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*Full Length Research Paper*

# Assessment of four recombinant mycobacterial antigens as serodiagnostic markers for pulmonary tuberculosis

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**An accurate laboratory diagnosis is essential for the effective management of pulmonary tuberculosis patients. This study is precisely aimed at developing an immunodiagnostic assay, using recombinant mycobacterial antigens for the accurate laboratory diagnosis of pulmonary tuberculosis. An indirect ELISA was standardized to measure serum IgG antibody levels against four recombinant mycobacterial antigens (ESAT-6, PlcA, HspX and Tb8.4) in sputum culture-negative, smear negative patients with pulmonary tuberculosis. The sensitivity of the ELISA with PlcA, HspX, Tb8.4 and ESAT-6 were 41.7, 63.3, 45 and 53.3% respectively. A higher sensitivity (76.7%) in ELISA could be achieved when a multiantigen cocktail, consisting of all the above four antigens, was used. These antigens did not give false positive results in the sera of healthy control subjects. The specificity of PlcA, HspX, Tb8.4, ESAT-6 and multiantigen cocktail in 'disease controls' were 95.7, 93.5, 94.6, 93.5 and 94.6%. High specificity and sensitivity of ELISA with these recombinant mycobacterial antigens in cocktail indicates that they can be used for the diagnosis of pulmonary tuberculosis particularly in patients.**

**Key words:** *Mycobacterium tuberculosis*, recombinant mycobacterial antigens, serology, tuberculosis.

## INTRODUCTION

Pulmonary tuberculosis is a major global health disease. Approximately one-third of the world's population is currently exposed to *Mycobacterium tuberculosis* (Frieden et al., 2003). In both developing and developed countries, there is a resurgence of tuberculosis (TB) among patients with HIV as well as in 'latently' affected individuals (Zumla et al., 2000). By and large, a diagnosis of pulmonary tuberculosis in majority of patients is made based on the relevant clinical manifestations and sup-ported by compatible radiological features in thorax. However, a confirmative diagnosis of pulmonary tuberculosis continues to depend upon the direct demonstration of Acid-Fast Bacillus (AFB) in sputum specimens by sputum specimens by Ziehl-Neelsen staining or isolation of M. tuberculosis by culture methods. Despite the advances in bacteriological techni-ques, such as BACTEC MGIT-960, the isolation rate of *M. tuberculosis* is infrequent and less sensitive in majority of TB patients. Alternate diagnostic parameters have been evaluated as adjuncts in the diagnosis. These include estimation of tuberculostearic acid (French et al., 1987), adenosine deaminase (Laniado-Laborin, 2005), nucleic acid amplification (Bennedsen et al., 1996; Haldar et al., 2007) and different immunodiagnostic assays (Chiang et al., 1997; Del Prete et al., 1998). Immunoassays to detect antibody in sera and body fluids in TB patients appear promising in terms of operational advantages in the laboratory as well as due to their reproducibility. In an attempt to improve sensitivity and specificity several immune-oreactive mycobacterial antigens have been evaluated in the ELISA during the past two decades. These include

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**Table 1**. List of primers used in amplifying genes from *M. tuberculosis*.

| Primer | Direction | Sequence |
|---|---|---|
| Esat-6 (RV3875) | Forward | 5'GGAATTCCATATGACAGAGCAGCAGTGGAATTTC3' |
| Esat-6 (RV3875) | Reverse | 5'CCCAAGCTTGGGCTATGCGAACATCCCAGTGACG3' |
| *plcA* (Rv2351c) | Forward | 5'GGGGTACCCCGATGTCACGTCGAGAGTTTTTG3' |
| *plcA* (Rv2351c) | Reverse | 5'CCCAAGCTTGGGTCAGCTGCACAGCCCGC3' |
| *hspX* (Rv2031c) | Forward | 5'GGGGTACCCCGATGGCCACCACCCTTCCCGTTC3' |
| *hspX* (Rv2031c) | Reverse | 5'CCCAAGCTTGGGTCAGTTGGTGGACCGGATCTG3' |
| *tb8.4* (Rv1174c) | Forward | 5'GGGGTACCCCGATGAGGCTGTCGTTGACCGCA3' |
| *tb8.4* (Rv1174c) | Reverse | 5'CCCAAGCTTGGGTTAATAGTTGTTGCAGGAGC3' |

purified protein derivative antigen (Zeiss et al., 1984), Antigen 5 (Ma et al., 1986), glycolipids (Reggiardo et al., 1981), 38 KDa protein (Bothamley and Rudd, 1994) complex mycobacterial protein A60 (Cocito, 1991) 16 KDa protein (Raja et al., 2002). In the present study, an indirect ELISA has been developed to measure specific IgG antibodies in sera of pulmonary tuberculosis patients using four recombinant mycobacterial antigens, PlcA (Rv-2351c), HspX (Rv2-031c), Tb8.4 (Rv1174c), ESAT-6 (Rv-3875) and its multi-antigen cocktail. These antigens were purified in large-scale by cloning and expression of their genes in *E. coli*. Technical aspects highlighting their characterization as well as their accuracy in an immunoassay for the laboratory diagnosis of pulmonary tuberculosis and its field application has been highlighted.

## MATERIALS AND METHODS

### Patients

In this study, 194 sera were collected from the following patients. These patients were admitted to the Hospital for Tuberculosis and Chest Diseases, Pulayanarkotta, Thiruvananthapuram, India (n = 141), and at SCTIMST, Thiruvananthapuram, India (n = 53). There were 158 male and 36 female patients and their age ranged from 19 to 79 years. Their 'disease status' was recorded from the respective case records. (a) 26 sera were collected from sputum culture positive patients with pulmonary TB ('confirmed' pulmonary TB) (b) 34 sera were collected from sputum culture negative, smear negative patients with pulmonary tuberculosis. These patients showed compatible radiological features of pulmonary tuberculosis in thorax and they also showed optimum clinical response to anti-tuberculosis chemotherapy. Hence these 34 patients were regarded as 'probable' cases of TB. The control group consisted of (c) 42 sera collected from voluntary blood donors attending the Department of Transfusion Medicine of this institute and (d) 92 sera from patients with non-tuberculous pulmonary diseases and other respiratory diseases - bronchial carcinoma (n = 59), bacterial pneumonia (n = 11), and bronchial asthma (n = 22). All the sera specimens were coded and aliquots were stored at – 70°C.

### Cloning of *M. tuberculosis* open reading frames into *E. coli* expression vectors

### Materials

*E. coli* BL21 (DE3) pLysS, pET-32a (+) and pET-28a (+) (Novagen, Madison, WI). *Nde*I, *Kpn*I, *Hind*III (NEB, MA,USA). pGEM-T Easy vector, Taq DNA polymerase, T4 DNA Ligase and Nickel–nitrilo-triacetic acid (Ni–NTA) affinity agarose beads (Promega Corpo-ration, Madison, WI). The DNA sequencing kit used was ABI PRISM Big Dye Terminator cycle sequencing Ready Reaction kit, Version 2.0 (PE Applied Biosystems, CA, USA). Anti- HIS antibody and PCR gel elution kit (GE healthcare biosciences, NJ, USA). Microtitre plate (Dynatech Laboratories, Alexandria, VA). Antibodies and substrate for ELISA were procured from Sigma Aldrich, MO, USA. Primers were designed using Primer Premiere 5 (PREMIER Biosoft International, CA, USA). All the chemicals were of molecular biological grade. Ultra-pure de-ionised water was used in this study.

### High-level expression and affinity purification of recombinant proteins

PCR primers were designed to amplify the full-length open reading frame of four genes from genomic DNA of the *M. tuberculosis* $H_{37}$Rv. Genes were amplified using specific primers (Table 1). The 5' oligonucleotide contained a *Kpn*I restriction site and the 3' oligonucleotide contained *Hind*III restriction site. PCR product was cloned into pGEMT easy vector as per manufacturer's instructions. Sequencing was performed and all possibilities of mutation were checked. Insert was released from pGEMT easy vector with KpnI and *Hin*dIII enzymes and later sub-cloned into pET-32a prokaryotic expression vector, similarly digested with *Kpn*I and *Hin*dIII for directional cloning. To reduce the end size of the expected protein a thioredoxin (trx) tag was previously removed from pET-32a vector by single restriction with *Nde*1 and re-legation.

Recombinant pET-32a with specific genes was used in transforming *E. coli* BL21 (DE3) pLysS cells for expression. Recombinant (His tag) proteins (PlcA and Tb8.4) were purified to near-homogeneity from the insoluble inclusion body from isopropyl D-thiogalactopyranoside-induced batch cultures by affinity chroma-tography using ni-nitrilotriacetic acid (NTA) agarose beads in the presence of 8 M urea as per manufacturer's instructions. HspX was found in the soluble protein, and purified by native conditions, but

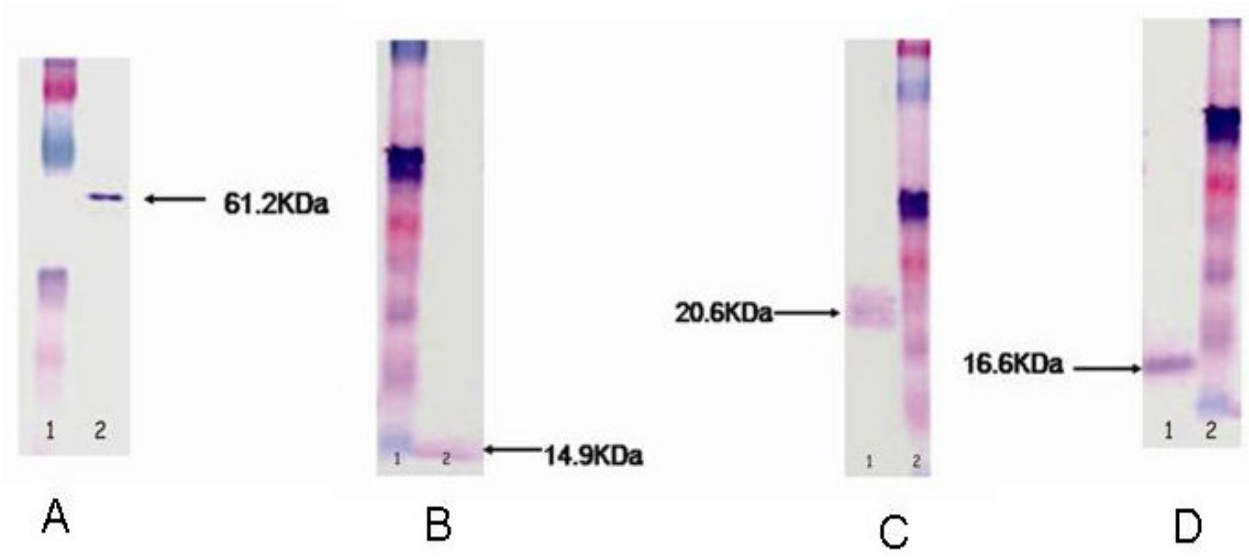


**Figure 1.** Immunoblot of PlcA (A), ESAT-6 (B), HspX (C), Tb8.4 (D). (A) Immunoblot showing His-tagged PlcA purification: lane1: Kaleidoscope prestained standard protein marker (Bio-Rad), lane 2: Purified PlcA. (Primary antibody: anti-HIS antibody; 10% SDS-PAGE); (B) His-tagged ESAT-6 purification: Kaleidoscope prestained standard protein marker, lane 2: Purified ESAT-6. (Primary antibody: anti-HIS antibody; 15% SDS-PAGE); (C) His-tagged HspX purification: lane1: purified HspX; lane 2: Kaleidoscope prestained standard protein marker. (Primary antibody: anti-HIS antibody; 12% SDS-PAGE); (D) His-tagged Tb8.4 purification: lane1: purified Tb8.4; lane 2: Kaleidoscope prestained standard protein marker. (Primary antibody: anti-HIS antibody; 15% SDS-PAGE)

yield of HspX after purification was much lower than other proteins, hence HspX was also purified under denaturing conditions.

Gene coding for ESAT-6 protein with *Nde*I and *Hind*III sites was cloned in pET-28a expression vector for high yield expression, while the steps of cloning was similar to that of pET-32a, except that pET-32a was selected based on ampicillin resistance while kanamycin resistance was used in selection of transformants with pET-28a. ESAT-6 was present as inclusion bodies and was purified with Ni-NTA purification method under denaturing conditions.

Purity of the recombinant proteins was assessed by SDS-PAGE, followed by immunoblotting with monoclonal anti-His antibody (Figure 1). Protein expressed was assessed by comparing the size with known protein markers. This migration was slightly higher than the molecular mass of the native molecule, which was expectedbecause of the addition of the histidine tag sequence and a thrombin site added to the recombinant protein to facilitate purification. Proteins were quantified by Bradford's assay (Bradford, 1976).

**Estimation of antigen-specific IgG antibody in sera samples:**

ELISA was standardized for the measurement of IgG in sera against the four recombinant antigens (ESAT-6, PlcA, HspX and Tb8.4). Briefly, each well in round-bottom microtitre plate was coated with individual recombinant mycobacterial protein (ESAT-6, HspX and Tb8.4- 500pg/well; PlcA -350pg/well) for 2 h at room temperature (RT), following which the wells of the microtitre plate were quenched at RT with 1% bovine serum albumin (BSA) in 0.15 M phosphate-buffered saline in Tween-20(PBS-T). Subsequently the sera samples from healthy and disease controls as well as from TB groups were serially diluted in 1% BSA in PBS and 100 µl (1:5000 dilution in BSA/PBS-T) was added to each well and incubated overnight at 4˚C. The plates were then washed thoroughly and incubated for 2 h at RT, with 100 µl of (1:1000) anti-human IgG-alkaline phosphatase conjugate (Sigma chemicals; St. Louis USA). The colour reaction was developed by the addition of a substrate containing para-nitrophenyl phosphate (1 mg/ml in Diethanolamine buffer) and the plates were incubated for 30 min at RT. 3N Sodium hydroxide (25 µl) was added to the wells to stop the reaction. The absorbance in each well in the microtitre plate was read at 405 nm using a micro- titter ELISA reader (Bio-Tek instruments, USA).

ELISA was performed in a batch of 10 specimens. Serum from a sputum culture-positive patient with pulmonary TB was used as a positive control. Inter-observer variation, reproducibility of assay and batch-to-batch variation of recombinant antigens were evaluated by using two different batches of antigen in the same serum sample at two different occasions.

ELISA was also simultaneously performed in all the serum specimens using a cocktail of all the four recombinant antigens in equal concentration (200 pg/ml each). The optimum concentration of each antigen in cocktail was calculated by a dose-response curve. The technical procedures adopted were identical as described for individual recombinant antigens.

## RESULTS

Standardization of ELISA showed that ESAT-6, HspX and Tb8.4 at 500 pg/well and PlcA at a concentration of 350 pg/well at 1:5000 dilution of sera gave the 'best' discrimination to distinguish 'disease' control and TB patients. Hence these concentrations were uniformly applied in ELISA.

The mean absorbance values of anti- PlcA, HspX, Tb8.4, ESAT-6 and multiantigen antibody titre in TB and

**Table 2.** The mean OD in tuberculous and control sera specimens.

| Antigens | Tuberculosis (mean ±SD) (n = 60) | Diseased control (mean ±SD) (n = 92) | Healthy control (mean ±SD) (n = 42) |
|---|---|---|---|
| PlcA | 0.722 ± 0.38 | 0.431 ± 0.103 | 0.284 ± 0.099 |
| HspX | 0.883 ± 0.461 | 0.399 ± 0.206 | 0.278 ± 0.106 |
| Tb8.4 | 0.811 ± 0.392 | 0.463 ± 0.167 | 0.279 ± 0.112 |
| ESAT-6 | 0.868 ± 0.291 | 0.488 ± 0.197 | 0.361 ± 0.176 |
| Multiantigen | 0.915 ± 0.52 | 0.522 ± 0.177 | 0.357 ± 0.098 |

**Table 3.** Results of ELISA using four mycobacterial antigens and multiantigen cocktail.

| Antigens | Sensitivity (%) | | | Specificity (%) | | |
|---|---|---|---|---|---|---|
| | Confirmed TB (n = 26) | Probable TB (n = 34) | Total TB (n = 60) | Healthy controls (n = 42) | Disease controls (n = 92) | Total non-TB controls (n = 134) |
| PlcA | 61.5 | 26.5 | 41.7 | 100 | 95.7 | 97 |
| HspX | 69.2 | 58.8 | 63.3 | 100 | 93.5 | 95.5 |
| Tb8.4 | 57.7 | 35.3 | 45 | 100 | 94.6 | 96.3 |
| ESAT-6 | 50 | 55.9 | 53.3 | 100 | 93.5 | 95.5 |
| Multiantigen | 77 | 76.5 | 76.7 | 100 | 94.6 | 96.3 |

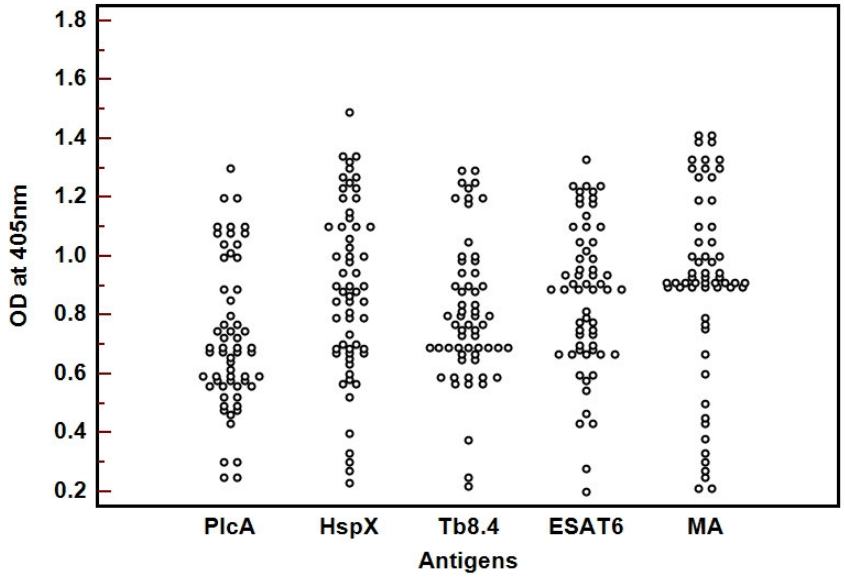


**Figure 2.** Antibody response of TB patients.

control groups are summarized in Table 2. The 'cut-off' value to demarcate TB patients from the disease control group was determined using the following criterion: mean absorbance of disease controls + 2 SDs. Accordingly a serum specimen in the ELISA was considered as positive when the absorbance is greater than 0.637, 0.811, 0.797, 0.882 and 0.876 respectively for antigens- PlcA, HspX, Tb8.4, ESAT-6 and multiantigen complex. Based on this criterion, the sensitivity of PlcA, HspX, Tb8.4, ESAT-6 and multiantigen cocktail were 41.7, 63.3, 45, 53.3 and 76.7% respectively. A higher sensitivity in ELISA was obtained with a multiantigen cocktail consisting of all the four antigens. Not a single false positive reaction in ELISA was recorded in any one of the 42 BCG vaccinated healthy individuals. The specificity of PlcA, HspX, Tb8.4, ESAT-6 and multiantigen cocktail in disease' control patients were 95.7, 93.5, 94.6, 93.5 and 94.6% (Table 3). An antibody response of TB (confirmed and probable) patients against individual antigens and multiantigen (MA) cocktail is given in Figure 2.

## DISCUSSION

In this study, an attempt has been made to isolate and characterise four mycobacterial recombinant antigens, PlcA, HspX, Tb8.4 and ESAT-6. PlcA is a membrane-associated phospholipase C1 of *M. tuberculosis.* This is also been referred to as Mtp40 antigen (Raynaud et al., 2002). The immunological activity of PlcA is regarded to be restricted to pathogenic *Mycobacterium* species. ESAT-6 is the early-secreted antigenic target 6-kDa protein, specific for *M. tuberculosis* complex and reported to be absent from *M. bovis* BCG (Munk et al., 2001). ESAT-6 is well known to elicit strong antibody responses and was reported to stimulate delayed type hypersensitivity skin reactions in guinea pigs (Brusasca et al., 2001). HspX (*acr* /16-kDa antigen / alpha-crystallin) is regarded to be more sensitive than ESAT-6, CFP10 and antigen 85 in terms of sensitivity for TB diagnosis (Shin et al., 2008). Tb8.4 is an immunodominant T-cell antigen of *M. tuberculosis* and this antigen predominantly elicit cell-mediated immunological responses in human beings and animal models (Coler et al., 1998).

Earlier published reports highlighted the potential application of mycobacterial antigens including ESAT-6 in evaluation of T-cell mediated immune responses in TB patients (Cardoso et al., 2002; Liu et al., 2004). There are few reports that would highlight the evaluation of humoral immune responses induced by the recombinant mycobacterial antigens. In our data, we found, the individual recombinant antigens were less sensitive in eliciting IgG antibody and yielded false negative even among sputum culture positive pulmonary tuberculosis patients and this limited their application in ELISA.

To overcome the low sensitivity, a multiantigen cocktail containing all these four mycobacterial antigens were used. Multiltiantigen cocktail in ELISA gave higher sensitivity than individual antigens. Multiantigen did not give any false positive results in healthy controls. However positive results were obtained in the sera of four out of 51 patients with bronchial carcinoma. Reactivation of tuberculous lesions is well known to occur in some patients with bronchial carcinoma (Dacosta and Kinare, 1991).

Therefore, it is likely that IgG antibody in the sera of four patients with bronchial carcinoma may be due to the reactivation of old tuberculous lesions in lungs. With a sensitivity of 76% and a specificity of 95%, we regard that the multiantigen cocktail consisting PlcA, HspX, Tb8.4 and ESAT-6 antigens can be used for the routine diagnosis of pulmonary tuberculosis particularly in patients in whom bacteriological methods did not contribute to the diagnosis. HspX gave the highest sensitivity among individual antigens. This result is in concordance with the study done by Shin et al. (2008). All the four recombinant mycobacterial antigens gave high specificity.

Pulmonary tuberculosis is still a common infectious lung disease in many developing countries. One of the reasons for the high mortality rate in TB patients is due to the delay in making a laboratory diagnosis. The results of this study will add one more armoury for the laboratory diagnosis of pulmonary tuberculosis. However, its use in early diagnosis of tuberculosis needs to be explored. This immunodiagnostic system can also be used in clinical specimens from extra- pulmonary tuberculosis such as CSF, pericardial fluid and synovial fluids. Prior to their application, currently attempts are made to enhance the specificity of these recombinant mycobacterial antigens in larger populations of specimens in patients with non-tuberculous pulmonary diseases. It is also being envisaged to undertake a multicentric evaluation of this recombinant mycobacterial antigen in patients coming from different geographic locations. The ultimate goal is to develop a uniformly acceptable immunodiagnostic system for the early diagnosis of pulmonary tuberculosis so that the patients can get the benefit of chemotherapy for this potentially curable disease.

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