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Dynamic IgG antibody response to immunodominant antigens of *M. tuberculosis* for active TB diagnosis in high endemic settings

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ABSTRACT

Background: Even though various techniques have been developed for rapid diagnosis of tuberculosis (TB), still there is an immense need for a simple, cost effective, highly sensitive and specific test. Hence, one of the possibilities is identification of *Mycobacterium tuberculosis* specific antibodies in infected serum by using specific antigens.

Methods: We tested 10 recombinant *M. tuberculosis* antigens to evaluate IgG levels among Healthy control subjects (HCS), Healthy household contacts (HHC) and pulmonary TB patients (PTB) by ELISA.

Results: The median IgG levels specific to all the antigens are higher in PTB than HHC and HCS. Amongst single antigens, 38-kDa antigen has showed maximum sensitivity of 50% than any other antigens at 95.5% specificity. Among the two antigen combination, 38-kDa + Rv1860 has showed maximum sensitivity of 66.6% with specificity of 92.2%. The same antigen combination (38-kDa and Rv1860) predominantly identifies smear negative and culture positive TB patients with 68% sensitivity and 92.2% specificity. Most of the antigens have exhibited higher antibody titre in cavitary TB than non cavitary. With regard to latent TB infection (LTBI) identification, Rv1860 has exhibited maximum sensitivity of 53.3% with 95% specificity.

Conclusions: IgG response to combination of recombinant mycobacterial antigens (38-kDa, Rv1860, Rv2204c and Rv0753c) presents good specificity with acceptable level of sensitivity for TB diagnosis.

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1. Introduction

According to World Health Organization (WHO) global tuberculosis (TB) 2015 report, one-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of human TB. It is estimated that ~9.6 million people developed TB and 1.5 million died from TB in 2014 [1]. India alone accounted for 24% of total cases among the 95% of TB deaths that occurred in low- and middle-income countries [1]. Targets for the post-2015 global TB strategy include a 95% reduction in TB deaths and a 90% reduction in TB incidence by 2035. To achieve these targets, implementation of new diagnostic tools is utmost important. There are two major gaps in the existing diagnostic tools: lack of simple and accurate point of care (POC) test for TB that can be used for rapid diagnosis at the primary care level; and lack of a biomarker (or combination of biomarkers) that can be used to identify latently infected individuals who will benefit from preventive therapy. Although acid-fast staining of bacilli in sputum smear is a simple and relatively fast test, it has reduced sensitivity and requires >104 bacilli per ml of sputum for the reliable detection of active TB [2]. Culture test is considered as the gold standard diagnostic test for active TB, however results take weeks to obtain and it is expensive and need a well-equipped laboratory, trained staff, and an efficient transport system to ensure viable specimens. Moreover about 5–10% active TB cases often gives false negative results [3]. Although chest X-ray can be useful for the diagnosis of active TB, it is not specific.

To make the diagnosis of TB as more accurate, rapid and convenient, new diagnostic techniques such as molecular methods and immune reactions based on cell-mediated-immune (CMI) or humoral immune response have been investigated. Polymerase chain reaction (PCR) based molecular methods for detecting *M. tuberculosis*-specific nucleic acids, especially WHO endorsed GeneXpert MTB/RIF have revolutionized the diagnosis of active TB and rifampicin resistant TB. However, they are costly and require technological investment [4]. Recently introduced T cell immune based assay, Interferon Gamma Release Assays (IGRA) have been successful for detection of *M. tuberculosis* infection, by virtue

Abbreviations: M. tb, *Mycobacterium tuberculosis*; LTBI, latent tuberculosis infection; WHO, World Health Organization; DOTS, Directly Observed Treatment Short Course; TST, tuberculin skin test; IGRA, interferon-gamma release assay; PPD, purified protein derivative; HCS, healthy control subjects; HHC, healthy house-hold contacts; PTB, pulmonary tuberculosis; QFT-GIT, Quantiferon TB Gold In Tube assay; TMB, tetramethylbenzidine.

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of their higher sensitivity and specificity than TST [5]. Although, IGRAs show great sensitivity in detecting latent TB infection (LTBI) and active TB cases, their performance is affected in immunosupressed individuals [6]. In addition, IGRA cannot differentiate between LTBI and active TB disease [5,7], which forbid their usage in high endemic settings.

Lack of effective diagnostic tests is responsible for the delay in TB diagnosis, which leads to progression of the disease and ultimately becomes the potential source for *M. tuberculosis* transmission. Humoral immune-based tests confer advantages over the conventional methods due to their speed (results may be available within hours) and simplicity [8]. It has been demonstrated that identification of *M. tuberculosis* specific antibodies in the infected serum by enzyme linked immune sorbent assay (ELISA) is highly sensitive and reproducible technique [9]. Serodiagnosis is characterized by convenient sampling, low costs, easy operation, and rapid determination which can be implemented in clinical laboratories in resource-constrained settings, where access to diagnostic instruments is limited and cost efficiency has high priority [10].

1.1. Rationale and hypothesis of the study

Selection of antigen and its antibody isotype are the key elements for any successful serological assays. Most of the serological assays for active TB diagnosis use a single antigen (38-kDa protein), which may not be recognized uniquely by the host immune system. Since, there is significant heterogeneity in antigen recognition and response, the diagnostic efficacy of single antigen is hampered. However, it can be improved by identification and inclusion of cocktail antigens which will react with the sera of most of the infected individuals [11,12]. Currently, number of *M. tuberculosis* antigens has been characterized for serodiagnosis of active TB. Unfortunately, none of the antigens showed as the promising candidate for active TB diagnosis. Identification of additional immunodominant antigens to enhance the performance of sero-diagnostic tests is of great interest in the field of TB diagnosis. Therefore, it is necessary to test immuno-dominant antigens for improving the accuracy of TB identification by serodiagnosis. Many immuno-dominant antigens have been identified by various stringent techniques for serodiagnosis of TB disease [13–15]. In this context, our laboratory has identified various secreted *M. tuberculosis* antigens based on their ability to provoke cellular immune response [16]. Most of these antigens act as both T cell and B cell antigens. Amongst, we have selected 10 antigens (Rv2204c, Rv3716c, Rv0753c, Rv0009, Rv1860, Rv2626c, Rv3914, Rv1908, Ag85a and 38-kDa) for this study, since there is no serodiagnostic data on most of the selected antigens. In this study, we have cloned, over expressed and purified these 10 antigens and evaluated IgG response among Healthy control subjects (HCS), Healthy house-hold contacts (HHC) and pulmonary TB patients (PTB) by ELISA. We checked the usefulness of these antigens in the identification of active TB and differential diagnosis of latent and active TB. In addition, we also analysed dynamic IgG response to the selected antigens for the identification of different stages of active TB based on smear and cavity status.

2. Materials and methods

2.1. Antigen preparation

The details of recombinant proteins, expression vectors and expression strains of *Escherichia coli,* used in this study were showed in Table 1. As described previously, the expression and purification of recombinant proteins were done by standard protocols followed in our laboratory [17–19].

Table 1
Recombinant antigens of *M. tuberculosis* used in this study.

Protein name	Gene number	Molecular mass (kDa)	Expression vector	Expression strain of *E. coli*
38-kDa	Rv0934c	38	pET23a	BL21(DE3)pLysS
Ag85a	Rv3804c	36	pET23a	BL21(DE3)
mpt32	Rv1860	32.7	pET23a	BL21(DE3)
Rv2204c (hp)	Rv2204c	12.5	pRSET-A	BL21(DE3)
mmsA	Rv0753c	55	pRSET-A	BL21(DE3)
PpiA	Rv0009	16	pRSET-A	BL21(DE3)pLysS
Trxc	Rv3914	12.5	pET23a	BL21(DE3)pLysS
Rv2626c (hp)	Rv2626c	15.5	pET23a	BL21(DE3)pLysS
KatG	Rv1908	80.5	pET23a	BL21(DE3)pLysS
Rv3716c (hp)	Rv3716c	13.3	pRSET-A	BL21(DE3)pLysS

hp - hypothetical protein.

2.2. Study population

This study was approved by institutional ethical committee established by National institute for Research in Tuberculosis (NIRT), Chennai. All the subjects provided written informed consent before drawing the blood. Subjects with previous history of TB, those under immunosuppressive therapy were excluded from this study. All the subjects were confirmed as human immunodeficiency virus (HIV) negative by routine acquired immunodeficiency syndrome (AIDS) tests. HIV positive individuals, patients with diabetes, cancer, autoimmune diseases or other conditions that may affect the immune system of the individual, pregnant women, children, and individuals with indeterminate Quantiferon-TB Gold In-Tube test (QFT-GIT) results were also excluded from the study. A total of 270 subjects were included in three groups.

2.2.1. Healthy control subjects (HCS)

Ninety serum samples were collected from control subjects. All the enrolled HCS were from families where there was no history of TB and know exposure to TB; and they were recruited from offices, schools, colleges and slum areas in Chennai city and nearby villages. Active TB among HCS were ruled out by negative sputum smear microscopy, had normal chest X-ray and without clinical symptoms. Tuberculin skin test (TST) and QFT-GIT were performed in healthy controls to rule out LTBI in controls.

2.2.2. Healthy house hold contacts (HHC)

Ninety study subjects were recruited from families where there was one sputum positive case (index case) staying in the same household for at least 3 months immediately before the start of the anti TB treatment of the index case. These study subjects were identified by visiting the households of adult smear positive pulmonary TB patients who were enrolled for treatment in Revised National Tuberculosis Control Program (RNTCP) centres, Chennai. Disease free status was ruled out by negative sputum smear, normal X-ray and without clinical symptoms. Latent infection was confirmed by both TST and QFT-GIT. Among the HHCs, 58 were showed as positive by both TST and QFT-GIT. In the present study both TST and QFT-GIT positive HHCs subjects only considered as latently infected individuals. The subjects of this group were followed up for possible breakdown to TB and at the end of 6 months found to be healthy.

2.2.3. Pulmonary TB patients (PTB)

For this study, 90 Pulmonary tuberculosis patients (PTB) were recruited from RNTCP centres, Chennai, India. Two spot and one overnight sputum specimens were collected from each patient. The presence of active TB was defined as Ziehl-Neelsen-stained sputum smear positive or positive for mycobacterial culture or positive for chest X-ray. Among the 90 PTB patients, 65 patients were positive for both smear and culture; and 25 were negative for smear and positive for culture. Out of 90 patients, cavities were found in the lungs of 13 patients and 40 did not show cavities in the lungs. The cavity status of majority of the patients (37) was unknown. TB patients with multidrug-resistant tuberculosis (MDR-TB) infection, active TB patients with more than

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