Evaluation of Human Serological Response to Recombinant TB10.4 Antigen of 
*Mycobacterium tuberculosis*

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**ABSTRACT**

**Introduction:** Development of effective immunodiagnostic methods for detection of *Mycobacterium tuberculosis* infection is crucial. Serodiagnostic methods, based on antibody response to specific antigens could provide promising approaches for rapid, economical and easy to perform diagnostic tests which are crucial for tuberculosis (TB) control. In this study, the level of IgG antibody responses against recombinant TB10.4 and Bacille Calmette-Guerin (BCG) in sera from TB patients and vaccinated healthy controls were evaluated.

**Methods:** Indirect ELISA was used to assess the anti-TB10.4 IgG levels. Serum samples were obtained from vaccinated healthy controls, confirmed TB positive patients, TB cases under antibiotic treatment, and cases with atypical mycobacteria infection. The ratio of test sera optical density to the optical density of pooled negative control was taken as cut off value. **Results:** Using indirect ELISA method, anti-TB10.4 was detected in 64.5%, 93.5%, 85.7% and 100% of the sera from vaccinated healthy controls, confirmed TB positive cases, TB cases under antibiotic treatment and cases with atypical mycobacteria infection, respectively. The relative sensitivity for TB10.4 was calculated as 84.32%. **Conclusion:** In this study, the use of TB10.4 protein showed high sensitivity, but low specificity in detection of anti-*M. tb* antibodies in TB patients. These results suggest that further studies should be undertaken to identify the optimal combinations of antigens for the sensitive and specific serodiagnosis of TB.

**KEYWORDS:** Serodiagnosis, TB10.4 antigen, *Mycobacterium tuberculosis*

**INTRODUCTION**

Tuberculosis (TB) remains a major health problem with 2 billion infected individuals worldwide of which 8.6 million develop active disease and 1.3-1.6 die annually [1-3]. Despite the falling incidence rate since the 90’s, the burden of the disease is still high. Infection with *M. tb* in majority of human cases is asymptomatic, establishing a latent state with an approximately 10% life-time risk of developing active disease which would significantly increase in immune-compromised patients [4]. Generally, TB is a slowly progressing chronic disease that remains undiagnosed for many years and its most common form in adults is chronic pulmonary TB, whereas extra-pulmonary TB is especially common in children and HIV-co-infected patients [5].

A successful TB control program requires adequate case detection and the ability to classify the disease status accurately which have been hampered by the lack of full understanding of the underlying immunological responses against the disease [1, 6].

The gold standard and the most frequently used methods for diagnosis of active TB are detection of *M. tb* in sputum smear and culture, however smear requires a bacterial concentration of approximately 10,000/ml and has a varied sensitivity of 20-60%. Meanwhile sputum culture, although more sensitive and capable of detecting as little as 150 bacteria/ml, may take as long as 2 months in order to obtain results [7-8]. Recently, a WHO-endorsed sputum-based PCR method has been introduced with a sensitivity of 98% in smear positive and 68% in smear-negative TB, producing results in less than 2 h which can identify all the resistance-inducing mutations that produce rifampicin resistance phenotypes as well [9]. In addition to the drawbacks related to each individual test, all these processes require sputum containing *M. tb*, although many active TB patients as well as those with extra-pulmonary disease may not present *M. tb*-positive sputum [10, 11].

Host’s immune response to mycobacterial antigens such as tuberculin skin test (TST) which measures a delayed-type hypersensitivity reaction at the site of injection or IFN-γ release assays (IGRAs), measuring IFN-γ secretion of peripheral blood cells have also been used for TB diagnosis.

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However, vaccination, latent infection with TB or infection with non-tuberculous mycobacteria could produce immune reactivity and cannot reliably distinguish between these states and the active TB. This makes the identification of reliable biomarkers for detection of active TB crucial, especially since the early detection is very important in reducing the TB incidence rate [6]. The intracellular location of the bacterium and its very complex relationship with the immune system have largely been responsible for failure in developing antibody-based detection kits for the diagnostic purposes [1]. The evaluation of various serological tests available in the market has shown that the antibody detection methods are unreliable for TB diagnosis. Such methods produce imprecise and inconsistent results, giving highly variable values in terms of sensitivity and specificity which has led to a policy statement by WHO to ban their use for pulmonary and extra-pulmonary TB diagnoses [12, 13]. The detection of M. tb-specific antibodies has been an important diagnostic aid. However, serological assays as simple to perform and cost-effective tools that are now believed improvable, have generated great interest in identification and validation of markers that can be used for case detection and control monitoring [12, 14]. Furthermore, although the BCG vaccine prevents the occurrence of serious TB infections such as meningitis, it is hardly effective in the process of TB reactivation in patients who have been previously exposed to the bacillus [15]. This lack of efficacy as well as the emergence of multi-drug resistant (MDR), extensively drug resistant (XDR) and totally drug resistant (TDR) strains have made the control of TB a major global challenge urgently requiring effective diagnostic tools, therapeutics and vaccines [16]. Therefore, new vaccine candidates to replace or augment BCG in fight against TB are needed improvable, have generated great interest in identification and validation of markers that can be used for case detection and control monitoring [12, 14]. Furthermore, although the BCG vaccine prevents the occurrence of serious TB infections such as meningitis, it is hardly effective in the process of TB reactivation in patients who have been previously exposed to the bacillus [15]. This lack of efficacy as well as the emergence of multi-drug resistant (MDR), extensively drug resistant (XDR) and totally drug resistant (TDR) strains have made the control of TB a major global challenge urgently requiring effective diagnostic tools, therapeutics and vaccines [16]. 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The relative sensitivity and specificity of the tests were calculated using the following formula:

Sensitivity = true positives / (true positives + false negatives) x 100

Specificity = true negatives / (true negatives + false positives) x 100

Evaluation of the serum IgG level responses to the recombinant TB10.4 antigen in the groups under study was made in comparison to BCG vaccine (Pasteur Institute of Iran) which was used at approximately 25μg/well as the coating antigen.

**Statistical analysis**

Two-tailed z test was used for data analysis.

**RESULTS**

The optimum coating concentration of recombinant TB10.4 was determined as 25μg/well while higher amounts of antigen showed no significant difference in OD and adversely affected the specificity. Test samples were diluted 100-fold and the secondary antibody dilution of 1:25000 gave the highest specificity. The cutoff value for the test was calculated according to the optical density ratio of the test to the negative control sera. All calculated ratios smaller than or equal to 1.0, were considered as negative results.

The presence of specific antibodies to BCG and the recombinant TB10.4 was determined by i-ELISA which showed that 42 (64.61%) sera of the healthy vaccinated individuals produced low antibody response to BCG whereas only 16 individuals in this group had low antibody response to TB10.4 and the difference was statistically significant (p < 0.01). Furthermore, the total incorrect identification of bacteriologically confirmed cases (negative, suspected positive or negative) was significantly higher (p< 0.01) when BCG was used as the detecting antigen (Table 1).

<table>
<thead>
<tr>
<th>Test groups (no.)</th>
<th>%Suspected -ve</th>
<th>%-ve</th>
<th>%Suspected +ve</th>
<th>%+ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG</td>
<td>TB 10.4</td>
<td>BCG</td>
<td>TB 10.4</td>
<td>BCG</td>
</tr>
<tr>
<td>Vaccinated non-TB cases (65)</td>
<td>6.15</td>
<td>1.61</td>
<td>64.61</td>
<td>24.91</td>
</tr>
<tr>
<td>Confirmed +ve samples (77)</td>
<td>7.79</td>
<td>1.29</td>
<td>9.09</td>
<td>2.59</td>
</tr>
<tr>
<td>Cases under antibiotic treatment (14)</td>
<td>7.14</td>
<td>7.14</td>
<td>21.42</td>
<td>-</td>
</tr>
<tr>
<td>Cases with atypical mycobacteria (5)</td>
<td>-</td>
<td>-</td>
<td>60</td>
<td>-</td>
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All sera from cases infected with atypical mycobacteria reacted with TB10.4, whereas only 2 of these sera gave positive results with BCG. Sensitivity of T.b10.4 was 84.32% compared to 68.42% for BCG which was statistically significant (p<0.01). Specificity of this antigen was low compared to BCG as all cases of atypical mycobacterial infections gave positive results.

**DISCUSSION**

Successful control of TB requires rapid and accurate case identification and to improve TB diagnosis, development of an effective serological test would be of great help. It has been shown that IgG antibodies increase in patients with active TB and their utilization for diagnosis of active disease has been proposed [25]. However, despite all efforts, no commercial sero-diagnostic test with satisfactory sensitivity and specificity has become available. It has been demonstrated that the antigen used is one of the factors affecting these criteria [26]. In this study, IgG responses to the recombinant TB10.4 protein were detected in over 93% of the patients with active TB. However, the IgG response to BCG was significantly lower which was unexpected, since the use of complex antigens is associated with high sensitivity. This lack of correlation could have been due to the suboptimal cutoff values selected for the tests resulting in lower sensitivity of BCG coated ELISA or may have been caused by the lower antibody avidity for the surface of live M. tb. [27].

The high level of cross-reactivity between TB10.4 protein (64.51%) and sera from healthy control group in this study is in contrast to the results reported by Weldingh et al. who detected no IgG response against this protein among the healthy individuals with or without prior BCG vaccination [14]. The reason for this discrepancy was not ascertained; nevertheless all BCG vaccine strains including the Pasteur Strain carry 3 copies of the gene encoding this protein [19] which could have been the reason for the IgG response in the control group. Furthermore, the homologues of the tb10.4 have been identified in the other species belonging to the M. tb complex which could explain the positive results obtained with the group of patients infected with atypical mycobacteria [19]. In addition, contamination of the Ni-NTA-purified recombinant TB10.4 with antigens of the E. coli host which is abundant in the normal human flora might have caused the false positive results in the control group.

High variability in antibody responses to M.tb among individuals has been shown and has been associated with factors such as bacterial load, formation of immune complexes between circulating mycobacterial antigens and the antibodies produced, differential in vivo expression of different M. tb antigens as well as variation in pathogenesis of the infecting strain leading to low antibody activity and consequently false negative tests [28, 29]. To overcome some of these difficulties in sero-diagnosis of M.tb, the use of poly-antigenic preparations in place of mono-antigenic tests has been recommended.

In this study, the use of TB10.4 protein showed high sensitivity, but low specificity in detection of anti-M. tb antibodies in TB patients. These results suggest that further studies should be undertaken to identify the optimal combinations of antigens for the sensitive and specific serodiagnosis of TB.
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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES
