Original Article

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 being developed and recent studies have shown that during the early stages of TB infection, strong immune responses were generated against a number of secretary proteins with clear evidence for presentation of these antigens to T cells through MHC class I and II pathways [16, 17]. A highly immunogenic antigen belonging to this group is TB10.4 which is encoded by Rv0288 gene, located on ESX-3 gene cluster. This low molecular weight protein that belongs to ESAT-6 family has shown potentials as a vaccine candidate and its fusion with Ag85B is undergoing clinical trials and has been shown to induce a higher secretion of IFN-γ in TB patients than ESAT-6 [18, 19]. Furthermore, its use as a component of a protein cocktail for skin tests as an alternative to conventional tuberculin has been suggested [20]

A recent study measuring IgG response to several *M.tb* antigens in pulmonary TB patients, recent TB contacts with latent TB infection (LTBI) and healthy subjects has concluded that the assessment of serum IgG responses to selective purified *M.tb* antigens may help to improve diagnosis of active TB, particularly for sputum-smear negative patients or recurrent cases while these may also help to differentiate between active TB and latent TB infections [21].

In this study, the level of serum antibodies against recombinant TB10.4 was evaluated in healthy BCG vaccinated individuals, bacteriologically confirmed TB patients with or without antibiotic treatment and patients infected with atypical mycobacteria infection and the results were compared with BCG immunogenicity, using indirect ELISA method.

MATERIALS and METHODS

Expression and purification of recombinant TB10.4 protein

Transformed *E. coli* BL21 (DE3) with the expression vector pET102/D containing the coding region for TB10.4 protein was induced with isopropyl-1-thio-β-D-galactoside (IPTG) and the expressed protein was purified as described by Gholoobi *et al*. [22].

Sample collection

Sputum and blood samples were collected from presumptive TB patients referred to Mycobacteriology Department of Pasteur Institute of Iran from April 2012 to April 2013. Blood samples were incubated at 4˚C for 24 hours and after centrifugation at 3000 rpm for 10 minutes, the separated sera were stored at -20˚C till use.

Fluorescent and Ziehl-Neelsen staining were performed directly on all collected sputa which were then decontaminated with the N-acetyl-L-cysteine-sodium hydroxide method and concentrated by centrifugation prior to culturing on Lowenstein-Jensen medium [23]. The Cultured samples were incubated at 37˚C for a minimum of 45 days and the tubes showing no growth after this period were reported negative. Furthermore, FLASH-PCR detection kit (DNA technology, Russia) was used for detection of M. tuberculosis and/or *M. bovis* infection, according to the manufacturer's instructions.

Indirect ELISA

Sera from 77 cases which were confirmed bacteriologically by smear staining, culture or PCR, 14 from TB patients under antibiotic treatment, 5 with atypical mycobacterial infections and 65 from vaccinated non-TB cases were used for indirect ELISA (i-ELISA) using recombinant TB10.4 and BCG. Pools of 5 sera from negative, unvaccinated cases and 5 sera from TB-positive patients were used as negative and positive controls, respectively. The concentrations of i-ELISA reagents were optimized using checkerboard titration method [24] with pooled negative and positive sera (data not shown). The reagents concentrations providing the highest discrimination between the negative and positive sera were considered optimal.

Briefly, 96-well microtiter plates (MaxiSorp; Nunc, Denmark) were coated with 5-30 µg/ml of the rTB10.4 in 100 µl phosphate buffer saline (PBS, pH~7.5) per well and incubated at room temperature for 2 h. The plates were washed with PBS to remove unbound antigen and blocked with 0.05% skimmed milk in PBS containing 0.01% Tween 20 (TPBS) for 1 h at 37˚C. The sera were diluted 10-100 folds in blocking solution and 100 µl of the diluted sera were added to each well and incubated for 2 h at room temperature. The plates were then washed thrice with TPBS and incubated with peroxidase-conjugated goat antihuman antibody (Sigma, Germany) ranging from 1:100 to 1:25000 for 2 h and developed with 3, 3'-5, 5'-tetramethylbenzidine (TMB; Sigma, Germany). The reaction was stopped by the addition of 1N HCL and the plates were read at 450 and 630 nm using an ELISA reader (Titertek, Finland).

The cut off value was based on the signal to noise ratio of the mean OD values of the negative pooled sera. After subtracting the background, OD values greater than the negative pooled sera were considered positive. Samples giving ODs equal to or in upper 10% of the OD values of negative control were considered invalid and were classified as suspected negatives and positives, respectively.

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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) x100
Specificity = true negatives / (true negatives + false positives) x100
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. 1. i-ELISA results of different sera samples using BCG or TB10.4 as the detecting antigens

	%Suspected -ve		%-ve		%Suspected +ve		%+ve	
Test groups (no.)	BCG	TB 10.4	BCG	TB 10.4	BCG	TB 10.4	BCG	TB 10.4
vaccinated non-TB cases (65)	6.15	1.61	64.61	24.91	3.07	9.67	26.15	64.51
Confirmed +ve samples (77)	7.79	1.29	9.09	2.59	5.19	2.59	77.92	93.53
Cases under antibiotic treatment (14)	7.14	7.14	21.42	-	21.42	7.14	50	85.71
Cases with atypical mycobacteria (5)	-	-	60	-	-	-	40	100

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 Tb10.4 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

1. Tucci P, Gonzalez-Sapienza G, Marin M. Pathogen-derived biomarkers for active tuberculosis diagnosis. Frontiers in microbiology. 2014;5:549. doi:10.3389/fmicb.2014.00549.
2. Pukazhvanthen P, Anbarasu D, Basirudeen SAK, Raja A, Singh M. Assessing humoral immune response of 4 recombinant antigens for serodiagnosis of tuberculosis. Tuberculosis. 94;622-33.
3. Wallis RS, Doherty TM, Onyebujoh P, Vahedi M, Laang H, Olesen O et al. Biomarkers for tuberculosis disease activity, cure, and relapse. The Lancet Infectious diseases. 2009;9(3):162-72. doi:10.1016/S1473-3099(09)70042-8.
4. Young DB, Perkins MD, Duncan K, Barry CE, 3rd. Confronting the scientific obstacles to global control of tuberculosis. The Journal of clinical investigation. 2008;118(4):1255-65. doi:10.1172/JCI34614.
5. Jain A. Extra pulmonary tuberculosis: a diagnostic dilemma. Indian journal of clinical biochemistry : IJCB. 2011;26(3):269-73. doi:10.1007/s12291-010-0104-0.
6. Walzl G, Haks MC, Joosten SA, Kleynhans L, Ronacher K, Ottenhoff TH. Clinical immunology and multiplex biomarkers of human tuberculosis. Cold Spring Harbor perspectives in medicine. 2015;5(4). doi:10.1101/cshperspect.a018515.
7. Davies PD, Pai M. The diagnosis and misdiagnosis of tuberculosis. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2008;12(11):1226-34.
8. Horne DJ, Royce SE, Gooze L, Narita M, Hopewell PC, Nahid P et al. Sputum monitoring during tuberculosis treatment for predicting outcome: systematic review and meta-analysis. The Lancet Infectious diseases. 2010;10(6):387-94. doi:10.1016/S1473-3099(10)70071-2.
9. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F et al. Rapid molecular detection of tuberculosis and rifampin resistance. The New England journal of medicine. 2010;363(11):1005-15. doi:10.1056/NEJMoa0907847.
10. Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. Nature reviews Immunology. 2011;11(5):343-54. doi:10.1038/nri2960.
11. Bacakoglu F, Basoglu OK, Cok G, Sayiner A, Ates M. Pulmonary tuberculosis in patients with diabetes mellitus. Respiration; international review of thoracic diseases. 2001;68(6):595-600.
12. Steingart KR, Flores LL, Dendukuri N, Schiller I, Laal S, Ramsay A et al. Commercial serological tests for the diagnosis of active pulmonary and extrapulmonary tuberculosis: an updated systematic review and meta-analysis. PLoS medicine. 2011;8(8):e1001062. doi:10.1371/journal.pmed.1001062.
13. Weldingh K, Rosenkrands I, Okkels LM, Doherty TM, Andersen P. Assessing the serodiagnostic potential of 35 Mycobacterium tuberculosis proteins and identification of four novel serological antigens. Journal of clinical microbiology. 2005;43(1):57-65. doi:10.1128/JCM.43.1.57-65.2005.
14. World Health Organization (WHO). Commercial sero-diagnostic tests for diagnosis of tuberculosis. Policy statement. 2011 Geneva: World Health Organization.
15. Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV et al. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. Jama. 1994;271(9):698-702.
16. Kassa D, Ran L, Geberemeskel W, Tebeje M, Alemu A, Selase A et al. Analysis of immune responses against a wide range of Mycobacterium tuberculosis antigens in patients with active pulmonary tuberculosis. Clinical and vaccine immunology : CVI. 2012;19(12):1907-15. doi:10.1128/CVI.00482-12.
17. Hoang TT, Nansen A, Roy S, Billeskov R, Aagaard C, Elvang T et al. Distinct differences in the expansion and phenotype of TB10.4 specific CD8 and CD4 T cells after infection with Mycobacterium tuberculosis. PloS one. 2009;4(6):e5928. doi:10.1371/journal.pone.0005928.
18. Davila J, McNamara LA, Yang Z. Comparison of the predicted population coverage of tuberculosis vaccine candidates Ag85B-ESAT-6, Ag85B-TB10.4, and Mtb72f via a bioinformatics approach. PloS one. 2012;7(7):e40882. doi:10.1371/journal.pone.0040882.
19. Skjot RL, Brock I, Arend SM, Munk ME, Theisen M, Ottenhoff TH et al. Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. Infection and immunity. 2002;70(10):5446-53.
20. Malaghini M, Thomaz-Soccol V, Probst CM, Krieger MA, Preti H, Kritski A et al. Recombinant antigen production for assays of intradermoreaction for diagnosis and surveillance of tuberculosis. Journal of biotechnology. 2011;156(1):56-8. doi:10.1016/j.jbiotec.2011.07.015.
21. Yun-Gyoung H, Ahreum K, Young Ae K, An Sik K, DaeYeon K, Yeun K et al. Evaluation of antigen-specific immunoglobulin G responses in pulmonary tuberculosis patients and contacts. J ClinMicrobiol 2015 doi: 10.1128/ JCM.03050-14.
22. Gholoobi A, Sankian M, Zarif R, Farshadzadeh Z, Youssefi F, Sadeghian A et al. Molecular cloning, expression and purification of protein TB10.4 secreted by Mycobacterium tuberculosis. Iran J Basic Med Sci 2010;13:189-93.
23. Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A et al. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clinical microbiology reviews. 2011;24(2):314-50. doi:10.1128/CMR.00059-10.
24. Pruvot M, Forde TL, Steele J, Kutz SJ, De Buck J, van der Meer F et al. The modification and evaluation of an ELISA test for the surveillance of Mycobacterium avium subsp. paratuberculosis infection in wild ruminants. BMC veterinary research. 2013;9:5. doi:10.1186/1746-6148-9-5.
25. Hur YG, Kim A, Kang YA, Kim AS, Kim DY, Kim Y et al. Evaluation of antigen-specific immunoglobulin g responses in pulmonary tuberculosis patients and contacts. Journal of clinical microbiology. 2015;53(3):904-9. doi:10.1128/JCM.03050-14.
26. Welch RJ, Lawless KM, Litwin CM. Antituberculosis IgG antibodies as a marker of active Mycobacterium tuberculosis disease. Clinical and vaccine immunology : CVI. 2012;19(4):522-6. doi:10.1128/CVI.05573-11.
27. Perley CC, Frahm M, Click EM, Dobos KM, Ferrari G, Stout JE et al. The human antibody response to the surface of Mycobacterium tuberculosis. PloS one. 2014;9(2):e98938. doi:10.1371/journal.pone.0098938.
28. Ridell M. Serodiagnosis of tuberculosis. The European respiratory journal. 1988;1(7):587-8.
29. Schierloh P, Klepp L, Vazquez C, Rocha RV, Blanco FC, Balboa L et al. Differential expression of immunogenic proteins on virulent Mycobacterium tuberculosis clinical isolates. BioMed research international. 2014;2014:741309. doi:10.1155/2014/741309.

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