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Expression and Purification of *Mycobacterium tuberculosis* Antigens for Use in Immunoassays for Serodetection of *M. tuberculosis* Infection in TB Patients

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Abstract.- Tuberculosis (TB) is a fatal and contagious disease. The annual death toll occurring from TB is approximately 2 million according to World Health Organization (WHO). The removal of disease from global face needs immediate treatment for which early diagnosis is pre-requisite. Existing tests for the diagnosis of TB are not efficient and robust. In the present study *Mycobacterium tuberculosis* specific six antigens namely cfp-10, esat-6 and hspx, along with three antigens which are components of immunodominant mycolyl transferases ag85a, ag85b, ag85c were expressed and purified to evaluate their potential use in immunoassays like Western blotting and multiplex microbead immunoassay. Protein expression of all six antigenic genes was optimized for time and different concentrations of inducer isopropyl β-D-1-thiogalactopyranoside. Protein products were confirmed by Western blotting and purified through immobilized metal affinity chromatography (IMAC) technique using columns having affinity for His-tag. Each fluorescently labeled set of microbeads were coated with one of the *M. tuberculosis* specific antigenic proteins and later on human plasma samples of reactivated TB patients along with healthy BCG as well as tuberculin skin test negative controls were tested for presence of antibodies against these antigenic proteins individually in a multiplex format. The results were generated in median fluorescence intensity form which detected antibodies against *M. tuberculosis* specific antigenic proteins only in reactivated TB patients. This system detected antibodies against four antigenic proteins in 100% of reactivated TB patients. Thus, *M. tuberculosis* antigens described in this study seem to have purified at the level to be used in the development of immunoassays for the detection of *M. tuberculosis* infection in TB patients of different categories like active and latent TB.

Key words: Tuberculosis, *M. tuberculosis*, antigens, antibodies, multiplex microbead, immunoassay, median fluorescence intensity, MMIA

INTRODUCTION

Approximately one third of the world's population is already infected with the *Mycobacterium tuberculosis* and the number of new tuberculosis (TB) cases arising each year is nearly 2 million. World Health Organization ranks Pakistan at 8th in high burden TB countries (WHO, 2009). The coughing and sneezing of patients causes spread of disease due to generation of infectious aerosols. This route of infection in human population emphasizes on early diagnosis of TB to eradicate and limit the disease. The unavailability of accurate, fast and efficient diagnostic methods for TB causes increase in the number of patients thus limiting control of disease (Perkins *et al*., 2006). In this prevailing condition it is highly critical to identify infected individuals and screen the immediate contacts for starting drug administration in order to control spread of TB.

The ideal test for TB diagnosis must be able to detect early infection with high sensitivity and specificity, must generate results rapidly, and must be inexpensive and causing little or no patient's discomfort. At least one of the above mentioned requirements are not fulfilled by the presently available diagnostic tests including sputum smear microscopic examination, culturing of sputum

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samples, PCR-based diagnosis, lipoarabinomannan detection in sera, Mantoux test and chest X-ray. An attractive solution to this problem presented by Dillon *et al.* (2000) is the detection of *M. tuberculosis* specific antibody in patient sera, as serodiagnostic test satisfies all requirements mentioned above. Therefore, a number of efforts were made to clone, express and purify immunogenic TB specific antigens to be used for immunoassays.

In this connection, Xie *et al.* (2002) cloned immunodominant antigen ag85a into plasmid pBK-CMV, afterwards recombinant shuttle was transformed into *Escherichia coli* (*E. coli*) XL1-blue MRF and IPTG was used as inducer for expression of protein. Stable expression of protein with molecular weight 32 kDa was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Xu *et al.* (2004) used polymerase chain reaction (PCR) to amplify 14 kDa, 37 kDa and esat-6 antigenic genes from genomic DNA. The amplified product was then cloned into pGEX 4T-1 expression vector. The recombinant plasmid was transformed into Bl21 strain and induced for expression giving good level of induction. Purification of recombinant protein was also done successfully by affinity chromatography.

Similarly, Boucau *et al*. (2009) amplified ag85c lacking stop codon with specific primers through PCR and cloned in pET-29, producing 6X His tag at C-terminus of ag85c. T7 expression cells were used to transform expression plasmid pET29-ag85c and cultured in terrific broth. Expression was induced with 1 mM Isopropyl thiogalactoside (IPTG) and expressed protein was purified by immobilized metal affinity chromatography (IMAC) using HisTrap column followed by ion exchange chromatography and size exclusion chromatography on a Sephadex 200.

In a study carried out by Shi *et al*. (2010) *M. tuberculosis* H37Rv genomic DNA was used to amplify the open reading frames (ORFs) of Rv2031c and fbpB by PCR. The PCR products were cloned into pProExHTb vector, and positive clones were confirmed by restriction analysis and sequencing. Both the hspx and ag85b were expressed with His tag at N-terminus by induction with 0.4 mM IPTG. The expressed recombinant protein was analyzed on SDS-PAGE after purification by Nickle-nitrilo tritilic acid (Ni-NTA) column chromatography and further confirmation was done through Western blotting.

Cfp-10, a 10-kDa protein, was cloned and expressed as a fusion protein in soluble form to be available for diagnostic purpose (Yousefi *et al*., 2009). *M. tuberculosis* H37Rv was used to extract DNA and PCR amplified products obtained by specific primers were confirmed and cloned into pET102/D expression vector, again confirmed by sequencing. This expression vector initially transformed in *E. coli* TOP10 and later in *E. coli* Bl21 expression strain for production of recombinant protein. IMAC was used for purification of soluble protein and expression of 28 kDa fusion protein was checked by SDS-PAGE. The 28kDa fusion protein consisted of CFP-10 core protein fused to a C-terminus His-tag of 3 kDa and an N-terminus His patch thioredoxin (13 kDa). N-terminus thioredoxin patch is utilized in pET 102/D vector for expression in soluble form.

M. tuberculosis H37Rv genomic DNA was also utilized for amplification of esat-6 gene (288 nucleotides in length) with specific primers by PCR (Farshadzadeh *et al*., 2010). pET102/D expression vector was used to insert purified PCR product and *E. coli* TOP10 strain used for transformation of recombinant plasmid. After extraction recombinant plasmids were transformed in expression strain *E. coli* Bl21. IPTG was used to induce expression, Ni-NTA agarose column to purify and SDS-PAGE to analyze the protein. Although ESAT-6 is a 6 kDa protein but was expressed at about 22 kDa reason being a His patch thioredoxin at N-terminus of size 13 kDa and 6X His tag plus v5 epitope at C-terminus of 3 kDa.

In 1976 Nassau *et al.* used enzyme linked immunosorbent assay (ELISA) for the first time for serodiagnosis of TB which provided 57 % sensitivity and 98 % specificity. Thereafter, a number of semipurified, purified and recombinant antigens were used in ELISA based TB detection in different studies. However, 38-kDa antigen was used most frequently and the sensitivity of the assays incorporating this antigen ranged from 16-80%, and was dependent upon the smear status of

patient alongwith population used (Weldingh *et al*., 2005). Uptil now there is not a single antigen available which alone has sufficient sensitivity and specificity to be used in a serodiagnostic potential. Besides, diagnostic tests using antibody detection are the better option but the only drawback is that all individuals infected with *M. tuberculosis* may not have antibodies to any single antigen used in the test because of different stages of TB prevailing in different patients (Lyashchenko *et al*., 1998). Therefore, a multi-antigen print immunoassay (MAPIA) was reported by Lyashchenko *et al*. (2000) for infectious diseases in humans. Then in 2007, the same researchers presented Prima TB STAT-PAK assay, a lateral flow test for TB detection in non-human primates. Both these tests were used to generate profiles of *M. tuberculosis* antibodies by printing multiple antigens on a nitrocellulose membrane through process of microaerosolization. Khan *et al*. (2008), however, used multiplex microbead immunoassay (MMIA) for profiling anti *M. tuberculosis* antibodies in non-human primates. Recently, Kumar *et al*. (2010) used a cocktail of CFP-10, AG85 complex, AG85A, AG85B and AG85C rather than using any single antigen in ELISA but did not get better results in terms of either sensitivity or specificity.

Based on these facts, this study was planned for over-expression of selected *M. tuberculosis* specific immunogenic antigens in bacterial expression system like pET, their purification by metal affinity chromatography utilizing His-tag, analysis and confirmation by SDS-PAGE and Western blotting respectively and then testing of efficiency of the system of expression and purification by immunoassay like MMIA and also possible use of selected proteins in detection of TB in human population.

We report here over expression of six *M. tuberculosis* specific antigens, their purification and potential use in development of MMIA for the detection of *M. tuberculosis* infection in TB patients.

MATERIALS AND METHODS

Expression of antigenic genes

Six plasmids *i.e.*, ag85a, ag85b, ag85c, cfp-10, esat-6 and hspx alongwith His-tag were obtained from TB Resource Centre, Colorado State University, Boulder, CO., USA. These were transformed into expression strain Bl21DE3pLysS (Invitrogen, USA) and expression of each gene was obtained under optimized conditions considering IPTG concentration, time and temperature of incubation. The culture was divided into aliquots and those were induced with different concentrations of IPTG from 0.1- 0.8 mM. After getting optimal expression of each of six antigenic proteins which were different in each case, cultures were induced at optimum IPTG concentration for different time durations like 0-8 hrs with one hour intervals. Later in experiments optimized concentration of IPTG and time was used to over-express proteins. The protein expression was confirmed by SDS-PAGE and Western blotting. The cellular localization of over-expressed proteins was checked by pelleting down 50 ml bacterial culture, resuspension in 1X binding buffer and sonication. The proteins accumulating in inclusion bodies at 37°C were induced at 24°C to get maximum amount of over-expressed proteins in soluble form. All six antigenic genes were then expressed in bulk using specific IPTG concentration, time and temperature determined separately for each antigenic gene and then expressions of all genes were reconfirmed by SDS-PAGE and Western blotting.

Purification of antigenic proteins

Affinity chromatography was used to purify His-tagged expressed proteins by using His binding resin obtained from Novagen (USA) following protocol provided by Supplier. The binding buffer was added in the pellet obtained from bulk culture. Then lysozyme, DNase and protease inhibitors were added in this mixture subsequently. The mixture was incubated at 37 °C for 15 minutes to aid in the lysozyme activity. Sonication was done at 40% power with 10 second pulse on and 20 second pulse off for a total 5 minutes. Centrifugation was used to remove cell debris and DNA. Supernatant was checked for presence of target over-expressed protein by running sample on SDS-PAGE. After estimation of total protein in sonicated supernatant by bicinchoninic acid assay (BCA), and estimation of target protein was done by comparison of its ratio with the total protein as seen from SDS-PAGE. The

lysate was filtered through 0.22um syringe filter to clear the lysate and prevent clogging of the purification column. One ml pre-charged His-bind resin was added to column and allowed to settle for 30-40 minutes. After this storage buffer was allowed to flow through. Column was washed with 3 ml nanopure water and for equilibration, 4 ml 1X binding buffer was allowed to flow through. Then filtered sonicated supernatant was loaded on to column and allowed to flow through. The wash buffer was added to the column for removing any nonspecific bound protein and allowed to pass through. Thereafter elution buffer was used to elute target protein. All fractions collected at each step i.e. flow through of sonicated supernatant, 1X binding buffer, 1X wash buffer and five elution fractions of 1X elution buffer each of ~1 ml were finally run on SDS-PAGE to check the steps of purification. The Western blotting was also performed to confirm the protein by using antibodies against His tag at N- and C-terminus of expressed proteins. For this purpose 5 ul of purified protein fractions 1, 2, 3, 4, Bl21DE3pLysS cells without plasmid (as negative control in Western blotting) alongwith molecular weight marker were electrophorased on SDS-PAGE. Over night transfer was done onto polyvinyl diflouride (PVDF) membrane in transfer buffer after soaking membrane for 1-2 minutes in methanol and then for 5 minutes in transfer buffer. After taking out PVDF membrane, blocking was done for 1 hr with skim milk powder and washed thrice with wash buffer i.e. phosphate buffer saline - Tween 20 (PBS-T). Incubation in primary antibody, which was anti-His antibody at 1:1000 dilutions, was done for 1 hr, membrane washed three times with wash buffer for 10 minute. Then incubated with secondary antibody (anti rabbit conjugated to horseradish peroxidase) for I hr. thereafter the membrane was washed and incubated for five minute in Amersham ECL plus Western blotting detection reagent. Subsequently membrane was dried on filter paper and photograph taken at fluorescent mode in typhoon scanner (GE Life Sciences, USA). The purified proteins were then dialyzed in PBS, pH 7.4 and used for bead coating to carry out MMIA.

Plasma samples collection

Plasma samples from human TB patients with positive sputum smear for acid fast bacilli (AFB) were collected from Federal TB Center Rawalpindi, Punjab, Pakistan. An approved protocol by the Ethics Committee of Pir Mehr Ali Shah Arid Agriculture University Rawalpindi (PMAS-AAUR) was followed in collection of samples. Healthy individuals were Bacillus Calmette-Guerin (BCG-ve) and tuberculin skin test negative (TST-ve). Total blood samples collected in this group were fifteen and diseased group sample comprised of five patients with reactivated TB disease. Three ml blood was drawn and immediately put into ethylene diamino tetra acetic acid (EDTA) coated tubes (Cat # 367835, BD Diagnostics, USA) and mixed several times by inverting tube. Later blood was centrifuged at 3000 rpm for 10 minutes to pellet down blood cells. Plasma was collected and aliquoted into 5 cell culture tubes 200 µl in each.

Activation of microbeads and coupling antigens to microbeads

Microbeads were purchased from Luminex Corporation (Austin, TX). All six purified antigenic proteins were chemically cross linked to microbeads following the protocol essentially described by Khan *et al.* (2006). Bead stock was resuspended by vortexing and sonicating in water bath sonicator for 15 to 30 sec (Branson 1510, Danbury, CT). Then 250 µl containing ~ $2.5x10^6$ beads was put in micro centrifuge tube (Cat # 1415-2500, USA Scientific, USA) and centrifuged at 21,000xg for 2 min. For cross-linking of proteins to beads, activation of the beads was done, after washing beads with nanopure water by adding 80 µl of activation buffer (100 mM monobasic sodium phosphate, pH 6.3) and mixing through vortexing and sonicating. Then 10 ul of 50 mg/ml sulfo-N-hydroxysulfosuccinamide (NHS; Pierce, Rockford, IL) was added and mixing done by vortexing. Later 10 ul of 50 mg/ml 1-etyhl-3- [3-dimethylaminopropyl] carbodiimide (EDC; Pierce, Rockford, IL) was added and again mixing was done by vortexing. All incubations were carried out in the dark. This mixture was then shifted to rotator shaker at room temperature for 20 min and centrifuged at 21,000xg for 2 min. The pelleted microspheres were washed twice with PBS, pH 7.4. The antigenic protein to be coated was centrifuged at 10,000 rpm to pellet down any salts and diluted

the protein in PBS in the desired concentration. Pelleted beads were resuspended for coating in the relevant antigen which has already been diluted in PBS buffer. Optimization of antigen coating concentration was performed by coating different microbead sets at different concentrations with same protein *i.e.*, 3 µg, 6 µg, 25 µg and 100 µg. The optimized concentration for coating of each antigen was different: for ag85a25 µg/ml; for ag85b12.5 µg/ml; for ag85c 25 µg/ml; for cfp-10 100 µg/ml; for esat-6 6.25 µg/ml; for hspx 6.25 µg/ml. For coupling, the activated beads and protein mixtures were incubated by shaking on a rotator for 2 hr at room temperature. The coating beads were then washed twice with 250 µl wash buffer (0.1% Tween-20 in PBS, pH 7.4), resuspended in 250 µl of blocking buffer (1 % BSA, 0.1 % Tween-20 in PBS, pH 7.4, 0.05 % sodium azide), and shaken on rotator for 30 min at room temperature. The antigen coated micro beads were then washed once with 1 ml blocking buffer, resuspended in 1ml blocking buffer and stored at 4°C for up to a week. For longer storage time, beads were kept frozen at -70°C for months.

Microbeads sets were also coated with BSA at 100 µg/ml (Pierce, Rockford, IL) and cell lysate at 100 µg/ml concentration to be used as negative controls in the multiplex assay, whereas R-phycoerythrin conjugated goat anti human IgG (Cat # 109-116-098, Jackson Immunoresearch Laboratories Inc.) at 20 µg/ml coated bead set was used as positive control for reporter molecule with streptavidin conjugated to R-phycoerythrin.

Multiplex microbead immunoassay (*MMIA*)

Multiplex microbead immunoassay was performed in 96-well, filter bottom plates designed for high throughput separations (1.2 µm Multiscreen, Millipore Corporation, Bedford, MA). The number of beads for each bead set coated with a specific antigen was 2,000 per well. For example, for a nine-plex assay, 2,000 beads of each of nine sets, coated with optimized concentration of one antigen, were mixed to get a total of 18,000 beads per well. Human plasma samples were diluted to 1:100 pre-plate in 2% Prionex (Cat # 24621-100, Polysciences Inc, USA) in the dilution cups and 50 µl of this diluted plasma was mixed with nine-plex bead mixture per well. The 96-well plate containing mixture was then incubated on a shaker for 1 hr at room temperature. After incubation, liquid was drained from plate bottom in a vacuum manifold designed to hold 96-well plates (Millipore Corporation, Bedford, MA). Beads were washed twice with 100 µl of wash buffer per well and draining was carried out by vacuum manifold. Plasma samples from TB negative human provided the baseline (background) median fluorescence intensity (MFI) for the MMIA. For detection of human IgG, R-phycoerythrin conjugated goat antihuman IgG was used as secondary antibody at a dilution of 1:1,000 in wash buffer, by adding 100 µl per well. After mixing beads were incubated for 1 hr at room temperature, and were washed twice after antibody treatment. Finally, resuspension was done in 100 ul PBS-T. The plate was read on Luminex 100 instrument (Cat # CN-L003-01, Luminex Corp, USA) provided with XY-platform for automated reading of 96-well plates.

Luminex-100 instrument operation and data analysis

The Luminex instrument was used at default settings for routine use as given in User's manual. Data in the form of MFI obtained from Luminex data collection software (Version 1.7) which allows routine operation of instrument and data acquisition. Calibration beads (BioRad, USA) were used to calibrate instrument for identification of 1-100 bead sets, and for reporter i.e. phycoerythrin detection. The data was processed in Microsoft excel software and analyzed for the presence of antibodies against each antigen. The cutoff for the assay was the mean of negative population plus 3 standard deviation of mean.

RESULTS AND DISCUSSION

Expression and purification

Six selected genes were expressed to optimize the IPTG concentration, time and temperature which came out to be specific for different protein molecules. The results of one gene are given here. Ag85a has already cloned into pET23b and was first confirmed by restriction analysis showing 385bp fragment on agarose gel

electrophoresis. The fragment size was also same as in literature provided by TB Resource Centre, Colorado State University, Boulder, CO., USA. The recombinant plasmid was then used to transform host expression strain Bl21DE3pLysS and as a result of IPTG induction AG85A was expressed having 32.5 kDa size on Tricine-SDS-PAGE. The optimum IPTG concentration was 0.7 mM and optimum time of expression was 4 hrs for AG85A as shown in Figure 1 and Figure 2 respectively. The optimum IPTG concentration was also found to be the same as observed by Anuradha *et al*. (2007), whereas, time of maximum induction in this reference study was different *i.e.* 6 hr when cultured in Terrific broth. Then cellular localization of protein was determined which showed accumulation of protein in inclusion bodies at 37°C (Fig. 3). Therefore to obtain the soluble protein product the ag85a gene was subsequently expressed at 24°C. Then the gene was over-expressed at 24°C in bulk culture and purification was carried out using Nickle-Nitrilo tritilic acid (Ni-NTA) column chromatography. The results are shown in Figure 4 and Figure 5. Western blot analysis was carried out by method described in protocol provided by Amersham ECL Plus (GE Healthcare, UK) and visualized by typhoon scanner to confirm the presence of purified antigenic protein and results are given in Fig. 6. In our study ESAT-6 protein size was approximately 13kDa which included T7 tag at N-terminus and His-tag at C-terminus of the desired 6 kDa recombinant protein from pET23b where as Farshadzadeh *et al.* (2010) showed expression of ESAT-6 at about 22 kDa including a His patch thioredoxin of size 13 kDa at N-terminus and 6X His tag plus v5 epitope of 3 kDa in C-terminus.

The antigenic proteins purified in this study were of good level of purity as analyzed on SDS-PAGE and Western blotting. Therefore over-expression and purification of different immunodominant antigenic proteins like AG85A, AG85B, AG85C, CFP-10, ESAT-6 and HSPX was successfully achieved. pET system of expression utilizing pET plasmid was used for expressing proteins in bulk quantities. The system is well known and IPTG is used for induction, which is cheap substrate making overall expression process affordable. The method used for purification that is

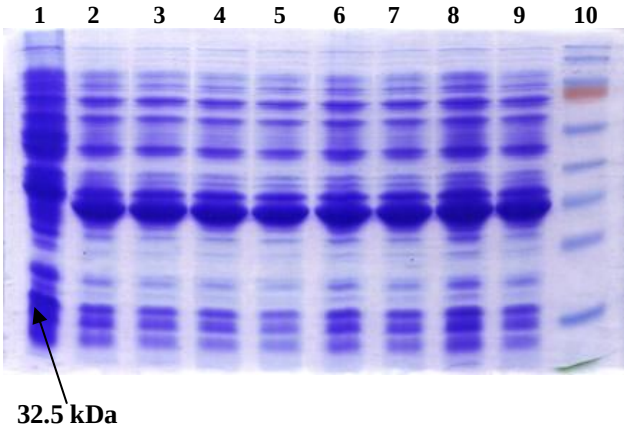


Fig. 1. 12 % SDS-PAGE showing the level of expression of ag85a when induced with various concentrations of IPTG at 37 °C for 5 hrs. Lane 1: Uninduced BL21 (DE3)pLysS cells having pET-ag85a, Lane 2-9: Induction at 0.1 – 0.8 mM IPTG, Lane 10: Protein Marker (Cat # SM0671, Fermentas USA).

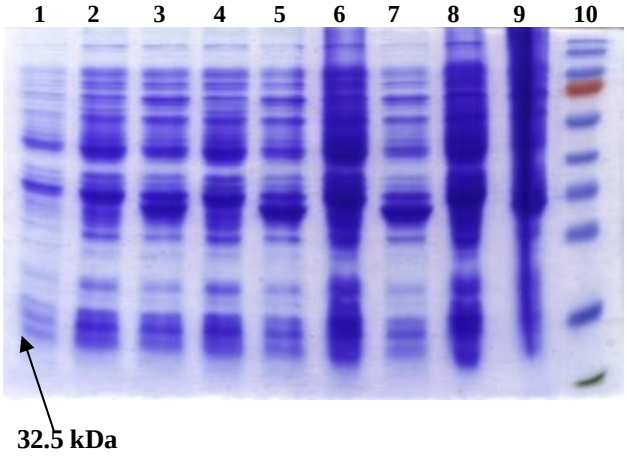


Fig. 2. 12 % SDS-PAGE showing expression level of ag85a with respect to time with 0.7 mM IPTG concentration. Lane 1: BL21(DE3)pLysS cells having pET-ag85a at 0 hr, Lane 2, 4, 6 and 8: Uninduced cells at 2 hr to 5 hrs, Lane 3, 5, 7 and 9: Induced cells at 2 hr to 5 hrs, Lane 10: Protein Marker (Cat # SM0671, Fermentas, USA).

IMAC is easy, user friendly and was successfully employed for purification of all six selected proteins. Therefore, it is speculated that this procedure could be used conveniently for the purification of *M. tuberculosis* specific antigens.

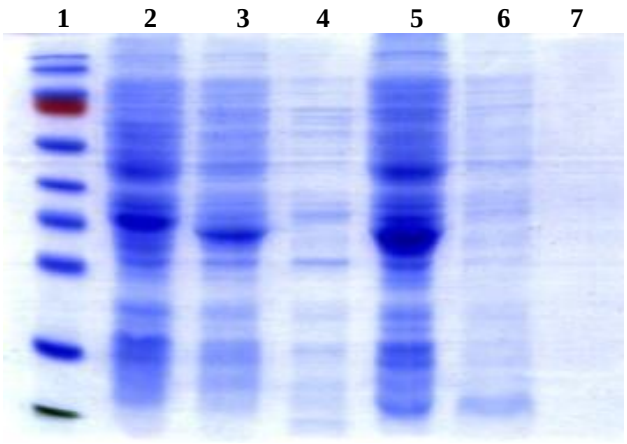


Fig. 3. 15 % SDS-PAGE showing cellular localization of AG85A. Lane 1: Protein marker (Cat # SM0671, Fermentas, USA), Lane 2: Uninduced BL21 (DE3)pLysS cells having AG85A, Lane 3: Induced cells, Lane 4: TCP after sonication, 5: IB after sonication, Lane 6 - 7: IB wash.

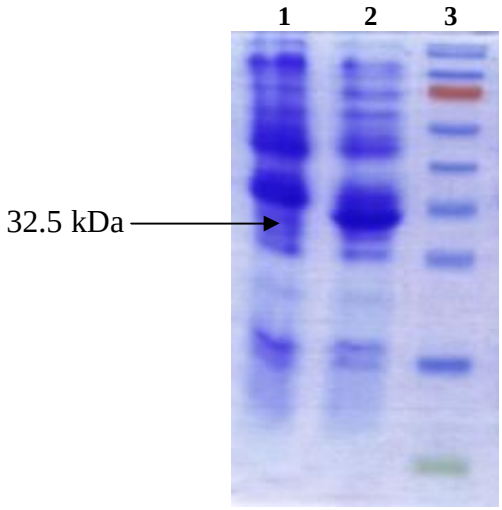
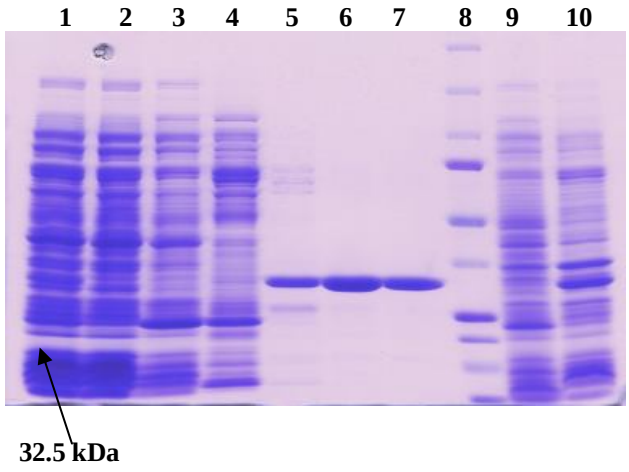


Fig. 4. 15 % SDS-PAGE showing bulk expressions of ag85a at 24 °C. Lane 1: Uninduced ag85a, Lane 2: Induced ag85a, Lane 3: Protein marker (Cat # SM0671, Fermentas, USA).

Multiplex microbead immunoassay

Antigens namely ESAT-6, CFP-10 and HSPX were specifically included to enhance specificity of the multiplex immunoassay because of their absence in most of non-tuberculous Mycobacteria and in BCG and their role in early diagnosis and latent TB (Silva *et al*., 2003; Khan *et al*., 2008). AG85 complex which is 30/32 kDa mycolyl transferase has also been included as it has recently been demonstrated as immunodominant marker of TB (Malen *et al*., 2008). This is a family



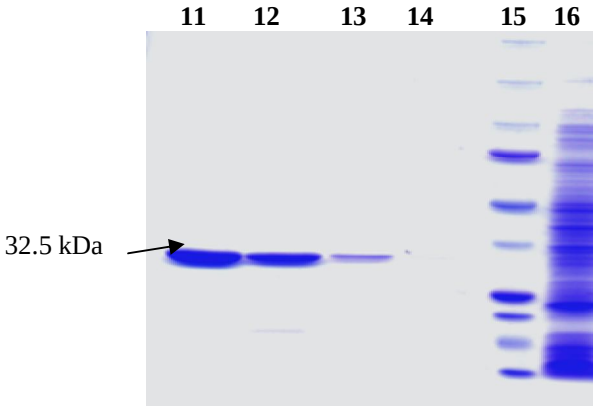


Fig. 5. 4-16 % SDS-PAGE showing the recovery of AG85A during His-tag purification. Fig. 5A Lane 1: Sonicated 0.45 um filtered supernatant, Lane 2: Flow through, Lane 3: 1X binding buffer wash, Lane 4: 1X wash buffer wash (60 mM Imidazole), Lane 5: 1X wash buffer wash (100mM Imidazole), Lane 6: 1X wash buffer wash (150 mM Imidazole), Lane 7: 1X AG85A elution Fraction-1, Lane 8: : Protein Marker (Cat # 161-0374, Fermentas, USA), Lane 9: Bl21(DE3)pLysS cells without plasmid, Lane 10: AG85A sonicated pellet. Fig. 5B Lane 11: AG85A elution Fraction 2, Lane 12: AG85A elution Fraction 3, Lane 13: AG85A elution Fraction 4, Lane 14: AG85A elution Fraction 5, Lane 15: Protein Marker (Cat # 161-0374, Fermentas, USA), Lane 16: Bl21(DE3)pLysS cells without plasmid.

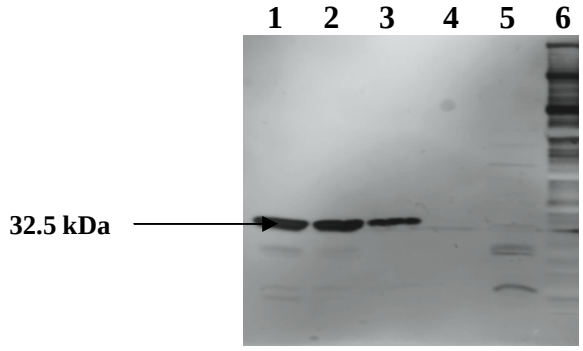


Fig. 6. Western blot showing the recovery of AG85A during His-tag purification. Lane 1: AG85A Fraction-1, Lane 2: AG85A Fraction-2, Lane 3: AG85A Fraction-3, Lane 4: AG85A Fraction-4, Lane 5: Bl21(DE3)pLysS cells without plasmid, Lane 6**:** Protein Marker (Cat # 161-0374, Fermentas, USA).

of three closely related proteins *i.e.*, AG85A, AG85B, AG85C found in all mycobacteria (Wiker *et al.*, 1986). The ratio of the MFI of antigen-coated bead sets to the MFI of BSA-coated beads in each reaction was calculated. This ratio is called the signal and gives measure of antibody detection. Luminex software was used for acquisition of the data and further processed by Microsoft Excel software. The reactivity of normal serum, termed background, for each antigen-coated bead set was also determined. For each bead set, the average of the signals from duplicate wells of three normal monkey sera was calculated, and three times the standard deviation value was added to the average. A positive control serum for all of the infectious agents was also used in each experiment. Samples were analyzed in duplicate; sera were considered positive for antibodies to an infectious agent if the signal from the relevant antigen coated bead set was greater than the background.

TST negative

In this group there were fifteen healthy controls collected from the healthy population of PMAS-AAUR. Two healthy controls were not considered during analysis as they showed high levels of antibodies even against controls *i.e.*, BSA and cell lysate. Furthermore, one more sample was also not included due to high antibodies against CFP-10. The remaining blood samples were found negative against all antigens when tested by multiplex. These results on one side showed that purified proteins coated on microbeads are of good purity, giving almost no back ground signal while on other side 80% (12 out of 15) control samples did not show presence of any basal level antibodies against the antigens used. This means patients will be detected positive by this test for specific antibodies only if these were present. Therefore the set of these six antigens seems to have potential to discriminate healthy controls from diseased ones.

Reactivated TB patients

There were five TB patients used to test the potential of purified protein and all five of them were found to be positive by MMIA. The MFI values recorded in this group were highest in order of: AG85A > AG85B > HSPX > AG85C >>>> ESAT-6 and CFP-10. In this group, 5, 5, 5, 1, 3 and 5 patients were detected positive against proteins used i.e. AG85A, AG85B, AG85C, CFP-10, ESAT-6 and HSPX respectively (Table I). Therefore, each antigen having a corresponding positivity of 100, 100, 100, 20, 60, and 100 percent was recorded. The total combined percentage of positivity for all six antigens came out 100 percent. The MFI signal of antibodies is shown in Table I.

Table I.- Antibody profiling of reactivated TB patients individuals in MMIA.

Patient No.	AG85A	AG85B	AG85C	CFP-10	ESAT-6	HSPX
1.	+++	+++	+++	-	-	+++
2.	+++	+++	+++	-	++	+++
3.	+++	+++	+++		++	+++
4.	+++	+++	+++	+	-	++
5.	+++	+++	+	-	+	+++

+, low positive; ++, medium positive; +++, high positive.

Table II.- Comparison of AFB microscopy with antibody detection by multiplex analysis.

Multiplex results	AFB positive (n=5) Reactivated TB
Negative	0 (0 %)
Positive (against 4 antigen)	5 (100 %)
Positive (against any 5 antigens)	4 (80 %)

All five TB patients analyzed in the study were AFB positive. When AFB positive patients were compared by MMIA, it was observed that in reactivated TB patients all 5 (100%) were positive for antibodies against four antigens used *i.e.*, AG85A, AG85B, AG85C and HSPX while only 1 and 3 patients were positive against CFP-10, and ESAT-6 antibodies giving 20% and 60% positive percentage respectively (Tables I and II).

This is the first report showing that purified proteins namely AG85A, AG85B, AG85C, CFP-10, ESAT-6 and HSPX when tested in multiplex assay have potential to detect antibodies against six antigens in AFB positive reactivated patients. Antibodies against four antigens (AG85A, AG85B, AG85C and HSPX) were detected in 100 % of the reactivated TB patients tested by multiplex. The data obtained in this study suggests that under optimized conditions antigenic genes can be over-expressed and after purification protein products are good enough in terms of quality to be used in MMIA. In fact, the subsequent use of these purified proteins in immunoassay like MMIA gave very promising results. Therefore this study provides a base for the development of a multiple antibody based diagnostic test for detection of *M. tuberculosis* infection not only in reactivated TB patients but also in other categories like latent and active TB patients.

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