

## T7 Phage Display Library a Promising Strategy to Detect Tuberculosis Specific Biomarkers

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### Abstract

One-third of the world's population is infected with tuberculosis, only 10% will develop active disease and the remaining 90% is considered to have latent TB (LTB). While active TB is contagious and can be lethal, the LTB can evolve to active TB. The diagnosis of TB can be challenging, especially in the early stages, due to the variability in presentation and nonspecific signs and symptoms. Currently, we have limited tools available to diagnose active TB, predict treatment efficacy and cure of active tuberculosis, the reactivation of latent tuberculosis infection, and the induction of protective immune responses through vaccination. Therefore, the identification of robust and accurate tuberculosis-specific biomarkers is crucial for the successful eradication of TB. In this commentary, we summarized the available methods for diagnosis and differentiation of active TB from LTB and their limitations. Additionally, we present a novel peptide microarray platform as promising strategy to identify TB biomarkers.

**Keywords:** Tuberculosis; TB; Latent TB; Biomarker; Serum antibody; Peptide microarray; T7 phage display

### Introduction

*Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), is surrounded by a cell wall rich in waxes and lipids which contributes to its high resistance to treatment and provides a survival benefit under unfavorable conditions either in the environment or in the host [1]. Mycobacteria require high oxygen tension to grow, thus the respiratory system provides the best environment for *Mtb* to grow and to spread through exhalation and expectoration [2]. Alveolar macrophages (AMs) in the lungs are the reservoir of *Mtb*. The host response to *Mtb* infection involves a complex interplay between mononuclear phagocytes, T and B lymphocytes. Initially *Mtb* are taken up into phagosomal compartments of macrophages, then undergo processing and presenting mycobacterial antigens to the surrounding T lymphocytes [3]. Activated T cells secrete cytokines and chemokines to keep the macrophages in an activated state and recruit other immune cells to the site of infection [4]. CD4<sup>+</sup> T cells are considered to play an important role in mediating a protective cellular immune response against *Mtb* by secreting cytokines and also serve as cytotoxic effector cells capable of directly lysing targeted cells. Additionally, B lymphocytes are a prominent component of tuberculosis granulomas and emerging evidence suggests that humoral immunity plays an important role in modulating immune responses against *Mtb* [5-7]. This suggests that serum antibodies are equally important as T cell based immunity in combating mycobacterial infections.

Tuberculosis (TB) remains the most common infectious disease and global health problem. The World Health Organization (WHO) estimated nine million new cases and 1.5 million deaths from TB in 2013 [8]. Based on the WHO data one-third of the world's population

is infected with *Mtb* complex and considered to have latent TB (LTB). The host's immune system typically prevents the organism from spreading beyond the primary site of infection, approximately 5 to 10% of these latent *Mtb* infections progress to active disease. Active TB is contagious and lethal with a mortality rate of greater than 50% in untreated individuals [8]. Therefore, early diagnosis of active TB is a crucial step in the success of treatment. The diagnosis of TB can be challenging, especially in the early stages, due to the variability in presentation and nonspecific signs and symptoms. In patients with smear negative TB early detection is even more difficult because of low numbers of bacilli in clinical samples [9]. Furthermore, there are limited tools available to predict treatment efficacy and cure of active tuberculosis, the reactivation of latent tuberculosis infection, and the induction of protective immune responses through vaccination. Therefore, the discovery of robust and accurate tuberculosis-specific biomarkers is crucial for the successful elimination of TB.

Here we summarize the conventional approach and new perspectives in TB detection. Additionally, we present a peptide microarray platform as a promising strategy focusing on the interaction between innate immunity and the humoral immune system to identify TB serum biomarkers. This may enable us to discover unknown epitopes targeting *Mtb* antigens leading to a better understanding of host defenses against *Mtb*.

Direct sputum smear microscopy is most commonly used to diagnose active TB. Microscopic examination of sputum is relatively rapid, inexpensive, and widely available for routine diagnosis of TB in developing countries. However, sputum smear microscopy is only 60-70% sensitive compared to the higher sensitivity of sputum culture for active TB. The sputum culture can take up to 8 weeks to obtain results and has a limitation in detecting extra-pulmonary TB. Importantly, this test cannot identify individuals with latent TB infection. Recently, the WHO endorsed the GeneXpertMTB/RIF test

for the detection of mycobacteria in sputum [10]. This test is a cartridge-based, automated diagnostic test that can rapidly identify *Mtb* DNA and its resistance to rifampicin, by nucleic acid amplification technique (NAAT) in less than 2 hours [10,11]. The cost and complexity of the GeneXpert is a concern for broad based implementation in low-resource settings. For rifampicin resistance detection, Xpert MTB/RIF provides accurate results and can allow rapid initiation of multidrug resistant treatment. This assay has lower sensitivity towards smear-negative pulmonary and extrapulmonary diseases [11-13]. However, this test cannot detect latent TB.

### Cytokine Based Tests Assessing Predominantly T cell Response

Two Interferon gamma release assays (IGRAs) approved by the U.S. Food and Drug Administration (FDA) are the QuantiFERON-TB Gold In-Tube test (QFT-GIT) and the T-SPOT TB Test (T-Spot) [14,15]. Both tests assess IFN $\gamma$  production of T cells after in vitro stimulation of whole blood or PBMCs with *Mtb* specific antigens such as ESAT6, CFP10 and TB7. The results can be obtained within 24 h. Major shortcomings of the QFT and T-Spot are the lack of its ability to distinguish between latent TB and active TB as well as low performance in endemic area in immunocompromised patients and children less than five years of age [16]. Dual-color Reverse Transcriptase-Multiplex Ligation dependent Probe Amplification (Dual-color RT-MLPA) is another assay that utilizes whole blood to evaluate the quantitative changes in gene expression profiles. This assay can differentiate between active TB, treated TB, latent infection or healthy controls, but needs further validation in larger prospective cohort studies [17].

### Serological Biomarkers for Active Tuberculosis

Serological tests based on the detection of circulating antibodies against *Mtb*-specific antigens have several theoretical advantages, as they are simple, cheap, and feasible for point of care diagnostics. These tests are developed using specific TB antigens. However, comparative available studies have sensitivities ranging from 0.09% to 59.7% with specificities from 53% to 98% [18,19]. Similarly, strategies to detect various circulating cytokines in serum or antibodies in urine samples as adjunctive biomarkers for the differential diagnosis of active and latent TB as well as non-tuberculosis mycobacteria diseases (NTM) [20-24] yielded less reliable results.

### Role of B Cells and Serum Antibodies in Host Defense Against *Mtb*

The protective role of B cells and humoral immune responses in tuberculosis has been regarded as inferior to the cellular immunity directed towards the intracellular pathogen *Mtb* [25]. Despite being a facultative intracellular pathogen, *Mtb* is potentially susceptible to various mechanisms of antibody-mediated immunity. Opsonization through Fc $\gamma$ R was shown to promote phagolysosomal fusion and intracellular killing [26,27]. Besides the well-known role of B cells in providing a robust T cell response against *Mtb* through secretion of various cytokines and chemokines, recent studies highlight the importance of B cells in the modulation of neutrophil function and granuloma formation [7,28-30]. In animal models, there is increasing evidence that mice with defective humoral immunity are more susceptible to TB. Additionally, passive transfer of antibodies to *Mtb* antigens protects mice against TB [31,32]. Interestingly, Ashenafi et al. [30] found that BCG-specific IgG-secreting peripheral plasmablasts

could be successfully utilized as a host-specific biomarker for TB diagnosis, even in subjects with impaired T cell function, including HIV positive subjects, and subjects with culture negative TB. Thus, the detection of *Mtb*-specific antibody responses in sera of TB patients provides an important step not only for TB diagnosis but also for the development of new vaccines and monitoring of the immune response after vaccination. There is a need for a new conceptual approach to understand the complex host immune response to *Mtb*, beyond cell-mediated immunity, including the mechanisms of humoral immunity and antibody formation.

Peptide microarray technology allows the testing of several thousand unique epitopes displayed as linear peptides on a slide to detect humoral immune responses in an unbiased fashion. In our lab, we developed a high throughput method using T7 phage display cDNA library [33]. This cDNA library was derived from mRNA isolated from bronchoalveolar lavage (BAL) cells and leukocytes of sarcoidosis patients. The combination of BAL cells and leukocytes likely contains all cellular players of the immune response against pathogens including antigen presenting cells (APC), T and B cells, and neutrophils. Thus, this method utilizes the immune repertoire, which can be immunoscreened using serum as the source of antibodies to identify disease-specific antigens (Figure 1).

This library was differentially biopanned with healthy control sera and sarcoidosis sera to enrich for sarcoidosis specific antigens. A microarray of these antigens was constructed and then immunoscreened with the sera of healthy controls and sarcoidosis patients. Surprisingly, by immunoscreening the same microarray platform with the sera of culture positive TB subjects, we identified 50 antigens that differentiated between TB, sarcoidosis and healthy controls [33]. Sarcoidosis is a granulomatous disease with striking clinicopathological similarities to TB [34,35]. Proteomic and genomic studies to identify sarcoidosis antigens led to identification of various *Mtb* related antigens including mycobacterial catalase-peroxidase (mKatG) indicating *Mtb* as a potential etiologic factor in sarcoidosis [34-41]. The fact that immunoscreening of our T7 phage cDNA library derived from polyA mRNA obtained from BALs and leukocytes of sarcoidosis patients could identify a panel of specific antigens to classify TB from sarcoidosis and healthy controls, suggests of the presence of TB antigens in cDNA library [33].

T7 phage display method has several advantages; the life cycle of T7 phage is far faster so that the process of cloning is more rapid. Additionally, several libraries can be pooled and screened in parallel. We used the bar coding strategy for the identification of T7 phage clones from a pool of T7 phage cDNA libraries [33]. Immunoscreening requires only minimal amount of serum and it is relatively cheap and non-invasive. Additionally, same platform can be immunoscreened with various categories of patients, including immunocompromised patients. In the process of immunoscreening, we have identified 164 antigens specific for smear negative but culture positive TB and exclusively recognized by antibodies present in TB sera. These results provide evidence that TB specific antibodies are generated and can be detected in TB patients and this may prove to be useful in developing biomarkers to diagnose TB as well as to differentiate between active TB and latent TB. Our hypothesis is that latent TB versus active TB has a different immune response profile to our T7 phage cDNA antigen library and that it can be characterized by a reasonable small set of biomarkers. These set of biomarkers will enable us to distinguish active TB from latent TB and non-infected sera. Furthermore, the specific TB antigens that were identified in our lab using T7 phage display cDNA

library may aid to develop a TB vaccine that will generate specific humoral response against *Mtb*.

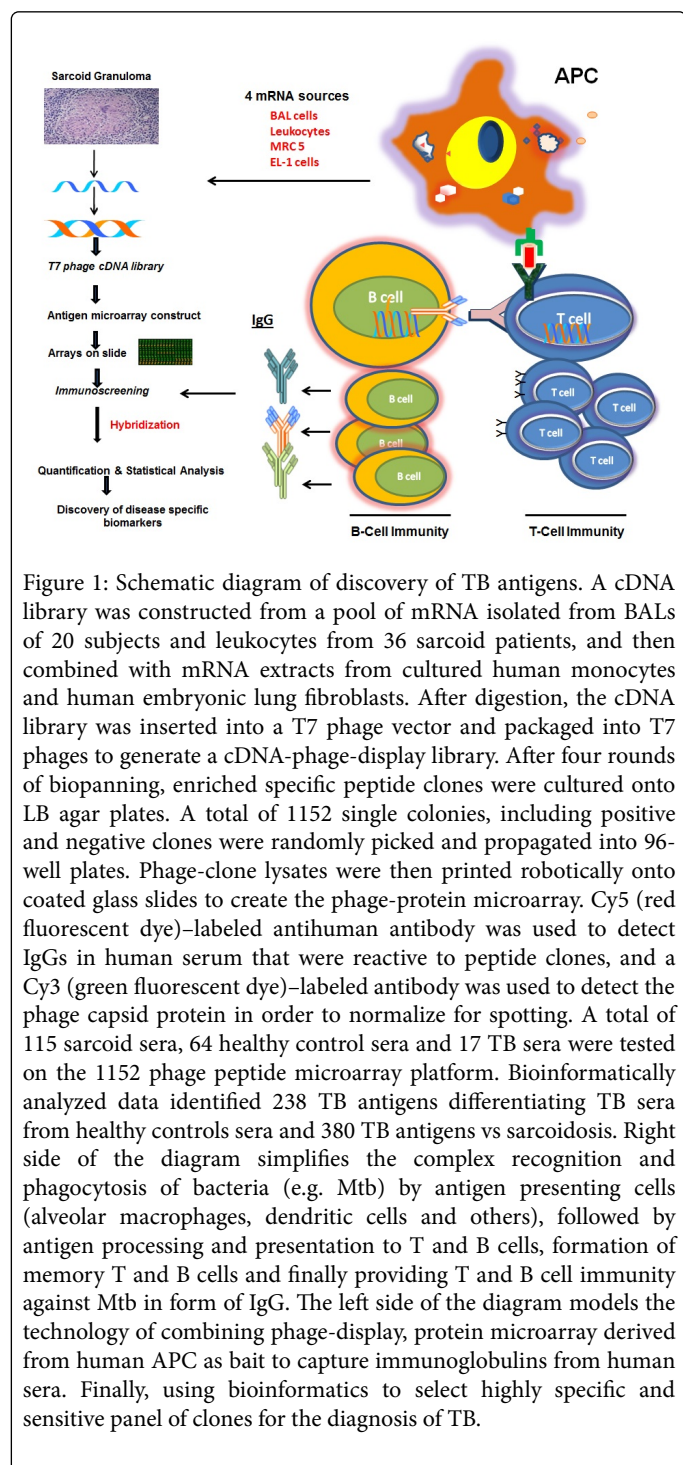


Figure 1: Schematic diagram of discovery of TB antigens. A cDNA library was constructed from a pool of mRNA isolated from BALs of 20 subjects and leukocytes from 36 sarcoid patients, and then combined with mRNA extracts from cultured human monocytes and human embryonic lung fibroblasts. After digestion, the cDNA library was inserted into a T7 phage vector and packaged into T7 phages to generate a cDNA-phage-display library. After four rounds of biopanning, enriched specific peptide clones were cultured onto LB agar plates. A total of 1152 single colonies, including positive and negative clones were randomly picked and propagated into 96-well plates. Phage-clone lysates were then printed robotically onto coated glass slides to create the phage-protein microarray. Cy5 (red fluorescent dye)-labeled antihuman antibody was used to detect IgGs in human serum that were reactive to peptide clones, and a Cy3 (green fluorescent dye)-labeled antibody was used to detect the phage capsid protein in order to normalize for spotting. A total of 115 sarcoid sera, 64 healthy control sera and 17 TB sera were tested on the 1152 phage peptide microarray platform. Bioinformatically analyzed data identified 238 TB antigens differentiating TB sera from healthy controls sera and 380 TB antigens vs sarcoidosis. Right side of the diagram simplifies the complex recognition and phagocytosis of bacteria (e.g. *Mtb*) by antigen presenting cells (alveolar macrophages, dendritic cells and others), followed by antigen processing and presentation to T and B cells, formation of memory T and B cells and finally providing T and B cell immunity against *Mtb* in form of IgG. The left side of the diagram models the technology of combining phage-display, protein microarray derived from human APC as bait to capture immunoglobulins from human sera. Finally, using bioinformatics to select highly specific and sensitive panel of clones for the diagnosis of TB.

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## Disclosure

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