Evaluation of Antigen Detection Test (Chromatographic Immunoassay): Potential to Replace the Antibody Assay Using Purified 45-kDa Protein for Rapid Diagnosis of Tuberculosis

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Background: The current strategy for combating tuberculosis (TB) is based on the early detection and treatment of patients to halt transmission. The present study was conducted to evaluate the diagnostic potential of three *Mycobacterium tuberculosis* antigens, 45-kDa, A60, and sonicated MTB antigen (SmTB-Ag), as antibody/antigen detection methods for the rapid and accurate diagnosis of TB. **Methods:** The SmTB-Ag and 45-kDa antigens were purified and A60 antigen was supplied by Anda-Biologicals, France. The 45-kDa and A60 antigens (for antibody detection procedures) and SmTB-Ag (for antigen detection test) were tested in the same study subjects. ELISA and immunochromatographic (rapid) test were performed on 201 sputum and

serum samples. Ninety-eight samples from TB patients and 103 samples from control individuals were studied. **Results:** The mean absorbance value of antibodies against 45-kDa antigen in the TB patients were (1.17 ± 0.44 , CI 1.09–1.26), significantly higher than in the non-TB group, (0.8 ± 0.28 , CI 0.74–0.85, $P < 0.05$). The sensitivities of tests using two antigens, 84% for the 45-kDa antigen and 65% for the A60 antigen, were lower than SmTB-Ag(93%). The rapid test yielded 93% sensitivity and 92% specificity. **Conclusion:** Findings highlighted the importance of antigen detection as a diagnostic tool. The rapid test evaluated in this study may be useful for diagnosis of TB. J. Clin. Lab. Anal. 28:70–76, 2014. © 2013 Wiley Periodicals, Inc.

Key words: TB diagnosis; MTB antigens; TB detection markers; ELISA; rapid test

INTRODUCTION

Tuberculosis (TB) remains a grave burden to public health. Early diagnosis of TB is not only vital for preventing the spread of the disease but also for timely initiation of treatment (1). Currently, the diagnosis of active TB mainly relies on clinical symptoms, radiological findings, and the detection of *Mycobacterium tuberculosis* (MTB) in clinical samples using smear staining and mycobacterial culture (2). An effective in vitro diagnosis of TB based on serological methods would be regarded as an attractive progress because immunoassays are simple, rapid, inexpensive, and may offer the possibility to detect cases missed by standard sputum smear Microscopy (3).

Serological tests for active TB are based on detection of antibodies elicited by antigens of MTB, which

are recognized by the humoral immune system. Many serological tests utilize an ELISA format, while several others are available as rapid point-of-care tests (4). The most common of these tests rely on detection of the humoral antibody immune response to MTB, or direct detection of antigens in specimens other than serum. Serological tests (antibody detection) for TB are

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widely used in developing countries (5, 6). However, the WHO recently announced its first negative policy in TB, against the use of current TB serological tests (7).

The focus of this study was to analyze and compare the potential of three MTB antigens, A60 (Anda-Biologicals, France), and two antigens were purified, sonicated MTB antigen (SmTB-Ag) and 45-kDa, as antibody/antigen detection markers in the serodiagnosis of active TB. The 45/47-kDa antigen from MTB is an immunodominant adhesion restricted to *Mycobacterium* genus and has been proposed as an alternative candidate to generate a new vaccine against TB or for diagnosis kits (8). The A60 is one of the best-known antigens used in the serologic tests, which are present in all mycobacteria, and has been the object of extensive investigation (9).

METHODS

Settings

This study was conducted in the Protein Chemistry Laboratory of Tuberculosis Department, Pasteur Institute, Tehran. Serum and sputum samples (three morning sputum samples per patients) from 201 adult subjects were collected from all study groups. All samples (serum and sputum) were obtained from these patients within days of their admission to the TB laboratory. The Sputa and sera were tested simultaneously and the analyses were performed blinded. Consent from all subjects was obtained before recruiting them into the study. In Iran, Bacillus Calmette-Guerin (BCG) vaccination is given within 1 week of the birth of child. The control population and Multidrug-Resistant-Tuberculosis (MDR) patients were tested for HIV at the AIDS Department of Pasteur Institute, Tehran. These subjects were negative for HIV. The study was approved by the Institutional Review Board at the Pasteur Institute, Tehran.

Study Subjects

TB group

This group consisted of 79 patients with clinically active TB whose diagnostic procedures are complete and 19 patients with not clinically active TB. The inclusion criteria required clinical, bacteriological, or radiographic evidence of current TB. Not clinically active TB is defined by a history of previous episode of TB or abnormal stable radiographic findings in a person with a positive reaction to tuberculin skin test, negative bacteriologic studies, and no clinical or radiographic evidence of current disease (10). To diagnose active TB, sputum microscopy and culture for MTB on Lowenstein-Jensen medium were done on sputum samples by staining with Ziehl-Neelsen stain and *N*-acetyl methods, respectively (11).

Non-TB group

The control population consisted of 75 patients with chest problems other than TB (asthma or other respiratory infections) and 28 healthy adults with no signs of clinical impairment and normal chest radiographs.

Crude *Mycobacterium* Antigens Extract

Bacteria (MTB-1126, Pasteur Institute, Tehran) were harvested by loop from the surface of the solid culture, washed and resuspended in phosphate-buffered saline (PBS) pH 7.4 containing phenylmethylsulfonyl fluoride (PMSF) 1 mM, Ethylenediamine-Tetraacetic Acid (EDTA) 20 mM, sodium azide 0.02%, triton X114 0.5%, glycerol 10%, sucrose 12.5 mM, DNase 1 µg/ml, and Dithiothreitol (DTT) 10 mM. The bacilli were subjected to sonication for 1 h at 50 Hz, using a cell sonicator (Bandelin, GM-220, Germany) in ice, and subsequently centrifuged at 5,000 rpm for 45 min at -4°C . Proteins were precipitated by adding refrigerated ethanol (-20°C) to the supernatant. After centrifugation at 13,000 rpm for 45 min at -15°C pellets were resuspended in PBS and then dialyzed for 24 h against saline (12). The protein content was measured by Bradford's method (13).

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) of Crude Extract

SDS-PAGE was performed with the Bio-Rad system for vertical electrophoresis in a Mini-Protean Tetra Cell unit (Bio-Rad Laboratories, Hercules, California) by the method of Laemmli (14). Samples were prepared in SDS-mercaptoethanol buffer by boiling for 5 min. Fifty micrograms per lane of crude *Mycobacterium* extract was loaded on 12% vertical polyacrylamide slab gels. The gel was run at a constant current of 10 mA until the tracking dye entered the resolving gel; the current was then increased to 15 mA. Proteins were visualized by staining with silver staining (15). Molecular weight marker in the range 14.4–116.0 kDa was used (Fermentas, Ontario, Canada). β -galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp981 (25.0 kDa), β -lactoglobulin (18.4 kDa), Lysozyme (14.4 kDa).

Gel Filtration

Gel filtration was carried out in glass chromatographic column (100×1.5 cm) with the use of Superdex 200 (bead diameter: 24–44 µm, Sigma). Column was packed by employing a Prista pump (Etatron, Rome, Italy). The suspended medium under vacuum was degassed and poured down the wall of the column using a glass rod. Phosphate

buffer (0.05 M sodium phosphate, 0.15 M NaCl) pH 7 was used for elution. The flow rate was 0.25 ml/min, with fractions of 1,000 μ l collected. Total sample concentration, void volume (V_0), and sample volume ($1.62\% \times V_t$) were 18.4 mg, 37 and 2 ml, respectively (16). The analytic column was calibrated using the following proteins as reference standards: carbonic anhydrase, approximate molecular weight 29,000; cytochrome c, approximate molecular weight 12,400; alcohol dehydrogenase, approximate molecular weight 150,000; blue dextran, approximate molecular weight 2,000,000; β -amylase, approximate molecular weight 200,000; and albumin bovine serum, approximate molecular weight 66,000 (molecular weight marker kit for gel filtration chromatography, range: 12,000–200,000, Sigma). The purified proteins were detected by monitoring their UV absorbance at $A_{280\text{nm}}$ using a spectrophotometer (Jenway 6305, UK).

Antibody Purification Procedure

Affinity chromatography was used to purify specific polyclonal antibodies against MTB, SmTB-Ag. This step was carried out on an affinity column based on the use of SmTB-Ag covalently coupled to Cyanogen bromide (CNBr)-activated Sepharose 4B (CNBr activated sepharose 4B, Pharmacia, Uppsala, Sweden) according to our technique as described previously (9).

Preabsorption Procedure

The antibodies and serum samples were treated with normal oral flora to remove any nonspecificity and cross-reactivity. Cross-reactive immunoglobulin was removed by incubation of antibodies and sera for 12 h at 4°C with tested sputum. Antibodies and sera were incubated overnight at 4°C in the presence of negative sputa (9).

Rapid Test—Antigen Detection

The rapid test detects antigen–antibody complexes if the sample contained TB bacilli. A total of 200 μ l of processed sputum was added to the reaction funnel and was allowed to absorb completely. A funnel was used to concentrate the sample in the middle of the filter. After washing with the PBS buffer, 150 μ l antibody solution (Ab against SmTB-Ag) was added to the test funnel. After another wash with the PBS buffer, 150 μ l protein gold conjugate was added. Finally, 150 μ l of wash buffer was added to remove all unbound conjugates. A red-spot development was observed immediately when the patient sputum contained mycobacterial antigens. The positive test is a central red-pink color that differentiates well from the surrounding area. Negative test yielded a white cen-

tral region. Distilled water and BCG were used as negative and positive controls, respectively.

Enzyme linked immunosorbent assay (ELISA) Test—Antibody Detection

The ELISA was carried out to detect MTB antibody in the sera of patients. Firstly, the coating of antigens (45-kDa and A60, 8 μ g/ml) was performed in carbonate–bicarbonate buffer (pH 9.6) overnight at 4°C. A60 antigen was supplied by Anda-Biologicals, France. Patient anti-serum appropriately diluted (1:200) in PBS 0.05% Tween 20 was added and incubated at room temperature for 2 h. The wells were washed with the PBS followed by addition of 100 μ l antirabbit-IgG conjugated to alkaline phosphates (Sigma) with 1:2,000 dilutions in PBS 0.05% Tween 20 and incubated at room temperature for 2 h. One hundred microliters, substrate, p-nitrophenyl-phosphate (Sigma) was added and incubated at room temperature for 10 min. The reaction was stopped by the addition of 100 μ l 1 M NaOH. Absorbances were read at 405 nm in a multiscan plate reader. (Stat Fax-2100, Awareness Technology Inc.) (12, 17).

Statistical Analysis

Statistical analysis was performed with SPSS version 18.0. Receiver operating characteristic (ROC curve) was used to calculate the cut off value (18). Comparisons between TB and non-TB groups were done using χ^2 test. A P -value <0.05 was considered significant. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated according to Grange and Laszlo method (19). The PPV and NPV were calculated for different prevalences of disease in Iran: 23 per 100,000 prevalence rate and 17 per 100,000 incidence rate of new cases of TB in 2010 (20).

RESULTS

Gel Filtration

Figure 1 illustrates the gel filtration results on a Superdex 200 column. The fourth peak (tube no. 61) of protein contents after gel filtration was applied to a SDS-PAGE.

SDS-PAGE

Each protein yielded a single band on SDS-PAGE and gels stained with silver staining. The SDS-PAGE of the purified MTB proteins is shown in Figure 2.

Fig. 1. Gel filtration of culture fluids of MTB on a Superdex 200 column. Optical density (OD) at 280 nm, indicating the protein content in eluate fractions.

Fig. 2. SDS-PAGE of the purified MTB proteins. Lane 1, low molecular weight marker. Lanes 2, 4, and 5, MTB proteins were separated by SDS-PAGE and were stained with silver staining. Lane 3, SDS-PAGE results for the void volume fraction after gel filtration of the 45-kDa antigen (proteins) on a Superdex 200 column.

ELISA and Rapid Test

The patients had a mean age 48.17 ± 15.80 years and 95 (47.3%) were males. The demographic data of the patients are shown in Table 1. The ELISA test was performed prior to and after sera preabsorption process, while the rapid test was carried out only after antibody preabsorption procedure. The ELISA and rapid test results against 45-kDa, A60 and SmTB-Ag antigens are presented in Table 2. Individual values of antibodies against 45-kDa and A60 antigens were used for the generation of ROC curves (data not shown). Consequently, the optimal cut off values of the optical densities of antibody responses to the 45-kDa and A60 antigens before and after preabsorption proce-

dures for diagnosing TB patients were 1.27, 0.89, 0.74, and 0.79, respectively (Table 2). In assay based on the detection of antigen, the grade of a positive test color dot varied from a dark reddish purple to scanty light pink. Among these antigens, the performance of the SmTB-Ag was found to be the most impressive (sensitivity 93%, specificity 92%, PPV 91%), whereas those of 45-kDa antigen and A60 antigen were slightly lower (Table 2).

The serum positivities for SmTB-Ag in cases of clinically and not clinically active TB were 93.7% and 89.5%, respectively, while the positivity for patients in the non-TB group was 7.8% (Table 2). Compared to 45-kDa and A60 antigens, these positivities were higher. Overall, the rapid test yielded 93% sensitivity and 92.2% specificity. The mean absorbance value of antibodies against 45-kDa antigen in the TB patients was $(1.17 \pm 0.44, \text{CI } 1.09\text{--}1.26)$ significantly higher than in the non-TB group ($0.8 \pm 0.28, \text{CI } 0.74\text{--}0.85, P < 0.05$). The difference absorbance value of antibodies against A60 antigen between TB and non-TB group was not significant ($P > 0.05$).

DISCUSSION

The focus of the present study was to test antigen and antibody detection procedures for the isolation of mycobacteria and to compare their performance with each other. The study demonstrated that the antigen detection method (rapid test) provided an excellent isolation rate of mycobacteria, which was much better than that obtained by antibody detection procedures (ELISA test).

In a study based on antibody detection by ELISA, measurement of IgG against MTB antigens revealed sensitivity of 71% and specificity of 100% for Myco G antigen,

TABLE 1. Demographic Data of Patients Studied

Characteristic					
Age	Mean $\pm$ SD (years)		Range (years)		Minimum / maximum (years)
	48.17 $\pm$ 15.80		67.0		20/87
					Total <i>N</i> = 201
Age group		Frequency <i>N</i> (%)			
<20		1 (0.5)			
20–40		71 (35.3)			
40–60		84 (41.8)			
60–80		39 (19.4)			
>80		6 (3.0)			
					Total <i>N</i> = 201
Gender		<i>N</i> (%) cases			
Male		95 (47.3)			
Female		106 (52.7)			
					Total <i>N</i> = 201
Patient group					
TB cases	Non-TB cases	TB cases	Non-TB cases	TB cases	Non-TB cases
<i>N</i> (%)	<i>N</i> (%)	<i>N</i> (%)	<i>N</i> (%)	<i>N</i> (%)	<i>N</i> (%)
98 (48.8)	103 (51.2)	Age (mean $\pm$ SD)		Gender <i>n</i> (%)	
		49.56 $\pm$ 17.52	46.85 $\pm$ 13.94	Male 48 (48.9) Female 50 (51.1)	Male 47 (45.6) Female 56 (54.4)

sensitivity of 43.5% and specificity of 96.3% for patho test, and sensitivity of 81% and specificity of 96.3% for combination of both tests (21). Xiu-Yun et al. have recently reported the specificities achieved by 38 kDa and 38E6 ranged from 82.0 to 93.0% in patients with non-TB respiratory disease and in controls (22). In another study, Okuda et al. analyzed three antigens, Tuberculous glycolipid (TBGL), Lipoarabinomannan (LAM), and A60, for serodiagnosis of TB. The percentages of patients positive in all three tests were 58.8% for active pulmonary TB and 71.4% for chronic TB (23).

These rates are similar to our findings. The discrepancy in findings between the present and an earlier study can be explained by the difference in the numbers of cases. However, the difference in sensitivity and specificity does not imply a significant difference in predictive values (especially the negative) because of the relatively low disease probability in Iran. That is to say, the clinical importance of the difference is limited. In addition, we explored the use of preabsorption process with normal oral flora, with the aim to improve the detection rate of TB. Using ROC analysis, a cut off value was determined, which defined the sensitivity and specificity against 45-kDa and A60 antigens. Under this cut off value, the false positive rate still remained in the TB and non-TB groups. The false positive cases showed by the control group may be explained as these patients may infect with some bacteria carrying some antigens that shared similar antigenicity with MTB sonicated antigen. Preabsorption procedures were attempted in order to eliminate nonspecific binding

of antibody and improve the diagnostic yield. For example, in the ELISA test, after preabsorption process, the percentage of sensitivity had risen to 84 and 65% based on coated antigen (45-kDa and A60), respectively. However, it could only improve the sensitivity and specificity by about 20%. This suggests that there is a significant increase in the amount of specific antibody to the 45-kDa and A60 antigens during the preabsorption procedures. Therefore, there is a need to analyze the data in greater detail in order to clarify and select an optimal cut off titer of best diagnostic yields. In summary, these findings indicate that 45-kDa-based assays are likely MTB-specific and warrant further exploration but the ELISA test results based on A60 antigen were disappointing, as this test was neither sensitive nor specific for diagnosis of TB.

Antigen detection of 200 sputum samples by a rapid immunochromatographic assay for the detection of MTB gave a sensitivity of 97.9%, specificity of 99.0%, PPV of 98.9%, and NPV of 98.0% (24). In another study, the developed fast-dot ELISA detected the *Mycobacterium* antigen in serum of TB patients with a sensitivity of 90.8% and 93.0% PPV (12). A polyclonal antibody specific to MTB proteins was produced by Haldar et al. and used in the detection of the antigen for rapid diagnosis of tuberculous meningitis in children. All assays had a specificity and sensitivity ranging from 92 to 98% (25). The present study demonstrates that the positivities for antigens 45-kDa, A60, and SmTB-Ag in case of not clinically active TB patients were 68.4, 26.3, and 89.5%, respectively. The

TABLE 2. Description of Antibody/Antigen and Comparison of Sensitivity, Specificity, PPV, and NPV in Sera and Sputa of Patients Groups Performed by ELISA and Rapid Test Along With Mean Absorbance, Range, Antigen Types, and Preabsorption Process

(Ab/Ag detection)	Patient population (N)	Positivity for Ab/Ag N (%)	Negativity for Ab/Ag N (%)	Absorbance (mean $\pm$ SD)	95% CI ^e	Range
Ab detection (45 kDa)	TB (98)	82 (83.6)	16 (16.4)	1.17 $\pm$ 0.44	1.09–1.26	2.10
	Clinically active TB (79)	69 (87.3)	10 (12.7)	1.01 $\pm$ 0.44	0.91–1.11	2.11
	Not clinically active TB (19)	13 (68.4)	6 (31.6)	0.97 $\pm$ 0.73	0.80–1.13	1.25
	Non-TB (103)	19 (18.4)	84 (81.6)	0.8 $\pm$ 0.28	0.74–0.85	1.47
	Non-TB pulmonary control (75)	15 (2.0)	60 (80.0)	0.94 $\pm$ 0.37	0.85–1.02	1.44
	Healthy control (28)	4 (14.3)	24 (85.7)	0.03 $\pm$ 0.48	0.84–1.22	2.07
Ab detection (A60)	TB (98)	64 (65.3)	34 (34.7)	0.92 $\pm$ 0.31	0.86–0.98	1.27
	Clinically active TB (79)	59 (74.7)	20 (25.3)	0.88 $\pm$ 0.38	0.79–0.96	1.23
	Not clinically active TB (19)	5 (26.3)	14 (73.7)	0.94 $\pm$ 0.45	0.72–1.16	1.40
	Non-TB (103)	27 (26.2)	76 (73.8)	0.82 $\pm$ 0.41	0.74–0.91	1.23
	Non-TB pulmonary control (75)	19 (25.4)	56 (74.6)	0.87 $\pm$ 0.36	0.78–0.95	1.43
	Healthy control (28)	8 (28.6)	20 (71.4)	0.83 $\pm$ 0.31	0.70–0.95	1.23
Ag detection (smTB-Ag)	TB (98)	91 (92.8)	7 (7.2)	–	–	–
	Clinically active TB (79)	74 (93.7)	5 (6.3)	–	–	–
	Not clinically active TB (19)	17 (89.5)	2 (10.5)	–	–	–
	Non-TB (103)	8 (7.8)	95 (92.2)	–	–	–
	Non-TB pulmonary control (75)	6 (8.0)	69 (92.0)	–	–	–
	Healthy control (28)	2 (7.1)	26 (92.9)	–	–	–
Ag 45 ^a (before absorption, cutoff 1.27)		Ag 45 ^a (after absorption, cutoff 0.89)	A60 ^a (before absorption, cutoff 0.74)	A60 ^a (after absorption, cutoff 0.79)	SmTb-Ag ^b (rapid test)	
Sensitivity	60 (%)	84 (%)	50 (%)	65 (%)	93 (%)	
Specificity	63 (%)	82 (%)	52 (%)	74 (%)	92 (%)	
PPV ^c	64 (%)	81 (%)	54 (%)	71 (%)	91 (%)	
NPV ^d	59 (%)	84 (%)	49 (%)	67 (%)	93 (%)	

^aAntibody detection (ELISA test).^bAntigen detection (rapid test).^cPositive predictive value.^dNegative predictive value.^e95% Confidence Interval.

reason for this positivity result can be explained by the patients previously prescribed course of chemotherapy. Interestingly, these patients were positive for tuberculin skin test and received insufficient anti-TB drug in the past and not covered under dot.

These observations are approximately in agreement with our results. Detection of antigen almost certainly confirms the presence of active disease. In contrast, detection of antibody does not always indicate active disease as the antibody response can be detected even 6 months after clearance of the infection (1). The TB patient does not produce antibodies against all antigenic substances in the cell wall of the tuberculous bacilli and the specificities of the antibodies differ among patients. Consequently, the use of more than a single antigen would improve the sensitivity of serodiagnosis for active TB (23). Thus, our data show that the 45-kDa antigens studied in this work induce higher humoral responses than the tests based on the A60 antigen. However, neither the 45-kDa nor the A60 antigen test used in this study is useful for routine diagnostic application by itself. This convenient antibody detection test may have value for diagnosis of

patients suspected of having TB in high-prevalence areas. However, the specificity of the test is too low for it to be useful as a screening test. Besides, recent publication has emphasized that available commercial serological (antibody detection) tests for TB are inaccurate and highly inconsistent (7).

These results show that the antibody detection method is not satisfactory, but that the results from antigen detection tests were very effective, for detecting active pulmonary TB. Antigen detection has the potential to be simplified into a point-of-care format. Although, WHO has strongly recommended against the use of current, commercial serodiagnostic tests for pulmonary TB, meta-analysis of noncommercial antigen/antibody detection tests and antigen discovery-related proteomic studies have identified several candidate antigens/antigen combinations potentially useful for pulmonary TB serological test (25, 26). Our results demonstrate that antigen detection is an attractive diagnostic modality for point-of-care tests as it requires a tiny volume of sample and could be a valuable tool as a routine test for the early and rapid diagnosis TB in developing countries.

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