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Development of a New Indirect ELISA Method for Detection of Anti-Tuberculosis Antibodies in Human Serum

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ABSTRACT

***Background*:** Tuberculosis is a crucial health problem. Establishing a rapid, reliable and still inexpensive diagnostic method for tuberculosis seems to be substantial in developing countries where TB has very high incidence rate.

***Methods*:** An Indirect Enzyme-linked immunosorbent Assay (ELISA**)** was established to detect serum antibodies against *Mycobacterium tuberculosis*. Three kinds of antigens were used to prepare the solid phase for antibody assay including: purified protein derivative (PPD), *M. tuberculosis* Bacilli, and *Mycobacterium bovis* Bacillus Calmette Guerin (BCG). Sera of two main following groups were investigated in this study: sera samples from smear-positive, culture-positive and Tuberculin Skin Test-positive TB patients and sera samples from smear-negative, culture negative and TST-negative healthy individuals.

***Results*:** Among the antigens used, BCG produced higher sensitivity and specificity in the assay. With PPD as the solid phase, higher sensitivity, but lower specificity was observed in comparison with BCG. Both, low response and noise (non-specific binding) were observed with TB bacilli as the solid phase in the assay.

***Conclusion*:** Using BCG solid phase system in this method resulted in higher sensitivity in comparison to single antigen solid phase systems. In addition, we were able to circumvent the problem of non-specific bindings in more popular multi-antigenic solid systems such as PPD. By using this new indirect ELISA, a rapid, reliable and still inexpensive diagnosis of tuberculosis might be possible. Although, further investigations are required to confirm our result.

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Introduction

Tuberculosis is a crucial health problem worldwide, based on report from World Health Organization (WHO), with 8.7 million cases in 2011 (1). The diagnosis of Tuberculosis (TB) is primarily based on the identification of *Mycobacterium tuberculosis*, and clinical and radiological evidence. Culture of bacteria is the diagnostic "gold standard" method. Although, it requires a long time and it is not always successful. On the other hand, in practice, only 40-50% of patients with pulmonary tuberculosis are smear positive with Ziehl-Neelsons direct microscpy method (2) and approximately 17% of TB transmission is through patients with smear-negative and culture-positive tuberculosis (3). Moreover, due to the high workload, laboratory workers, especially in developing countries, process an excessive number of slides resulting in a lower quality diagnostic service. Therefore, development of a serological test for the diagnosis of TB has been an interesting area of research since the report by Brown and Petrof in 1918 (4). In recent years several research teams, all over the world, have tried to setup immundiagnostic assays to detect antigen or antibodies of *M. tuberculosis* (MTB) in serum or other body fluids (5-11). However, the challenge is to develop a simple and inexpensive test with sensitivity better than that of direct microscopy which could be widely used in developing countries.

In this study, we have developed a new sensitive and reliable indirect Enzyme-linked immunosorbent assay (ELISA) method for detection of Anti-tuberculosis antibodies in human serum by examining three easily accessible and inexpensive solid phases.

Materials and Methods

Patient sera

Twenty serum samples from smear-positive, culture-positive and Tuberculin Skin Test (TST)-positive TB patients as well as twenty-four serum samples from smear-negative, culture negative, TST-negative healthy individuals were collected. The samples were provided by the Mycobacteriology Department of Pasteur Institute of Iran. Two control groups were also provided: Nineteen sera from non-TB individuals who were smear-negative, culture negative, and TST-negative were collected. Five of these nineteen sera were intentionally chosen to have high titres of rheumatoid factors (RF). The high RF samples were provided by Fardis Medical Laboratory, Karaj, Iran. Based on the ethics board of our institution, ethical approval was not required for our investigation since all the samples were already collected for another study under informed consent of the donors.

Materials

The BCG used in this study was BCG vaccine, a product of Pasteur Institute of Iran. The Purified Protein Derivative (PPD) was obtained from Razi vaccine & serum Research Institute in Iran. All other reagents & solutions were obtained from Sigma-Aldrich Corporation, UK.

Evaluation of the antigen concentrations for ideal coating

Various concentrations of BCG in carbonate / bicarbonate buffer (100 mM, pH 9.6) were prepared. ELISA plates were coated with the solution for 1.5 h at 37°C. The plates were

then washed three times with Tween-20 solution (0.1%) and blocked with a solution of 0.5% BSA in PBS buffer for 1h at room temperature. The plates were then washed four times with Tween-20 solution, and incubated with polyclonal rabbit anti-Tb antibody (10 µg/ml) in PBS buffer including 0.1% Tween-20 and 0.1% BSA for 1h at 37°C. ELISA plates were then washed 5 times with the washing solution and incubated with polyclonal anti-Rabbit antibody conjugated with HRP (Horseradish Peroxidase) (1: 3000) for 1h at 37°C. After six times washing with Tween-20 solution, OPD (orthophenylenediamine) substrate in 0.05 M phosphate-citrate buffer (pH: 5.0, containing 0.03% sodium perborate) was added to the plates. The color development was stopped using 100 µl of 1N H_2SO_4. OD was measured at 492 nm using an ELISA plate reader.

A solid phase system using *M. tuberculosis* bacilli was prepared with the same method used for BCG. The *M. tuberculosis* was obtained from clinical isolates and was inactivated in water bath at 80ºC for 20 minutes. The highest concentration of the bacterial cells used for coating was 5.0×10^6 / ml according to the Mc Farland method (12). Similar experiments were carried out using PPD as the solid phase.

ELISA Analysis

A serum sample with high titres of anti-TB antibodies was diluted 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 in PBS containing 0.01% Tween-20 and 0.1% BSA. One hundred microliter of the diluted serum was added to each well of PPD coated plates (10 µg/ml) and BCG coated plates (50 µg/ml) and incubated for 90 min at RT. Washing four times with the Tween-20 solution and incubated with diluted (1:5000) alkaline phosphatase (AP) conjugated anti-human IgG (and IgM for BCG coated plates). After five times washing, enzyme activity was determined with pNPP substrate (in DEA buffer 50 mM, pH: 9.6 containing 1mM $MgCl_2$). Optical density of the plates was read at 405 nm after 15 min. All sera were then tested as above using BCG coated plates.

Results

Figure 1 shows the results from three solid phase systems. All three experiments were carried out simultaneously. With BCG as the solid phase, better response was obtained in comparison with the PPD and TB coated plates. The OD increased with increasing BCG concentration, and leveled off at about 50 µg/ml *(Figure 1.A).*

Figure 2.A shows the dose-response curve of IgG antibodies from different serum samples in BCG ELISA system. It is clear from the curves that the OD increases in proportion to IgG increase in the infected serum sample. The 5 sera from non-TB patients, with a high titre of rheumatoid factor (RF^{3+}) showed a relatively higher background (non-specific binding) in comparison with the serum from the healthy individual, when used at low dilution (1:50) *(Figure 2.B).*

Discussion

Tuberculosis is a crucial health problem worldwide. A rapid and reliable test for diagnosis of TB is essential, especially in resource-poor countries.

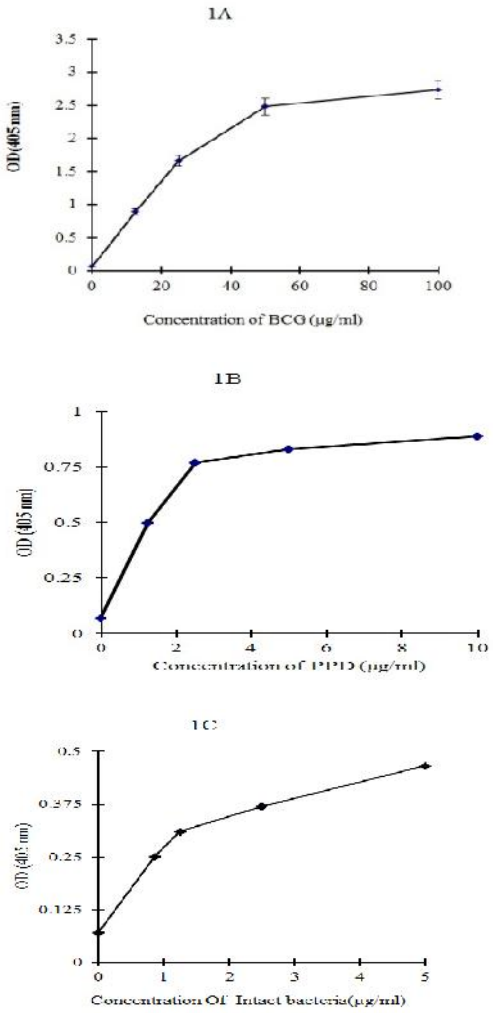

Figure 1. Efficacy of various solid phase systems to detect anti-TB Abs: **A.** BCG, **B.** PPD, **C.** Intact bacteria. All three experiments were carried out in parallel, simultaneously

Although bacterial culture is the diagnostic gold standard, it takes a long time and is not always successful. Moreover, direct microscopy lacks sufficient sensitivity. TST requires adequate experience to be measured & documented; a matter not fully considered by health workers (13). On the other hand, it is not rapid enough and it needs a return visit. Interferon-Gamma Release Assay, a test with potential to replace TST (14) is not widely available in developing countries where TB has a very high incidence rate.

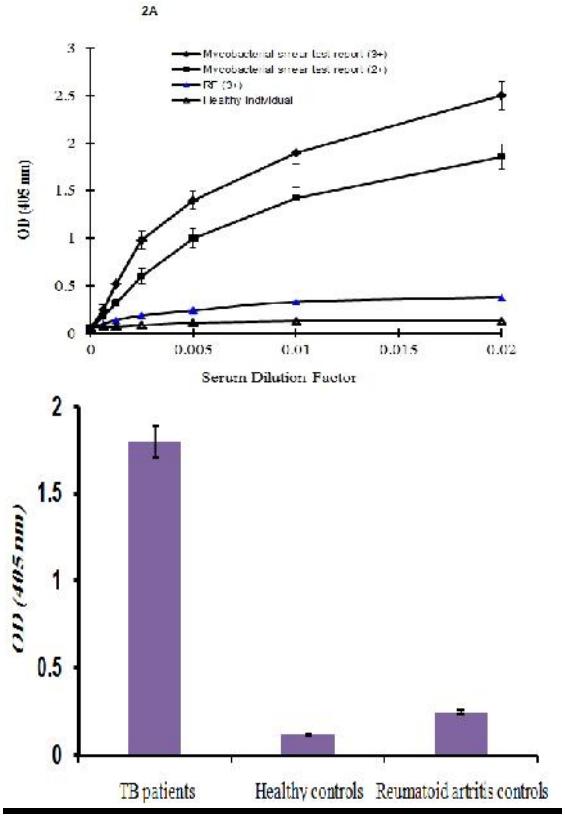

Figure 2. A. Dose-response curve of human IgG anti-TB Ab present in an infected serum with high titer of anti-TB antibodies, together with two kinds of control sera; **B.** The mean results of IgG anti-TB ELISA from three study groups.

Other commercially available serodiagnostic tests are not easily affordable in developing countries and their cost-effectiveness are under question (15, 16). Thus, we investigated to establish a rapid, inexpensive and reliable ELISA which can be a suitable parallel in TB diagnosis or screening procedure in developing countries. Since the sensitivity and specificity of the ELISA method for detecting antibodies in serum is seriously affected by non-specific binding of irrelevant immunoglobulins in antiserum to the solid phase (17), this problem should be minimized by different

strategies depending on the assay; In the case of anti-TB antibodies, by the application of multi-antigenic antigens (or multi-antigens) in order to enhance the sensitivity of the test, and using more specific types of antigens to reduce the non-specific binding of antibodies to the solid phase. We investigated BCG as the solid phase system in this study. Two control sera from non-TB individuals with and without rheumatoid factors were used to determine the background OD of the ELISA test. Sera with high titres of rheumatoid factors played a controlling role in our indirect ELISA since RF has been reported to cause interference and false-positive results in some indirect ELISA assays (18-21).

The sensitivity and specificity of the newly organized test was 90% and 93.5%, respectively. Three out of 19 of the non-TB serum with high titres of rheumatoid factors was determined as false positive cases, and two out of 20 sera from TB patients were determined as false negatives.

The specificity and sensitivity of our method is comparable to the results from previous research groups (22). The relatively high sensitivity of the present method is more likely due to the use of a specific multiantigenic system. In other similar studies (23-27), use of multiple antigens facilitated the development of a highly sensitive test for *M. tuberculosis* antibody detection. Similar use of BCG was already reported in diagnosing bovine tuberculosis and it was found that comparing to Ag85 & MPT-51, BCG gave the highest specificity and sensitivity (28). With the application of the new solid phase system, non-specific binding of serum immunoglobuin in a similar multi-antigenic solid phase system such as PPD (29) was considerably reduced, to an acceptable level.

More studies and on greater scales are required to be done to confirm the result of our studies so that a reliable and still inexpensive method will be translated to clinical application in some economically disadvantaged areas of the world.

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Conflict of Interest

None declared conflicts of interest.

References

1. WHO (World Health Organization). Global tuberculosis control:WHO Report Geneva 2010.
2. Dye C, Scheele S, Dolin P, *et al*. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* 1999; **282** (7): 677-86.
3. Behr MA, Warren SA, Salamon H, *et al*. Transmission of *Mycobacterium tuberculosis* from patients smearnegative for acid-fast bacilli. *Lancet* 199- 9; **353** (9151): 444-9.
4. Brown S, Petrof S. The Clinical Value of complement fixation in pulmonary tuberculosis based on a study of 540

cases. *Am Rev Tuberc* 1918; **2**: 525-40.

5. Glatman-Freedman A. Advances in antibody-mediated immunity against Mycobacterium tuberculosis: implica tions for a novel vaccine strategy. *FE MS Immunol Med Microbiol* 2003; **39** (1): 9-16.
6. Glatman-Freedman A, Martin JM, Riska PF, *et al*. Monoclonal antibodies to surface antigens of *Mycobacterium tuberculosis* and their use in a modified enzyme-linked immunosorbent spot assay for detection of mycobacteria. *J Clin Microbiol* 1996; **34** (11): 2795-802.
7. Glatman-Freedman A, Casadevall A, Dai Z, *et al*. Antigenic evidence of pre valence and diversity of Mycobact erium tuberculosis arabinomannan. *J Clin Microbiol 2004*; **42** (7): 3225-31.
8. Chan ED, Heifets L, Iseman MD. Immunologic diagnosis of tuberculosis: a review. *Tuber Lung Dis* 2000; **80** (3): 131-40.
9. Garg SK, Tiwari RP, Tiwari D, *et al*. Diagnosis of tuberculosis: available tec hnologies, limitations, and possibilities. *J Clin Lab Anal* 2003; **17** (5): 155-63.
10. Pereira Arias-Bouda LM, Nguyen LN, Ho LM, *et al*. Development of antigen detection assay for diagnosis of tuberculosis using sputum samples. *J Clin Microbiol* 2000; **38** (6): 2278-83.
11. Pereira Arias-Bouda LM, Kuijper S, van Deutekom H, *et al*. Enzyme-linked immunosorbent assays using immune complexes for the diagnosis of tuberculosis. *J Immunolo methods* 2003; **283** (1-2): 115-24.
12. Seeley JH, Vandemark P, Lee J., 1997.Medical microbiology and immunology. In: A laboratory manual of microbiology. W. H. Freeman and company, New York.
13. James SH, Dumois JA 3rd, Messina AF, *et al*. Healthcare worker knowledge of measurement and documentation of tuberculin skin test reaction. *Infect Control Hosp Epidemiol* 2009; **30** (12): 1230-2.
14. Pottumarthy S, Morris AJ, Harrison AC, *et al*. Evaluation of the tuberculin gamma interferon assay: potential to replace the Mantoux skin test. J Clin Microbiol. 1999; **37** (10): 3229-32.
15. Sohn H, Minion J, Albert H, *et al*. TB diagnostic tests: how do we figure out their costs? *Expert Rev Anti Infect Ther* 2009; **7** (6): 723-33.
16. Dowdy DW, Steingart KR, Pai M. Serological testing versus other strategies for diagnosis of active tubercu losis in India: a cost-effectiveness ana lysis. *PLoS Med* 2011 ; **8** (8): e100 1074.
17. Farajollahi MM, Cook DB, Hamzehlou S, *et al*. Reduction of non-specific binding in immunoassays requiring long incubations. *Scand J Clin Lab Invest* 2012; **72** (7): 531-9.
18. Barka NE, Agopian MS, Peter JB. False-positive IgM antibodies to Borrelia burgdorferi in indirect ELISA as a result of IgM rheumatoid factor. *J Infect Dis* 1990; **161** (6): 1312.
19. Ramos-Levi AM, Montanez MC, Ortega I, *et al*. A case of biochemical as-

say discrepancy: Interference with measurement of thyroid-stimulating hormone due to rheumatoid factor. *Endocrinol Nutr* 2012 [In press].

20. Bartels EM, Ribel-Madsen S. Cytokine measurements and possible interference from heterophilic antibodiesProblems and solutions experie nced with rheumatoid factor. Methods 2013 [In press].
21. Churchman SM, Geiler J, Parmar R, *et al*. Multiplexing immunoassays for cytokine detection in the serum of patients with rheumatoid arthritis: lack of sensitivity and interference by rheumatoid factor. Clin Exp *Rheumatol* 2012; **30** (4): 534-42.
22. al-Hajjaj MS, Gad-el-Rab MO, al-Orainey IO, *et al*. Improved sensitivity for detection of tuberculosis cases by a modified Anda-TB ELISA test. *Tuber Lung Dis* 1999; **79** (3): 181-5.
23. Houghton RL, Lodes MJ, Dillon DC, *et al*. Use of multiepitope polyproteins in serodiagnosis of active tuberculosis. *Clin Diagn Lab Immunol* 2002; **9** (4): 883-91.
24. Panda D, Lahiri A, Bhattacharyya I, *et al*. Humoral immune responses in different clinical forms of tuberculosis. *J Indian Med Assoc* 2001; **99** (8): 424, 426-7, 440.
25. Simonney N, Molina JM, Molimard M, *et al*. Comparison of A60 and three glycolipid antigens in an ELISA test for tuberculosis. *Clin Microbiol Infect* 1996; **2** (3): 214-22.
26. Whelan C, Shuralev E, Kwok HF, *et al*. Use of a multiplex enzyme-linked immunosorbent assay to detect a subpopulation of *Mycobacterium bovis*-infected animals deemed negative or inconclusive by the single intradermal comparative tuberculin skin test. *J Vet Diagn Invest* 2011; **23** (3): 499-503.
27. Sumi S, Radhakrishnan VV. Diagnostic significance of humoral immune responses to recombinant antigens of Mycobacterium tuberculosis in patients with pleural tuberculosis. *J Clin Lab Anal* 2010; **24** (5): 283-8.
28. da Silva EB, Silva BD, Leon JR, *et al*. Using BCG, MPT-51 and Ag85 as antigens in an indirect ELISA for the diagnosis of bovine tuberculosis. *Vet J* 2011; **187** (2): 276-8.
29. Gaborick CM, Salman MD, Ellis RP, *et al*. Evaluation of a five-antigen ELI SA for diagnosis of tuberculosis in cattle and Cervidae. *J Am Vet Med Assoc* 1996; **209** (5): 962-6.