Efficacy of PPE41, A Secretory Protein of *Mycobacterium Tuberculosis* (MTb) in the Detection of MTB Infections

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Abstract: *Mycobacterium tuberculosis* (MTb) causes pulmonary tuberculosis and extrapulmonary tuberculosis which remains a major cause of morbidity and mortality throughout the world. MTb infections are ubiquitous and could be fatal if untreated. The gold standard detection of tuberculosis (Tb) is the culturing of the MTb but the limitation is that it is time consuming. Other methods such as tuberculin tests are all known yield equivocal results. With this background we attempted to diagnose MTb infection using PPE41 protein. PE/PPE genome accounts for 10% of all MTb genome. PPE gene cluster encodes for more than 70 PPE genes of which PPE41 is a well-studied gene as an immunogen in several animal studies. MTb-PPE41 gene is about 604 bp and protein is 21.9 kDa and it is member of type VII secretion system (T7S). PPE41 has been studied as vaccine candidate however its utility value as diagnostic marker has not been studied extensively. To evaluate the efficacy of PPE41 protein in diagnosing MTb infection PPE41 gene was amplified and cloned into pRSET A vector and subsequently transformed into *E. coli XL10* gold. From this recombinant PPE41 protein was purified using IDA Excellose columns. Purified PPE41 protein was used to coat the ELISA plates. To these plates serum from confirmed cases of MTb was added which was followed by detection of antibody tagged to HRP. Results of this study revealed that 57% of the MTb positive sera reacted with coated PPE41 antigen. None of the control sera reacted with PPE41. Thus PPE41 appears to be a promising candidate for the diagnosis of MTb infections.

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Introduction

*Mycobacterium tuberculosis* (MTb) causes significant morbidity and mortality worldwide. MTb causes tuberculosis (Tb) which primarily affects the respiratory tract however dissemination of the organism to the other organ can lead to extra pulmonary tuberculosis in which other organs are affected\(^{1-2}\). Worldwide, 9.6 million people are infected with Tb of which 12% are co-infected with HIV. Of the 9.6 MTb victims, 5.4 million are men, 3.2 million were women and 1.0 million were children\(^{3}\). 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\(^{4-5}\). A reliable vaccine against MTb is still in the horizon though several vaccine preparations are still under evaluation\(^{6}\). 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 diagnose Tb. Currently available diagnostic methods 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 *Genexpert* and viii) serological methods. The sequencing of the MTb*H37Rv* genome was completed in 1997\(^{7}\).This has led to the identification of several major antigens of MTb such as secreted antigens like Ag85, MPT64, MPB70, LPPX, CEP 10, ESAT-6 and heat shock proteins (HSP) such as HSP 60 and HSP 70. Some of these proteins are found to be highly antigenic and suitable vaccine candidates against Tb\(^{8}\).

Proteins secreted into the extracellular environment by *MTb* are usual target of immune response in the infected host. The ESAT-6 antigen, a 6 kDa protein was purified from a strong stimulatory, low-molecular-mass fraction of culture filtrate\(^{9, 10}\). In spite of the availability of all these various techniques challenges in interpreting results still exist. Additionally, usages of serological methods have several advantages such as technical case, cost effectiveness, easy availability even in the developing countries. However, these methods suffer severe criticism of false positivity and the Government of India has started banning serology alone-based way of diagnosis of Tb\(^{11-12}\). Serology provides a useful tool for monitoring diseases and do not require culturing of pathogen which usually takes 4-6 weeks. Among serological tools, ELISA has been the method of choice because of this high sensitivity, simplicity, reproducibility and versatility in screening a large number of specimens\(^{13}\). These facts suggest that the existing serological assays possess problems which need to be removed by development of newer sensitive and specific serological methods\(^{14}\). In this back drop the current study was aimed to detect MTb antibodies among Tb individual by using ELISA protocol. In the current study the Prolin-Prolin-Glutamic acid 41 (PPE41) antigens was used to coat ELISA plates and its efficacy in diagnosing MTb infection was evaluated.
Material and Methods

Cloning of MTb-PPE41 gene

*M. tuberculosis* 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'-AGAGTGTCTGTCAGCG-3') primers.

Transformation of ligated plasmids

Amplified and ligated product was cloned into pRSET-A 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 transformants on low salt LB agar containing 100 μg/ml ampicillin.

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 400μl lysis solution was added and the tube was gently inverted 5-10 times and allowed to stand at room temperature for 5 mins. To neutralize, 300 μl of cold 3M sodium acetate (pH 4.6) was added and mixed gently by inverting 10-20 times. After being maintained on ice for 15 minutes, the tube was centrifuged at 12,000 rpm for 10 min. and the supernatant was transferred to another tube. To this chloroform:isoamyl alcohol (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.

Expression and purification of 6X His-rPPE41 in E.coliBL21 (DE3)

The recombinant plasmid pRSETA: rv2430c 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 PPE41 was induced by 1.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) in mid-log phase (OD600 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 PPE41 as inclusion bodies, was washed three times in a 0.4 ml of buffer B [50 mM Tris, 10 mM EDTA and 0.5%(v/v) Triton X-100, pH 8.0]. Then the PPE41 inclusion bodies were solubilized in 1 ml of denaturing buffer [6 M guanidine hydrochloride containing 1 mM EDTA and 100 µl of 1 M phenylmethylsulfonylfluoride (PMSF) to give a final PPE41 concentration of 0.5-1.0 mg/ml. The expressed recombinant protein was purified by capturing the 6X His-PPE41 using spin clean chelating IDA Excellose column (1 mg/ml). The column was washed with washing buffer [20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole pH 8.0]. The quality of the expressed and purified rPPE41 was checked by Tricine- SDS-PAGE followed by staining with coomassie brilliant blue R-250 stain.

**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 –20°C 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 reactive to PPE41 in the serum of active tuberculosis patients and healthy control subjects.

**MTb-PPE41 ELISA for the detection of MTb antibody:**

96-well polystyrene microtiter ELISA plates were coated with 100 µl of PPE41 antigen (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 / H2O2 were added to each well and incubated for 20 minutes. Then 0.1 ml of 1 N H2SO4 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.

**Results**

**Cloning of PPE41 gene and purification of MTb PPE41 from MTb:**

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 GGA TCC ATGCATTTCGAAGCG-3’) and
reverse (5’ - AGGAATTCAAGTCTCTGACGCG -3’) primers. Amplified product (Fig.3) was cloned into pRSET-A (Fig.1) in frame with N-terminal His tag. Cloned pRSET-A was grown in *E. coli* X10 gold at 37°C overnight. From the overnight culture MTb PPE41 proteins were isolated. As a confirmation plasmid DNA isolated from overnight culture was restriction enzyme digested using Bam HI/ Eco RI and the digested fragment of 604 bp is shown in the Fig.4.

![Circular map of pRSET-A](image1)

**Fig: 1 Circular map of pRSET-A**

![Agarose gel electrophoresis](image2)

**Fig:2 Agarose gel electrophoresis of genomic DNA from MTb**

Lane 1,2 and 3: MTbH37Rv DNA
Fig: 3 Agarose gel electrophoregram of PPE41 PCR amplicons
Lane 1: 100bp DNA Molecular weight marker
Lane 2:  *PPE41* - 604 bp

Fig: 4 Agarose gel electropherogram of Restriction digestion enzymes
Lane 1: 100bp DNA Molecular weight marker
Lane 2:  pRSET A: *PPE41* (Bam HI/Eco RI)
Detection of MTb infection by ELISA:

In order to evaluate the efficacy of MTb PPE41 recombinant protein as coating antigen in ELISA protocol we coated the plates with 100 µl of 1 µg/ml of purified MTb PPE41. Then the ELISA was performed as mentioned in the methods and the reading was taken at 405 nm. The cut-off value was mean±3 Standard deviation of the negative control. In the present study, immune reactivity of recombinant PPE41 to antibody in the sera from clinically proven Tb patients (n=200) and healthy controls (n=100) were analyzed. Of the tested 200 MTb patients, 113 (57%) showed reactivity to MTb antigen PPE41 (Fig.5 and 6). As expected none of the healthy controls showed any reactivity which is represented as <1 in the graph.

From this study we conclude that MTb PPE41 is a potential antigen that could be further characterized as a diagnostic marker. However it needs to be admitted that our method detected only 57% of the Tb cases and it failed to detect the remaining cases. Hence the method needs to be further optimized so that the method can detect all the Tb cases.

Fig.5: Number of TB samples reactive with PPE41 antigen. ELISA plates were coated with MTb-PPE41 protein antigen and patient serum containing the MTb antibodies were allowed to react. This reaction was further detected by anti human IgG tagged to HRP and plates were read with 450 nm. All the Tb patient sera were confirmed Tb cases both clinically and smear positivity. Healthy controls showed no reactivity and represented as <1 in the graph.
Fig. 6: Percentage of TB samples reactive with PPE41 antigen. ELISA plates were coated with MTb-PPE41 protein antigen and patient serum containing the MTb antibodies were allowed to react. This reaction was further detected by anti human IgG tagged to HRP and plates were read with 450 nm. All the Tb patient sera were confirmed Tb cases both clinically and smear positivity. Healthy controls showed no reactivity and represented as <1 in the graph.

Discussion

MTb infections and Tb remains 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 Baccilie Calmette-Guérin (BCG) vaccine which is an attenuated strain of M. bovis. But the preparation is good against meningeal and disseminated tuberculosis in children, but its effectiveness against pulmonary form in adolescents and adults is argued [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. Those genes that are incorporated in the vaccine constructs are also studied for their level of detection during diagnosis. Under these several genes have been studied for their diagnostic efficacy. Of these, PE/PPE41 gene cluster encodes 10% of MTb’s genome[18]. Of which ProlineProline Glutamic Acid (PPE41) has been described for its efficacy in inducing good immune response against MTb infection in animal studies[19]. In this study we evaluated the efficacy of MTb PPE41 antigen as a diagnostic marker of MTb infections. PPE41 is a gene which is a part of PPE gene cluster. PPE41 gene is about 604 bp long and its protein is about 21.9 kDa in size. For this we evaluated the efficacy of PPE41 in diagnosing MTb infection by testing the sera...
of patients suffering clinical Tb. Results unfolded that PPE41 based ELISA protocol detected only in 57% of cases where as it was ineffective against the remaining cases (false negativity). Thus the study implies that this protocol requires considerable modifications to diagnose better.

MTb utilize type VII secretion systems (T7S) to secrete proteins across their complex cell envelope. These organisms have up to five of these secretion systems, named ESX-1 to ESX-5[20]. RD1 gene locus of virulent MTb encode virulent genes namely early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) complex secreted via secretory locus called ESX-1[21]. ESX-1 locus also contains a pair of genes encoding PE/PPE proteins. PE/PPE proteins are coded by a family of genes that include PE, PPE and PGRS (or PE/PPE/PGRS) and these represent 10% of the coding capacity of the mycobacterial genome. Using the proteins of those gene products several serological assays were devised to diagnose MTb infection. These antigens were primarily chosen because of their broad spectrum immune induction activities in several animal models[22]. These protein antigens of MTb are devised to detect either MTb specific IgM or IgG antibodies. Among them commonly used antigens are 38 kDa PhosP[23], 30kDa antigen 6, 85B[24], 16 kDa Protein [25], lipoarabinomannan (LAM)[26], A60antigen [27], and Mtb81 [28] which were characterized, purified and independently tested with sera obtained from tuberculosis patients by different investigators. Previous studies have also used the following for the sero-diagnosis of MTb. They are ESAT-6, CFP10, Mtb8.4, MPT64, TB16.3 and Mtb8 [29]. Besides several commercial ELISA kits were also developed almost same time. All these studies detected MTb infections at varying degree but their strength of diagnosing all the 100% infections is still in the horizon.

There are no reports in the literature studying MTb-PPE41 as a coating antigen in an MTb ELISA. Because of that we could not do a direct comparison of our results with any. Though not exactly the same gene a closer antigen ESAT6 was as tested for its efficacy as ELISA coating antigen[30]. In that study they found that ESAT6 diagnosed 57% of Tb cases. Interestingly we also detected similar proportion of MTb cases. In another study conducted by Keertan Dheda et al. (2010)[31], lipoarabinomannan (LAM) was evaluated for the Tb diagnosis among HIV infected patients. They found that 6% of the Tb patients were diagnosed by this method. However the LAM-ELISA detected 21% of the cases when the patient was co-infected with HIV. When LAM ELISA was compared to our PPE41 ELISA, the latter had several fold higher upper hand in MTb diagnosis. Das PK et al.(1989) attempted to use whole bacteria sonicated lysate to coat the ELISA plate and found to be useful in diagnosing MTb infection however the exact percentages were not provided. Besides their attempt was to compare MTb and M. leprae [12]. These are the few reports in which MTb antigens were used for coating the ELISA plate for the diagnostic purpose.

MTb-PPE region contains more than 70 genes of which PPE41 is one of the most characterized protein. This protein has been well studied for its immunogenicity and its utility as vaccine candidate. But is efficacy as a diagnostic marker has not been studied much. It is with this idea MTb-PPE41 antigen was chosen for the evaluation as diagnostic marker for MTb induced Tb. The study showed sera from significant percentage of individuals (57%) reacted to PPE41 albeit could have been better. However this study needs further optimization to improve its sensitivity
and specificity nearing 100%. Thus PPE41 based diagnosis of MTb infection would serve as a preliminary platform to improve MTb diagnosis.

**Conclusion**

ProlinGlutamic Acid 41 (PPE41) is a member of T7S system which gets secreted from the virulent MTb. In several animal studies, PPE41 protein generated both cell mediated immunity and humoral immunity. Early diagnosis of Tb can completely cure the infection but the real problem is the diagnosis of Tb. False negativity is common during smear examination and tuberculin test. Other problem with tuberculin test is its false positivity among individuals who has been vaccinated with BCG. Also Mantoux test cannot distinguish infections whether they are due to virulent strains or avirulent counter parts. Gold standard is MTb culture but is a time consuming option. There is a need to diagnose MTb infection early and accurately. With this background the current study was conducted to use PPE41 as a diagnostic marker. Results indicated that perhaps PPE41-based ELISA detected 57% of the MTb cases while leaving a similar proportion undetected. Further studies are thus needed to optimize to serve us better. Thus PPE41 based system could be considered as a potential method to early diagnosis of MTb.

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