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Efficient diagnosis of *Mycobacterium tuberculosis* (MTB) infections by using membrane secretory proteins of MTB

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Abstract
Despite the high incidence of Tuberculosis (TB) in many developing countries, a specific and sensitive method for the diagnosis of TB is still lacking. *Mycobacterium tuberculosis* (MTB) surrounded by a unique diderm cell envelope that requires a specialized secretion pathway, known as the ESX or type VII secretion (T7S) pathway, to facilitate protein export. Several virulent proteins are secreted via T7S system and utility of such proteins in the MTB diagnosis has been documented. Three such proteins namely PPE41, MPT53, and TB18.5 were found to be highly specific for MTB and their usage as early diagnostic marker has not been reported. We evaluated the early diagnostic property of PPE41, MPT53 and TB18.5 proteins either singly or in combination. From this study we found that a cock tail of all 3 antigen combination detected maximum number of TB cases. Thus PPE41, MPT53 and TB18.5 proteins-based diagnostic methods could be optimized for the better early detection of MTB infections.

Keywords: *Mycobacterium tuberculosis,* Tuberculosis, PPE41, MPT53, TB18. 5, Culture filtrate proteins, membrane secretory proteins, T7S, ELISA

1. Introduction

Mycobacterium tuberculosis (MTB) causes significant morbidity and mortality worldwide and the primary infection caused by this organism is tuberculosis (TB) [1].Worldwide, 9.6 million people are infected with TB of which 12% are co-infected with HIV. Of the 9.6 MTB victims5.4 million are men, 3.2 million were women and 1.0 million were children [2]. TB is preventable by BCG vaccine however its efficacy varies with the geographical locations and in many countries including India BCG has been reported to be a complete failure [3, 4]. A reliable vaccine against MTB is still in the horizon though several vaccine preparations are still under evaluation [5]. TB is a curable disease however to achieve complete and robust cure early diagnosis of the disease is mandatory. These issues prompted us to develop an easy, reliable yet dependable diagnostic kit to diagnosis TB. Currently available diagnostic methods for TB are i) Chest X ray, ii) tuberculin test, iii) Tb interferon release assay, iv) sputum smear microscopy test, v) fluorescent microscopy, vi) culturing TB bacilli, vii) molecular biology tools such as Gene Xpert and viii) serological methods. Among the TB, pediatric TB is still complex and difficult to diagnose [6]. The sequencing of the MTB*H37Rv* genome was completed in 1997 [7]. This has led to the identification of several major secreted antigens of MTB such as PPE41, MPT53, TB18.5, Ag85, MPB70, LPPX, CEP 10, ESAT-6, HSP70, etc., [8] of which we evaluated the efficacy of using PPE41, MPT53 and TB18.5 antigens as early diagnostic marker of MTB.

Proteins secreted into the extracellular environment by MTB are usual target of immune response in the infected host. In MTB H37Rv, PE/PPE41 gene cluster encodes 10% of MTB's genome [9]. Of which Proline Proline Glutamic Acid (PPE41) gene which is about 604 bp long and its protein is about 23 kDa in size has been described for its efficacy in inducing good immune response against MTb infection in animal studies [10]. PPE41 is encoded by Rv2430c ORF which is a substrate for type VII secretion system [11]. MPT53 protein is a disulphide bond-forming protein E (DsbE) encoded by Rv2878c ORF which has 539 bp gene and 15kDa size protein [12]. TB18.5 is also known as MTSP17 encoded by ORF Rv0164 which has the gene size of 498 bp and 17 kDa size protein [13]. One of the advantages of these genes is that they are abundantly secreted by virulent MTB and not by other mycobacteria. That is the primary reason for testing these proteins as early diagnostic markers of TB infections.

In the present study we cloned PPE41, MPT53 and TB18.5 genes from H37Rv strain of MTB. Cloned plasmid constructs were transformed and proteins were purified and quantified. These proteins were used for coating the ELISA plates. Efficacy of these various proteins were evaluated either as single protein or a combination of two or a cock tail of three.

2. Material and Methods

2.1 Cloning of MTB genes

MTB H37Rv was inoculated in Lowenstein-Jensen (LJ) slants incubated at 37°C and incubated for 7-14 days until the colonies develop. After colonies are formed, bacterial cells were lysed and genomic DNA was isolated.PPE41 gene was amplified from MTB *H37Rv* at the open reading frame (ORF) Rv2430c using forward (5'-ACGGATCCATGCATTTCGAAGCG-3') and reverse (5'-AGAGTGTCTGTACGCG-3') primers. MPT53 was cloned from Rv2878c ORF using the specific primers (Forward 5'-ATGGATCCATGAGTCTTCGCCTG-3'; Reverse 5'-ATGAATTCGGACGTCAGCGCAGC-3') and TB18.5 was cloned from ORF Rv0164 from specific primers (Forward 5'-ATGGATCCATGACGGCAATCTCG-3'; Reverse 5'-ATGAATTCGCTGGCCGCCAGCTG-3'). Each gene was

amplified individually and their respective proteins were purified as described below.

2.2 Transformation of ligated plasmids

Amplified and ligated product was cloned into pRSET-A (Fig.1) and transformed into *E.coli XL10* gold as per the procedure of Chung and Miller (1988). Briefly, for 100 µl of competent cells (*E.coli XL10* gold) 500 ng of ligated DNA in TE was added and incubated on ice for 30 minutes. Heat shock was given at 42° C for 1 min. and returned to ice promptly for 5 min. Cells were transferred into 900 µl of LB broth (pH 6.1) and incubated at 37° C with constant shaking for 1 h. Recombinant transformants were selected by plating them on low salt LB agar containing 100 µg/ml ampicillin.

2.3 Isolation of Plasmid DNA

E.coli XL10 gold culture (1.5) containing pRSET a containing the insert was harvested by centrifugation in room temperature at 10,000 rpm for 1 min. To the cell pellet 200µl of cell suspension buffer was added and the cells were suspended thoroughly by vortexing. To the cell suspension 400 µl lysis solution was added and the tube was gently inverted 5-10 times and allowed to stand at room temperature for 5min. To neutralize, 300 µl of cold 3 M sodium acetate(pH 4.6) was added and mixed gently by inverting 10-20 times. After being maintained on ice for 15minutes, the tube was centrifuged at 12,000 rpm for 10 min. and the supernatant was transferred to another tube. To this chloroform: isoamylalcohol (24:1v/v) was added and emulsified by inverting 5-7 times, followed by centrifugation at 12,000 rpm for 10 min. at 4°C to break the emulsion. 500µl of aqueous layer was transferred to another tube and 1ml cold ethanol was added. The tube was inverted 5-8 times and kept on ice for 5 min. The tube was centrifuged at 12,000 rpm for 15 min. at 4°C and the supernatant was discarded by decantation. The DNA pellet was washed with cold 70% ethanol, partially dried and dissolved in 100µl of TE buffer and stored at -20°C.

2.4 Expression and purification of 6X His-rMTB gene in *E.coli* BL21 (DE3)

The recombinant plasmid was transformed into *E.coli* BL 21 (DE3) and the resulting cells were grown in LB medium containing 100 µg/ml ampicillin. The expression of TB gene was induced by 1.5 mm isopropyl-1-thio-β-D-galactopyranoside (IPTG) in mid-log phase (OD_{600} nm of 0.6-0.7) at 25°C for 5h. Cell pellets from 10ml cultures were re-suspended in 1ml of buffer A [50mm Tris 2mm EDTA and 0.1% (v/v) Triton X-100, pH 8.0] and lysed by sonication for 40 sec with a 30 sec rest period for four cycles. During sonication the sample tube was kept in ice to avoid excessive temperature. The insoluble fraction of the cell lysate, containing the TB protein as inclusion bodies, was washed three times in a 0.4 ml of buffer B [50 mmTris, 10mm EDTA and 0.5%(v/v) Triton X-100, pH 8.0]. Then the TB protein inclusion bodies were solubilized in 1 ml of denaturing buffer [6 M guanidine hydrochloride containing 1 mm EDTA and 100 µl of 1 M phenyl methyl sulfonyl fluoride (PMSF) to give a final TB protein concentration of 0.5-1.0 mg/ml. The expressed recombinant protein was purified by capturing the 6X His-TB protein using spin clean chelating IDA Excellose column (1 mg/ml). The column was washed with washing buffer [20mm sodium phosphate, 0.5 M NaCl, 250 mm imidazole pH 8.0]. The quality of the expressed and purified TB protein was checked by Tricine- SDS- PAGE followed by staining with Coomassie brilliant blue R- 250 stain.

2.5 Study population

For this study blood samples were collected from 200 patients infected with MTB (both clinical positive and smear positive) cases and 100 healthy control individuals. The TB samples were obtained from Govt. Hospital of Thoracic Medicine, Tambaram Sanatorium. From the blood, sera were separated and stored at –20ºC until use. This study was approved by Institutional Human Ethics Committee (Approval No: UM/IHEC/16-2013-I). ELISA was used to detect the IgG antibodies in the serum reactive to MTB antigen (s) coated on the ELISA plate.

2.6 ELISA for the detection of MTB antibody

96-well polystyrene micro titer ELISA plates were coated with 100 µl of TB protein (1 µg/ml) in carbonate-bicarbonate buffer (pH 9.0) overnight at 4˚C. The plates were washed thrice with washing buffer (PBS-Tween-20). Plates were blocked with PBS containing 1% BSA for 2 h at 37°C and washed 3x with PBS. Serum samples were diluted at 1:500 in sample dilution buffer and 0.1 ml of diluted serum were added to antigen-coated wells in duplicate and incubated for 30 min. at room temperature. Plates were washed 5x with PBS-Tween-20 and then incubated for 30 min. with 0.1 ml of 1:10,000 diluted goat anti-human immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) per well. Plates were washed with PBS-Tween 20 and 0.1 ml of TMB / H_2O_2 were added to each well and incubated for 20 minutes. Then 0.1 ml of 1 N H_2SO_4 was added to stop the reaction. Then the plates were read at 450 nm in an ELISA plate reader. Obtained optical density (OD) values measured for the TB positivity using cut-off value. Cut-off value was calculated as mean + 3SD of the negative control samples.

3. Results

3.1 Cloning and expression of MTB genes using pRSET-A vector

MTb H37Rv was inoculated in LJ slants incubated at 37°C and incubated for 7-14 days until the colonies develop. After colonies are formed, bacterial cells were lysed and genomic DNA was isolated which is shown in the Fig.2. PPE41 gene was amplified from *MTbH37Rv* at the open reading frame (ORF) Rv2430c using forward (5'-AC GGATCCATGCATTTCGAAGCG-3') and reverse (5' - AG GAATTCAGTGTCTGTACGCG -3') primers as described above. MPT53 was cloned from Rv2878c ORF using the specific primers (Forward 5'-ATGGATCCATGAGTCTTCGCCTG-3'; Reverse 5'-ATGAATTCGGACGTCAGCGCAGC-3') and TB18.5 was cloned from ORF Rv0164 from specific primers (Forward 5'-ATGGATCCATGACGGCAATCTCG-3'; Reverse 5'-ATGAATTCGCTGGCCGCCAGCTG-3') as described above. Amplified product (Fig.3) was cloned into pRSET-A in frame with N-terminal His tag. Cloned pRSET-A was grown in *E.coli X10* gold at 37ºC overnight. From the overnight culture MTb proteins were isolated. As a confirmation plasmid

vectors isolated from overnight culture were restriction enzyme digested using Bam HI/ Eco RI and the digested fragments are shown in the Fig.4. Not shown are the SDS-PAGE gel pictures which showed the each protein at the appropriate size in comparison with the molecular weight markers.

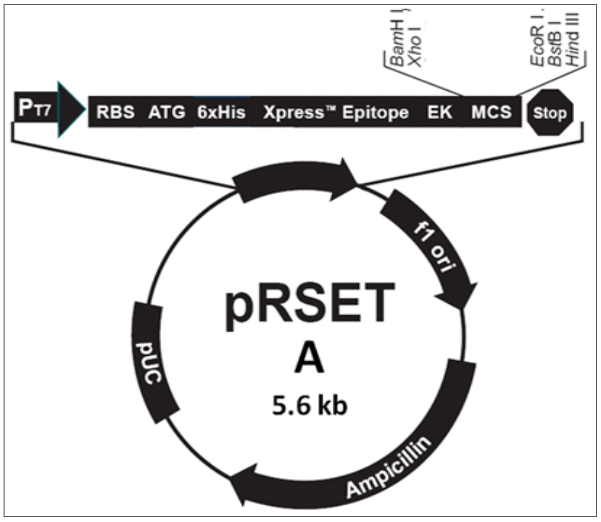

Fig 1: Map of pRSET-A vector with its MCS

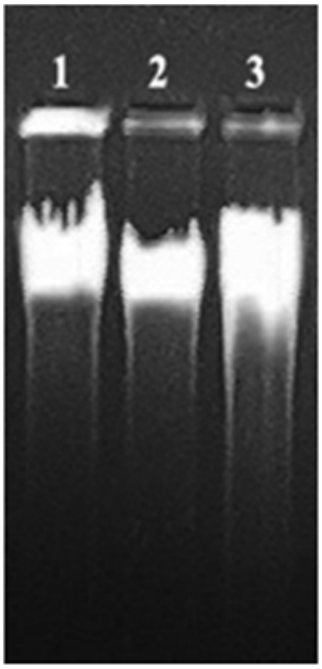

Fig 2: Agarose gel electrophoresis of genomic DNA from MTB: MTB *H37Rv* were grown in LJ media. Cultures were used for the genomic DNA isolation. Isolated genomic DNA were run in agarose gel electrophoresis. Lane 1, 2 and 3: MTB *H37Rv* genomic DNA.

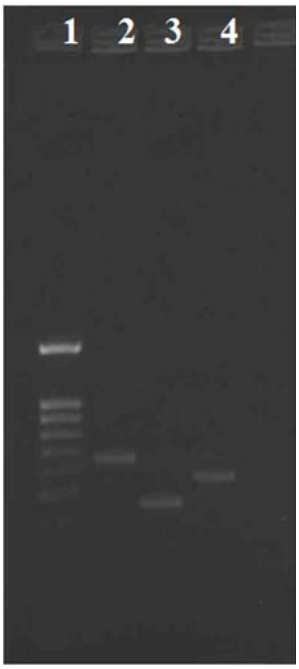

Fig 3: Amplicons of PPE41, TB18.5 and MPT53: Genomic DNA isolated from MTB were amplified by PCR using specific primer. Each amplified product was run in agarose gel electrophosresis; Lane 1-DNA ladder, Lane 2-PPE41 (604 bp), Lane 3-TB18.5 (498 bp) and Lane 4-MPT53 (539 bp).

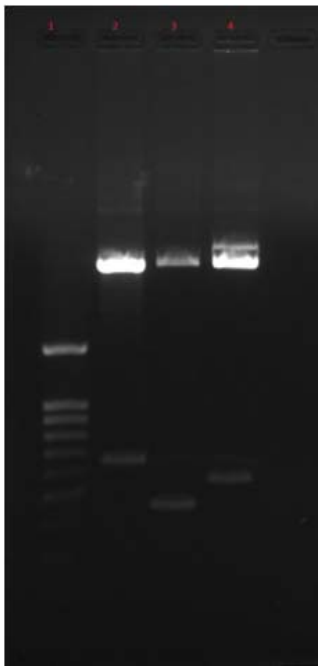

Fig 4: Restriction digestion analysis of cloned RSET vectors: Insert was confirmed by restriction digestion with Bam HI and EcoR1. Lane 1-DNA ladder, Lane 2-pRSET A::PPE41; Lane 3- pRSET A::TB18.5; Lane 4 pRSET A::MPT53.

3.2. Measurement of IgG antibody to Mycobacterial recombinant antigens by ELISA

In the present study, immunoreactivity of recombinant PPE41, MPT53, and TB18.5 to IgG antibody in the sera from TB patients and healthy controls were analyzed by ELISA. The mean OD obtained with healthy control at 450 nm plus 3 standard deviations (SD) was considered as cut-off value. In this study 100 healthy donor sera was tested for reactivity with recombinant MTB proteins. When the seroreactivity was evaluated in MTB patients, maximum reactivity was found with PPE41 protein which accounted for 57 (Fig-5). The other two antigen preparation showed minimum reactivity with TB positive sera. 29% patient sera reacted with MTP53 and 18% showed reactivity with TB18.5. Not shown in the figure is the

MTB antigen reactivity of control serum samples which showed no reactivity.

Fig. 6 shows the results of combining two MTB antigens in the detection system. This study increased the sensitivity of TB positivity. As shown in the figure the maximum reactivity was found with PPE41+MPT53 antigen combination with accounted for 74%. Antigen combinations PPE41+TB18.5 reacted with 69% of TB samples and MPT53+TB18.5 with only 59% of samples. To further increase the sensitivity, all the 3 MTB antigens were mixed (cocktail) and coated with ELISA plates. This method further increased the sensitivity to 88% (Fig.7). Of the tested 200 TB samples triple antigen ELISA detected a maximum of 176 MTB cases. None of the control sera reacted to this antigen combination. To check the reproducibility we repeated the experiments 3 times and we found similar results. This result suggest that cocktail of all the 3 antigens i.e. triple antigen system containing PPE41+MPT53+TB18.5 was the best in diagnosing MTB cases.

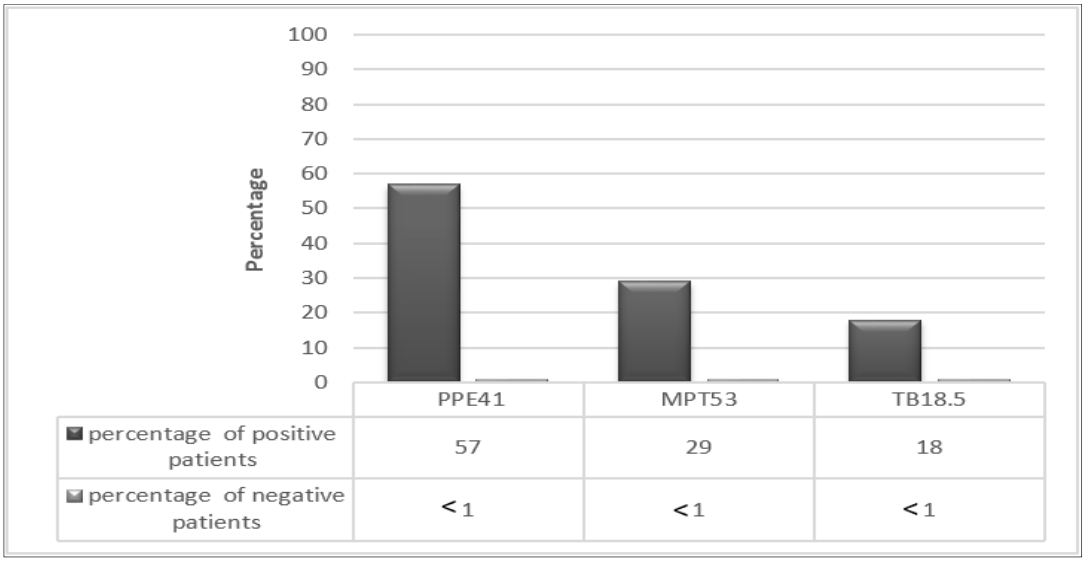

Fig 5: Percentage of TB samples reactive with single MTB antigen: Purified MTB proteins through columns were quantified and used for coating ELISA plates at a concentration of 0.1 µg/100 µl/well (i.e.1 µg/ml). Graph shows the percentage±SD of MTB samples reacted to each MTB proteins. None of the negative controls showed any activity which is represented as <1.PPE41 vs MPT53 p <0.001, MPT53 vs., TB18.5 p<0.01& TB18.5 vs PPE41 p <0.001 (Student's t-test).

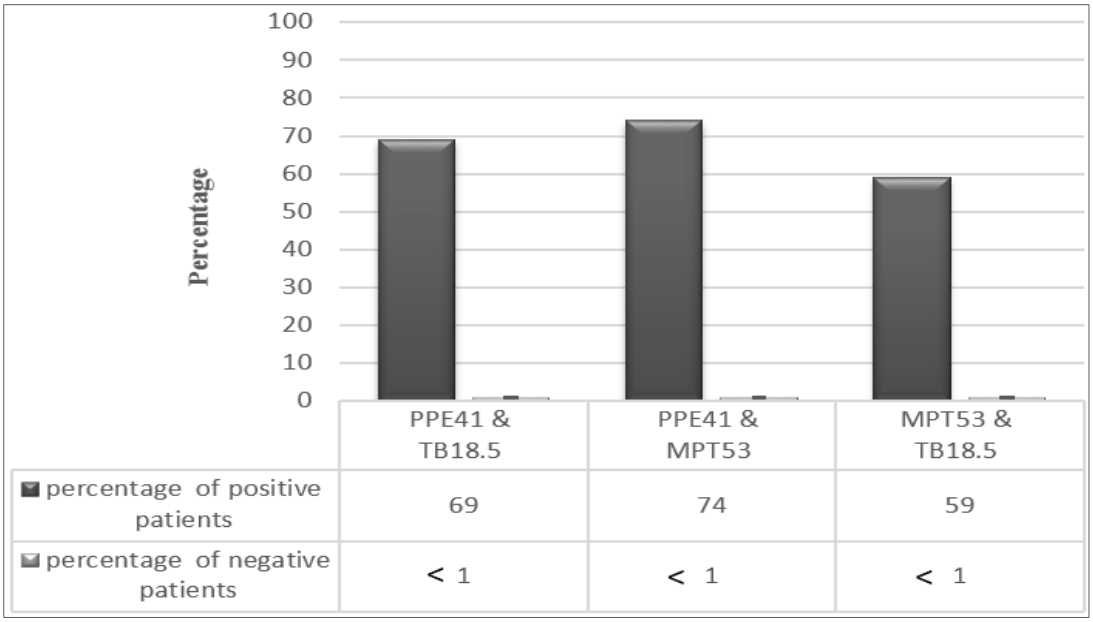

Fig 6: Percentage of MTB samples reactive with dual MTB antigens: ELISA plates were coated with 100 µl of 0.1 µg of each MTB antigen. Graph shows the percentage±SD of MTB samples reacted to each MTB proteins. None of the negative controls showed any activity which is represented as <1. (PPE41 + TB18.5 vs PPE41 + MPT53 p <0.01, PPE41 & MPT53 vs , MPT53 & TB18.5 p < 0.001, MPT53 & TB18.5 vs PPE41 & TB18.5 p< 0.001, Student's t-test).

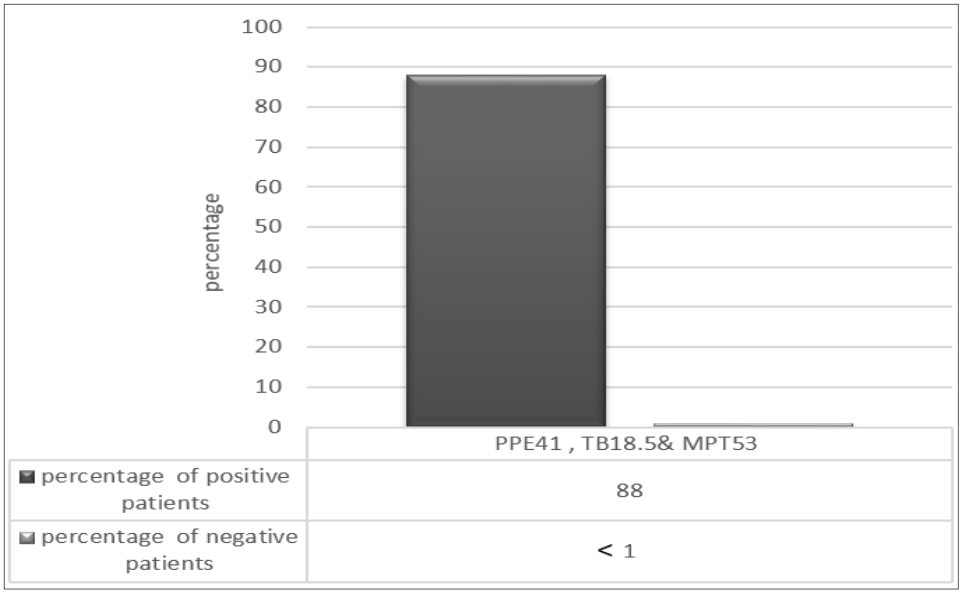

Fig 7: Percentage of MTB samples reactive with triple MTB antigens (cocktail): ELISA plates were coated with 100 µl of 0.1 µg of each MTB antigen. Graph shows the percentage±SD of MTB samples reacted to each MTB proteins. None of the negative controls showed any activity which is represented as <1. Maximum reactivity was observed with triple antigen system which showed 88%.

4. Discussion

TB remains as one of the dreaded global infectious diseases in spite of availability of anti MTB drugs and vaccines. MTB is susceptible to different lines of drugs though multidrug resistant (MDR) MTB and extensively drug resistant (XDR) MTB are not uncommon. Popular vaccine for MTb is Bacille Calmette-Guérin (BCG) vaccine which is an attenuated strain of *M. bovis*. Though BCG is effective against meningeal and disseminated tuberculosis in children, its effectiveness against pulmonary TB in adolescents and adults is equivocal [15]. Alternatively several other MTb vaccines are being tried of which the important ones are i) the therapeutic vaccine namely RUTI [16] and ii) prophylactic MTBVAV [17] are important developments. Currently available diagnostic methods for TB are i) Chest X ray ii) tuberculin test iii) Tb interferon release assay iv) sputum smear microscopy test v) fluorescent microscopy vi) culturing TB bacilli vii) molecular biology tools such as Gene Xpert and viii) serological methods. Of which serological methods are used world-wide because of technical simplicity, economical and easy less subjective. All the available diagnostic methods has its own merits and demerits and one such criticism for serological method is its false negativity and false positivity. This leaves us stranded that there is no accurate, ideal and reliable diagnostic method to diagnose TB and forces us to develop alternative methods with improved specificity and sensitivity. Our lab is one of the pioneering labs concentrating on developing ELISA based diagnostic methods for TB. In this report we evaluated the efficacy of 3 Mycobacterial secreted antigens namely PPE41, MPT53 and TB18.5 in diagnosing TB after cloning these genes from MTB-H37Rv strain. We tested the ability of these proteins separately or in combination. We found that a cocktail of all the 3 antigen could serve as diagnostic marker for TB diagnosis.

Efficacy of various MTB antigens in detecting MTB infections has been documented [14]. Review of the literature showed no previous report available which describes the efficacy of MTB proteins that we tested. In a study conducted by Welch, RJ (2012) a cocktail of MTB proteins namely MTB81, MTB8, MTB48, DPEP, the 38-kDa protein, and two additional proprietary antigens were tested for their efficacy [15]. In this study they found that 61.5% of the confirmed TB cases by their antigen cocktail preparation. When they combined this method and QuantiFERON-TB Gold In-Tube (QFT-GIT) the sensitivity improved to 86.7%. Though there was an improvement their sensitivity of their method, it is in par or slightly below the sensitivity of our method i.e.88%. In another study they found their sensitivity was 66.7% using a commercial ELISA (Strasbourg Cedex France) [16]. In an another study MTB antigen namely ESAT-6, 14 kDa, MPT63, 19 kDa, MPT64, and 38 kDa proteins were used as coating antigen for ELISA for the detection of MTB in tubercuous meningitis in CSF[17]. In clinical and culture positive CSF these antigens detected 72% of the cases suggesting that our method had a slightly higher edge over in diagnosis of MTB infections. Zhou, F. *et al.* (2015) used protein array system using glutathione S-transferase (GST)-fusion protein. In this study they found that this protein array system diagnosed 66.3% confirmed TB cases indicating the inferiority of their system [18].

Fujita, Y. *et al.* (2005) have used six lipid antigen isolated from M. bovis namely TDM-T, TDM-M, TMM-T, TMM-M, PL-1 and PL-2. They used these lipids in ELISA and found that they diagnosed 91.5 to 93.3% of the confirmed TB cases [19]. In another study Chan, ED, *et al.* (2000) used a lipoglycan namely lipoarabinomannan (LAM) as coating antigen [20]. In this study they found that 85-93% sensitivity in diagnosing TB. Even though these methods diagnose more clinical TB cases in general proteins are used predominantly since they are more potent immunogens. Besides that proteins not only used as diagnostic marker but also can be used as immunogens in vaccines. Because of the abundant false positivity and false negativity Government of India recently temporarily prohibited serology based diagnosis for the treatment.

However, serology cannot be completely abandoned because they offer easy and dependable diagnostic tool to detect MTB infection. Until then research should be continued to develop a novel yet reliable diagnostic serology method.

5. Conclusion

TB diagnosis is not 100% based on the assay system that currently available. Each system has its own merits and demerits. ELISA based diagnostic approach is the most preferred method because if it's technical simplicity, reliability and reproducibility. Various fractions of MTB namely proteins, lipids a lipoglycans has been tested as ELISA coating antigens. In this study we evaluated three recombinant proteins of MTB H37Rv namely PPE41, MPT53 and TB18.5 either separately or in different combination. Results of our study suggested that all these proteins can perhaps be used for diagnosis but at different degrees. As single protein, PPE41 outperformed the other antigens however a combination of all three proteins (cocktail) had the highest detection (88%) of MTB infections. Thus, antigen cocktail preparation can be used for the early diagnosis of MTB infections.

6. Acknowledgments

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