M. tuberculosis* excretory secretory antigens by peroxidase enzyme immunoassay, Ind. J. Tuberc. 57 (2010) 67–74.
[34] B. Samten, S. Fannin, K. Sarva, Yi Na, et al., Modulation of human T cell cytokines by the *Mycobacterium tuberculosis*-secreted protein Wag31, Tuberculosis 101 (2016) 99–104.
[35] A. Marei, A. Ghaemmaghami, P. Renshaw, M. Wiselka, et al., Superior T cell activation by ESAT-6 as compared with the ESAT-6-CFP-10 complex, Int. Immunol. 17 (2005) 1439–1446.
[36] T. Ulrichs, M.E. Munk, H. Mollenkopf, S. Behr-Perst, et al., Differential T cell responses to *Mycobacterium tuberculosis* ESAT6 in tuberculosis patients and healthy donors, Eur. J. Immunol. 28 (1998) 3949–3958.
[37] K.R. Steingart, N. Dendukuri, M. Henry, I. Schiller, et al., Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a metaanalysis, Clin. Vaccine Immunol. 16 (2009) 260–276.
[38] X.Y. He, J. Li, J. Hao, H.B. Chen, et al., Assessment of five antigens from *Mycobacterium tuberculosis* for serodiagnosis of tuberculosis, Clin. Vaccine Immunol. 18 (2011) 565–570.
[39] H.C. Teixeira, C. Abramo, M.E. Munk, Immunological diagnosis of tuberculosis: problems and strategies for success, J. Bras. Pneumol. 33 (2007) 323–334.
[40] B.L. Wang, Y. Xu, C.Q. Wu, Y.M. Xu, et al., Cloning, expression, and refolding of a secretory protein ESAT-6 of Mycobacterium tuberculosis, Pro Express Purif. 39 (2005) 184–188.
[41] O. Parkash, B.P. Singh, M. Pai, Regions of differences encoded antigens as targets for Immunodiagnosis of tuberculosis in humans, Scand. J. Immunol. 70 (2009) 345–357.
[42] S. Mahmoudi, S.M.M. Ghazi, R.H. Sadeghi, B. Pourakbari, Cloning, expression and purification of *Mycobacterium tuberculosis* ESAT-6 and CFP-10 antigens, Iran. J. Microbiol. 5 (2013) 374-37.
[43] J. Weiner, S.H. Kaufmann III, Recent advances towards tuberculosis control: vaccines and biomarkers, J. Intern. Med. 275 (2014) 467–480.
[44] T. Broger, R.B. Roy, A. Filomena, C.H. Greef, S. Rimmele, et al., Diagnostic performance of tuberculosis-specific IgG antibody profiles in patients with presumptive TB from two continents, Clin. Infect. Dis. 64 (2017) 947–955.
[45] S. Velayudhan, S.A. Porcelli, Recent advances in defining the immunoproteome of Mycobacterium tuberculosis, Front. Immunol. 4 (2013), https://doi.org/10.3389/fimmu.2013.00335.
[46] G. Delogu, M.J. Brennan, Comparative immune response to PE and PE_PGRS antigens of Mycobacterium tuberculosis, Infect. Immun. 69 (2001) 5606–5611.
[47] M. Romano, L. Rindi, H. Korf, D. Bonanni, P.Y. Adnet, et al., Immunogenicity and protective efficacy of tuberculosis subunit vaccines expressing PPE44 (Rv2770c), Vaccine 26 (2008) 6053–6063.
[48] A.M. Abdallah, T. Verboom, E.M. Weerdenburg, N.C. Gey van Pittius, P.W. Mahasha, et al., PPE and PE_PGRS proteins of Mycobacterium marinum are transported via the type VII secretion system ESX-5, Mol. Microbiol. 73 (2009) 329–340.
[49] A. Jones, M. Pitts, Al Dulayymi JR., J. Gibbons, A. Ramsay, D. Goletti, et al., New synthetic lipid antigens for rapid serological diagnosis of tuberculosis, PLoS One 12 (2017) e0181414.
[50] D.B. Young, H.P. Gideon, R.J. Wilkinson, Eliminating latent tuberculosis, Trends Microbiol. 17 (2009) 183–188.