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DIAGNOSTICS

Assessing humoral immune response of 4 recombinant antigens for serodiagnosis of tuberculosis

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SUMMARY

Serodiagnostic potential of four recombinant proteins (38 kDa[Rv0934], MPT64[Rv1980c], Adk[Rv0733], and BfrB[Rv3874]) was evaluated in Healthy control subjects (HCS), Healthy household contacts (HHC), Pulmonary tuberculosis patients (PTB), and Human immuno deficiency virus & Tuberculosis co-infected patients (HIV-TB). All the antigens tested individually for the detection of serum IgG by indirect ELISA. All the four antigens have a significantly higher antibody response in PTB compared to healthy controls ($P < 0.05$). The sensitivity of individual antigens ranged from 20% to 52.5% for the prefixed specificity of 95%. When results of all 4 antigens were combined the sensitivity was increased to 75% and specificity was reduced 89% in HCS. In smear- and culture-positive (S+C+) PTB, four antigen combination gives maximum sensitivity (89.6%) with 89% specificity. In smear negative culture negative (S−C+) PTB, three antigen combination (38 kDa with MPT64 and BfrB) gives maximum sensitivity (69.5%) and specificity (91.6%). In HIV-TB, 4 antigen combinations give the maximum sensitivity of 51.2% with 89% specificity. Combining serology (Four antigen combination) with smear was able to increase the sensitivity from 70% to 92.5% in culture positive PTB. So, we propose that this serology test can be used as adjunct test along with smear for rapid diagnosis of PTB.

1. Introduction

Mycobacterium tuberculosis is the most successful human pathogen and is the primary cause of tuberculosis (TB) in the world. Globally, about 2 billion people are infected with *M. tuberculosis*, 8.3–9 million develop active disease, with 1.3 million die from TB every year. WHO estimates that 8.6 million new cases of TB occurred in 2012 (122 per 100,000 population) [1]. Nearly 40% of the Indian population is infected with the TB bacilli. India alone accounts for an estimated one fourth (26%) of all TB cases worldwide [1]. Over 90% of the tuberculosis cases occur in the developing countries, where clinical diagnosis of tuberculosis is based primarily on microscopic examination of smears for acid-fast bacilli.

Acid-fast bacillus smears are positive only during advanced tuberculosis, when there are >5000 bacilli per ml of sputum [2]. Moreover, sensitivity of microscopic method is low, in extra pulmonary TB (EPTB), paediatric cases, and patients co infected with HIV [3]. Although mycobacterial culture is much more sensitive than microscopy and usually considered the gold standard for TB diagnosis, it is time consuming (6–8 wks) and importantly, in 10–20% of positive cases, the bacillus is not successfully cultured which ultimately affects its sensitivity [4]. Recent advances in solid media culture optimized the culture conditions for antibiotic susceptibility testing and obtained results in less than seven days but optimization of each step involved found to be complicated [5].

In recent years, *in vitro* assays that measure T-cell release of interferon γ (IGRA) in response to *M. tuberculosis* specific antigens such as early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) have been carried out to detect active and latent TB infection (LTBI). However, their performances are affected in immunosupressed cases such as HIV infected individuals [6]. In addition, IGRAs cannot differentiate latent and active TB disease [7]. Hence the usage of IGRA in high TB endemic countries is still limited.

It has been estimated that approximately 90% of TB patients were able to produce antibody against *M. tuberculosis* proteins [8,9]. Antibody based serological assays are considered to be attractive as they are simple to perform, cost-effective and easily implementable

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even in developing countries. The detection of *M. tuberculosis* specific human antibodies has been an important diagnostic aid in the diagnosis of TB. Efforts to exploit antibodies for TB diagnosis were carried through the decades and promising antigens have been identified in recent years [10,11].

First generation antibody detection tests were based on crude mixtures of constituents and products of *M. tuberculosis*, such as Culture Filtrate Antigens (CFAs) and purified protein derivative (TST) [12]. Since the antigens used in these preparations were shared by different bacterial species, which led to low specificities [13]. This problem of low specificity was improved by using purified and recombinant antigens specific for the TB complex [11].

To develop serological methods for the diagnosis of TB, different mycobacterial antigens such as antigen 5 (38-kDa antigen, Rv0934), P32 antigen (Rv3804c), 30-kDa antigen (Ag85B, MPT59, Rv1886c), cord factor, 88-kDa antigen (MTB81, Rv1837c), a 27-kDa antigen (MPT51, Rv3803c), ESAT-6, CFP-10 and LAM have been evaluated [14]. Among these antigens, ESAT-6, CFP-10 are *M. tuberculosis*-specific region of deletion 1(RD1) antigens. Serological assay using these antigens in high endemic settings like china and India exhibited high specificity ranged from 96% to 100% respectively [15–17].

It is a well known fact that the antibody responses to *M. tuberculosis* antigens are heterogeneous among individuals. For that reason, detection of antibodies against single antigens usually has a low sensitivity for diagnosis of TB.

Recently, the WHO published a policy statement regarding commercial serodiagnostic tests for diagnosis of tuberculosis. Based on a bivariate meta-analysis of commercially available tests, including 67 studies, the authors of the WHO statement concluded that *M. tuberculosis* antibody tests should not be used for the diagnosis of pulmonary and extrapulmonary *M. tuberculosis* infections [18]. Perhaps, the Expert Group strongly encouraged further research to identify new/alternative serological tests with improved accuracy. It is now generally recognized that serodiagnosis may be improved by the identification and inclusion in a cocktail of a number of antigens that react with the sera from a high proportion of infected individuals [19]. Genomic sequencing studies have helped in identifying 3924 protein coding open reading frames (ORF) in a virulent *M.tb* strain [20]. Among those, only very few have been screened for diagnostic purposes. A plenteous of antigens of *M.tb* with unknown functions has not been studied for their immunodiagnostic role. Thus, the requisite improvements in detection rate may be achieved by the screening of additional immunodiagnostic *M. tb* antigens, which could be included in the multiantigen cocktails.

In the current study, we evaluated the serodiagnostic potential of 4 recombinant proteins (PstS1, MPT64, Adk, and BfrB). The 38 kDa (PstS1) is an *M.tb* species specific secreted glycoprotein having epitopes shared very less with other mycobacterial antigens [12]. Serodiagnostic potential of 38 kDa was evaluated by many investigators [12,21–23]. The specificity of 38 kDa was tested in a vertical study by immunodetection on western blots revealed that it may contain B-cell specific epitopes, which confers for its immunodominance that can be detected by serology [24,25]. The sensitivity and specificity of 38 kDa have been reported to ranging from 37% to 74% and 98%–78% respectively largely depending on the sputum smear status of the patient and the patient population used in the studies and disease manifestations [22,26]. So, in this current analysis we included that as one of the standard reference antigen.

MPT64 is another antigen tested in our analysis. MPT64 (Rv1980c) is an RD2 gene product and a secreted protein of *M. tuberculosis.* A growing body of evidence implicates that MPT64 antigen of *M.tb* has either been used or investigated for its use in biomarker, diagnostic agents for TB diagnosis [27–29]. The sensitivity and specificity of MPT64 in those studies ranged from 74% to 80% and 34%–74% respectively [19,30]. MPT 64 has also been included as one of the component in the fusion protein antigen for improved diagnostic performance. Recently Feng et al. [31], reported that a poly protein comprises of 38 kDa and MPT64 are suitable for diagnosing active tuberculosis with sensitivity of 70.4% with 91.5% specificity by combining three isotypes (IgG,IgM and IgA).

In our earlier immunological analysis, 10 of the 2-Dimensional liquid phase electrophoresis (LPE) separated culture filtrate fractions of *M.tb* were able to induce higher IFN-γ response in latent tuberculosis population compared to active TB patients [32]. Those fractions were considered as "contact specific" (CS) fractions. Sixteen antigens which were able to identified in those fractions. Adk was one among the three novel T cell antigens present in the contact specific fractions. Moreover, Adk and BfrB were among the 24 novel T cell antigens in identified overall in that study [32]. Followed by that study, we did immunological and insilico characterization of Adk antigen [33]. In that study, using bioinformatics tools, we reported that Adk possessing high affinity peptides to both MHC class I and class II alleles and has the highest population coverage (97.24%) [33]. BfrB was earlier reported as novel B cell antigen [34]. The two antigens selected for the current study have been reported to be strong targets for humoral and cell-mediated immune responses [35,36].Thus, in this present study, we wanted to explore the B cell response of Adk and BfrB.

2. Materials and methods

2.1. Study population

This study was approved by Institutional Ethical Committee of National Institute for Research in Tuberculosis (NIRT), Chennai. All the study subjects were informed about the study procedure and the volunteers were assessed for this study.

A total of 448 subjects under five groups were included in this study.

2.1.1. Healthy control subjects (HCS) ($n = 100$)

Apparently normal healthy subjects without any clinical symptoms of TB were selected from families where there were no cases of pulmonary tuberculosis living in the same household, or in the neighbourhood. The study subjects were recruited from offices, schools, colleges and slum areas in Chennai city and nearby villages. Since our settings is endemic to TB, first to rule out the suspicion of active TB disease, all the subjects were asked to give three sputum samples and subjected to radiological and clinical examination. All the study subjects were negative for sputum smear microscopy. They had normal X-ray and clinical examination was also found normal. After registering the eligible subjects ($n = 100$), blood was drawn from all the study subjects. In order to rule out latent TB infection, TST and QFT-IT were carried out after drawing the blood. There were only 48 subjects out of 100 HCS recruited were negative for both TST and QFT-IT. This implies that these subjects have not been exposed to TB and unlikely to get infected by TB. For analysis, we assorted these subjects as healthy control subjects since they were devoid of TB infection.

2.1.2. Healthy house hold contacts (HHC) ($n = 108$)

Study subjects were identified by visiting the households of adult smear positive pulmonary TB patients who were enrolled for treatment at NIRT. Apparently healthy household contacts (HHC) were selected from families where there was at least one case of sputum positive pulmonary tuberculosis living in the same household, for at least 3 months immediately preceding the start of