

Humoral Immune Response Against 38-Kda and 16-Kda Mycobacterial Antigens in Tuberculosis

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ABSTRACT

Several enzyme-linked immunosorbent assays based on mycobacterial antigens have been used for the rapid diagnosis of tuberculosis, although, demonstration of *Mycobacterium tuberculosis* in a smear or culture is the most reliable method. In this study, diagnostic value of 16-kDa and 38-kDa mycobacterial antigens was investigated in the patients whom they were diagnosed as tuberculosis by clinic and/or bacteriologic findings in Turkey.

Patozym TB complex plus commercial enzyme-linked immunosorbent assay kit was used for measuring IgG against 38 kDa and 16 kDa recombinant antigens. Humoral immune response was analyzed in a group of 179 tuberculosis patients (143 smear-positive, 19 smear-negative, 8 lymphadenitis and 9 pleuritis), 15 inactive tuberculosis cases, and in control groups consisting of 40 healthy volunteers and 20 pulmonary diseases other than tuberculosis.

Sensitivity, specificity, positive predictive value, negative predictive value of the test were detected 52.5%, 93.3%, 95.9%, and 39.7%, respectively in TB cases. Three cases were found positive (20%) in inactive tuberculosis cases.

The ELISA test has presented a very good specificity, an acceptable level of sensitivity and PPV. It was thought that it could be used in combination with other methods to increase diagnostic accuracy, especially for culture-negative TB cases which are difficult to diagnose.

Key words: Immune response, mycobacterial antigens, tuberculosis.

INTRODUCTION

Approximately one third of the world's population is infected with *Mycobacterium tuberculosis*, and it is estimated that 8 million new cases and 3 millions deaths occur each year [1].

The standard diagnosis of TB is still made by clinical examination, direct sputum microscopy, and mycobacterial culture. To improve the diagnosis of tuberculosis (TB), more rapid diagnostic techniques have been investigated in recent years. Despite the increasing development of rapid identification techniques of mycobacteria, there is still need for a simple, sensitive, and specific test for TB. The development of an easy and inexpensive diagnostic tool for TB is an important goal, particularly in the view of a global increase in the number of cases of active TB, primarily affecting the developing countries (2-4).

The demand for a more rapid diagnostic procedure has focused on the possibility of demonstrating specific antibodies. Detection of antibodies is considerably simple and cheap, so serodiagnosis of TB has been extensively investigated [5]. Specificities of the serologic tests have improved when highly purified and recombinant antigens specific for the TB complex have been introduced [2].

Many mycobacterial antigens have identified such as 71, 65, 38, 23, 19, 16, 14, and 12 kDa proteins. 38 kDa is an immunodominant lipoprotein antigen, isolated as a component of antigen 5 by affinity chromatography, specific only for the *M. tuberculosis complex*. It is the most extensively studied antigen. 16 kDa antigen is an immunodominant antigen, frequently called 14 kDa, related to the family low molecular weight heat-shock proteins. This antigen contains B-cell epitops specific for the *M. tuberculosis complex* [6, 7].

Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive and reproducible serological technique. The method does not require sophisticated instrumentation, is simple,

and the reagents that it employs are in expensive. So that ELISA can be performed in every laboratory. The use of ELISA method with highly specific antigens is the good option for TB serodiagnosis [2, 8, 9].

Turkey is a country with moderate prevalence (40 cases per 100000 persons) and incidence (26 cases per 100.000 persons) of TB [10]. The objective of this study was to assess the clinical usefulness of detecting specific IgG via commercially available immunoassay test in the diagnosis of TB in Turkey.

MATERIALS AND METHOD

Setting

This study was conducted in a tertiary reference center of pulmonary diseases and tuberculosis in Western part of Turkey and approved by Hospital Ethical Committee.

Study subjects

Serum samples were collected consecutive patients followed by one ward in the hospital between September 2003 and May 2005. All patients were immunocompetant, HIV negative and none of them drug abuser. Cases were consisted of adult (min. 16 years) population.

Tuberculosis group. One hundred and seventy-nine samples from human deficiency virus seronegative individuals were studied. The inclusion criteria were defined according to WHO criteria for TB patients [10].

Inactive tuberculosis group. The patients had been treated either with complete course of antituberculosis or had presented with chest roentgenograms showing a fibrocalcified pulmonary tuberculosis lesion. All chest roentgenograms of the patients were stable for at least 6 months, and sputum smear and culture negative for TB. Fifteen serum samples were tested in this group.

Control group. Control group consisted of 60 samples from two main populations. Twenty serums were involved to the study from patients who are admitted to our hospital with pulmonary diseases other than tuberculosis. The diseases included pneumonia, chronic obstructive pulmonary disease, bronchiectasis, and lung cancer. The other group consisted of 40 healthy blood donors' serums documented by a general health examination

All serum samples were collected and stored at -40 C until use.

The serum samples and the positive, negative, and cutoff controls included in the kit were tested in duplicate.

BCG vaccination and tuberculin skin test (TST) status

BCG vaccination is mandatory in Turkey. TST is not a certain diagnostic tool due to BCG vaccination programme and endemicity of TB in the country. In this study, TST were not performed to all patients as comparative data for serologic status of patients. However, TST values were noted from the patients whom TST had applied during the hospitalization.

Numbers of BCG scars were recorded for all patients.

Serological test

Commercially available ELISA kit, the PATHOZYM-TB complex plus (Omega Diagnostics, Alloa, Scotland) was used. In the PATHOZYM-TB complex plus kit, the 38 kDa antigen, which is obtained by recombinant technology, is mixed with the 16 kDa recombinant protein. The study was performed according to the manufacturer's instructions. In brief, diluted (1/50) serum was distributed in microtiter wells and incubated for 60 min at 37°C. Unbound serum was removed by washing with a buffer solution. The wells were subsequently incubated with peroxidase-labeled antihuman conjugate at 37°C for 30 min. After another wash cycle, peroxidase substrate tetramethylbenzidine containing hydrogen peroxide was added to the wells and the colorimetric reaction was prolonged for 15 min in the dark at 37°C until stop

reagent was added. The absorbance values at 450 nm were recorded. Four standards (with 2, 4, 8, and 16 serounits/ml) were provided to generate a semilog reference curve. Because the sera were diluted 1/50, the units extrapolated from the curve were multiplied by 50 to obtain serounits for result interpretation. According to the manufacturer's instructions, a result was considered positive when the level of antibodies in a sample was higher than 200 serounits/ml.

The serum samples and the positive, negative, and cutoff controls included in the kit were tested in duplicate. Results are expressed as the number of serological units of specific IgG per milliliter and were read from a semi logarithmic reference curve, which was prepared by using the standard solutions included in the kit.

Statistical analysis

Chi square test was used for significance of difference of positivity percentages (in comparison with TB groups). Fisher's exact test was for significance of difference of positivity percentages in comparison with the "others" group (Inactive TB group, control groups). Sensitivity, specificity, and positive and negative predictive values (PPV and NPV) were calculated by standard methods. Difference was accepted significant when p value was less than 0.05.

RESULTS

In this study, 179 TB patients were enrolled as TB groups. Fifteen serum samples as inactive TB patients and 60 serums as controls were also studied. Numbers of cases, demographic characteristics and differences in ages of patient groups with controls were seen in table 1. TB group and control group age difference was not statistical different ($p > 0.05$). TB patients group' age means were not indicated statistically difference with each other, either ($p > 0.05$).

Pulmonary diseases subgroup of control cases mean age was older than healthy controls (p<0.05).

Table 1- Demographic data of TB and control groups.

Study groups	No. of cases	Mean age \pm SD (range)	p*
TB group	179	42.03 \pm 16.7 (16-74)	> 0.05
Smear (+)	143	39.25 \pm 16.63 (16-	> 0.05
Smear (-)	19	74)	> 0.05
TB pleuritis	9	40.3 \pm 16.96 (28-58)	< 0.05
TB lymphadenitis	8	28.42 \pm 3.09 (22-38)	> 0.05
		48.87 \pm 17.68 (25-	
		72)	
Inactive TB	15	63 \pm 10.09 (46-73)	< 0.05
Control groups	60	46.06 \pm 14 (24-78)	-
Non TB pulmonary dis.	20	55.95 \pm 11.52 (25-	-
Healthy controls	40	78)	-
		41.12 \pm 7.6 (24-56)	
TOTAL		44.58 \pm 16.2 (16-78)	

* p value was calculated for difference between ages of TB patients and controls.

Mean values of antibody titers of 38 kDa and 16 kDa mycobacterial antigens in smear positive and negative patients were detected 337.94 \pm 285.12 and 375.79 \pm 276.10 serounits/ml, respectively. Maximum, minimum and mean values of antibody titers for each examined group were showed in figure 1 and figure 2. Mean value of TB lymphadenitis was lower than other TB groups (p< 0.05). Difference between smear positive and negative pulmonary TB cases' means were not significant (p>0.05). Pulmonary TB and TB pleuritis

patients' mean values were significantly higher than inactive TB and control cases. Difference between inactive cases and controls was not statistically significant ($p>0.05$).

Sensitivity was detected 52.5% in total TB patients. Antibodies were detected higher than the cut off level in three inactive cases serums (20%). One patient (5%) in pulmonary diseases controls and three healthy control (7.5%) serums were found positive. Sensitivities, specificities, negative and positive predictive values and diagnostic accuracy rates of test in TB cases were seen in table 2. Difference between sensitivities of smear positive and negative pulmonary TB groups is not significant ($p>0.05$). Difference between pulmonary and extrapulmonary TB groups is not significant, either ($p>0.05$). Significant differences between tuberculosis and both inactive and control groups were calculated ($p<0.05$).

Table 2- Sensitivities, specificities, negative and positive predictive values of test in TB cases.

Cases (n)	Number of sero-positive cases	Sens.	Spec.	PPV	NPV	Diag. occ.
Sm (+) Pulm. TB (143)	78	54.55	93.33	95.12	46.28	66.01
Sm (-) Pulm. TB (19)	12	63.16	93.33	75	88.89	86.08
TB pleuritis (9)	2	22.22	93.33	*	*	*
TB lymphadenitis (8)	2	25	93.33	*	*	*

Sm (+) Pulm. TB: Smear positive pulmonary TB, Sm (-) Pulm. TB: Smear negative pulmonary TB, Sens.: Sensitivity, Spec.: Specificity, PPV: Positive predictive value, NPV: Negative predictive value. Diag. Occ.: Diagnostic accuracy. *: Statistical values didn't present due to low number of samples.

In two subpopulation of control cases (healthy and pulmonary diseases other than TB), there was no differences OD means and serologic positivity rate ($p>0.05$).

TST results were observed in totally 53 patients as 45 smear (+) and 8 smear (-); 27 seropositive and 26 seronegative patients. Mean TST values were found 14.5 ± 6.5 mm and 17 ± 3.9 mm in smear (+) and (-) patients, respectively ($p > 0.05$). Five (9.4%) patients' TST diameters were less than 10 mm. Four out of these five patients were seropositive. Mean diameter of TST of seropositive and seronegative patients were determined 13.8 ± 7.3 mm and 16 ± 4.6 mm, retrospectively ($p > 0.05$).

BCG scar were not observed in eleven patients. All BCG negative patients were found TST positive. Five (45.4%) BCG negative patients were seropositive, as well as six (54.6%) BCG negative patients were seronegative ($p > 0.05$).

DISCUSSION

This study shows that humoral response to mycobacterial antigens could be significant indicator for active pulmonary TB and could help diagnosis of TB.

According to the literature, serological tests have high specificity and a much lower sensitivity of in the adult population. The sensitivity of the tests depended also on the phase of the disease and on the presence of mycobacteria in sputum. In chronic and culture positive cases, antigenic stimulation persists and if the patient is genetically able to mount an immune response to the specific antigens, antibody levels are expected to be elevated [2].

The 38-kDa antigen is the most frequently studied serological antigen and is also a core component in different commercial TB serological tests [11, 12]. The recognition frequency reported for the 38-kDa antigen varies tremendously (from 16 to 94%), largely depending on the smear status and disease manifestation. The specificities of the test reported previously coincide (from 88 to 100%). The sensitivities of the test reported vary from 33 to 89% for smear-positive TB patients, 16 to 54% for smear-negative TB patients [5, 13, 14-17]. Some of literatures were given in table 3 for a quick look among main investigations. Studies have

reported lower sensitivities with the recombinant version of the 38-kDa antigen, with sensitivities by the ELISA technique ranging from 16 to 36% for smear-negative patients and from 36 to 67% for smear-positive patients [5, 7, 19]. Chiang et al [8] have reported that the 38 kDa antigen possessed sensitivity 64%, but while the specificity was set to 100%, sensitivity were detected as the highest (27.65%) among all antigens tested. Samanich et al. [19] detected antibodies to the 38-kDa antigen from pulmonary cavitary TB patients as 57.8% ratio. In Turkey, in a most recent study, Erer et al [20] reported 46.5 % sensitivity with 100% specificity in pulmonary TB.

Table 3- Different literatures about sensitivities of antibody responses against 38 kDa Ag and 16 kDa Ag in various TB patient populations.

Reference [No]	38 kDa Ag		16 kDa Ag		38 kDa + 16 kDa Ag	
	<i>N</i>	%	<i>N</i>	%	<i>N</i>	%
	Meena [16]	39	79	-	-	-
Chiang [8]	312	64.1	-	-	-	-
Wilkinson [17]	292	38	-	-	-	-
Welding [11]	48	19	-	-	-	-
Erer [20]	48	19	-	-	-	-
Uma [12]	N/A	61	-	-	-	-
Bothamley [14]	25	84*	-	-	-	-
	27	82**	-	-	-	-

	41	90 [#]	-	-	-	-
Samanich [19]	19	57.8	-	-	-	-
	122	73 ^a				
Wilkins [4]	122	70 ^{**}	-	-	-	-
	59	25	59	44	-	-
Lyashchenko [18]						
	42	45*	42	33*	-	-
Silva [5]	15	33 ^{**}	15	13 ^{**}	-	-
	51	59	51	50.9	-	-
Beck [7]						
	24	61*	-	-	24	59*
Demkow [2]	243	27 ^{**}	-	-	243	23 ^{**}
	52	42 ^a	-	-	52	42 ^a
	-	-	-	-	17	58.8*
Imaz [23]	-	-	-	-	41	29.3 ^{**}
	-	-	-	-	29	31*
Julian [3]	-	-	-	-	11	0 ^{**}
	-	-	-	-	30	56 ^b
Demkow [6]						

(n) Number of study population, * Smear positive patients, ** Smear negative patients,

^aExtrapulmonary TB, ^bBone and joint tuberculosis, #Tuberculosis without bacteriological confirmation

Although antibody response to the 38-kDa in pulmonary TB has been extensively studied, but there are only few reports about the utility of the 38-kDa-based serological tests in extrapulmonary TB. Antibodies were detected 12 to 56% for extrapulmonary TB patients [12]. Wilkins described competition ELISA assays based on 38 kDa monoclonal antibody high sensitivity and specificity. They detected antibodies to 38 kDa antigen in 73% of cases of extrapulmonary TB at 98% specificity [4].

Combination of antigens was also found to be more useful in the serodiagnosis [21].

Combined use of antigens maximizes the effectiveness of serodiagnosis, but, it is not possible to detect all antibodies as well as this could be cause low specificity. Okuda et al [9] indicated that maximum sensitivity was obtained when seven tests were combined, but reciprocal dropping specificity to 55% in controls. The combinatory use of the 38-kDa and 16-kDa may increase the test sensitivity compared with the 38-kDa alone [22]. Seropositivity for 38 kDa and 16 kDa together was found by Julian et al [3] as 31% in smear-positive, 0% in smear-negative and 8% in extrapulmonary TB and by Imaz et al [23] as %58.8 in smear-positive and 29.3% in smear-negative TB. Beck et al [7] have detected antibodies as 50.9% and 59% rate against 16-kDa and 38 kDa antigens in the samples of TB patients. Demkow et al [2] has found 59%, 23%, 40% and 43% rates for culture-positive, culture-negative, TB nodes and TB pleuritis, respectively. For inactive tuberculosis cases, some authors have reported the positive rates as between 2.9% and 25% [5, 8].

The TB patient does not produce antibodies against all antigenic substances in the cell walls of the tuberculous bacilli, and the specificities of the antibodies differ among patients.

Lyashchenko et al [18] pointed out that person-to-person variation of antigen recognition, rather than recognition of particular antigens, is a key attribute of humoral immunity in human TB. Heterogeneity of antigen recognition by serum antibodies during TB explains the failure

to detect specific antibody responses in TB patients when only a few purified antigens of *M. tuberculosis* were used [24]. However, significant variance in the serologic results could be obtained by using even the same antigen with samples from different populations. The diverse antibody response to *M. tuberculosis* may be governed by HLA types [9]. Houghton et al [25], who pointed out that the frequency of recognition by use of the same recombinant 38-kDa antigen preparation ranged from 35 to 82% with samples from smear-positive from four different geographic areas. Weldingh et al [11] had observed a large variation in sensitivity when they tested the same antigens with sera from TB patients living in Denmark and sera from TB patients living in Uganda; and in accordance with earlier results [26]; they found that the highest proportion of positive antibody responses was by serum samples from regions where TB is highly endemic, in addition to the differences in ethnic backgrounds, it is likely that there is a difference in the stage of disease at which the patients from areas of high and low endemicity are admitted to the hospital, and this parameter may also influence the magnitude of the antibody response mounted by the patients.

In most studies, response to the mycobacterial antigens were more common in patients with active TB who were smear-positive than with smear-negative disease (reviewed in literature 5]. In present study, response to the 38 kDa and 16 kDa antigens was obtained higher than in smear-negative than smear-positive patients, in contrary to literature. According to literature, serologic response depends on dissemination of pulmonary lesions, in addition to phase of disease, duration of antigenic stimulation and HLA types of patients [2, 3, 9, 18, 23]. In our TB groups, dissemination of lesions were not assessed and classified for this study. Probably, higher serologic reactivity could be resulted due to patients having disseminated lesions in smear-negative pulmonary TB group, coincidentally. In smear-negative TB group, diagnosis takes much more time than bacteriologic confirmed cases, so that antigenic stimulation could be presented longer and humoral response would have been stronger.

Sensitivity for extrapulmonary TB cases was found concordant with the most literature.

Although in these groups statistical power was low due to the insufficient number of cases, as a result of low sensitivity, diagnostic usefulness seemed weak.

Detecting TB activity is very difficult to assess whom had got chest roentgenograms showing a fibrocalcified pulmonary tuberculosis lesions when pulmonary disease symptoms were presented. Especially, in smear-negative TB patients, diagnosis is more problematic. In this point, serology might be useful. In order to inactive cases would be accepted as control group, sensitivity, specificity, PPV, and NPV were found 63.1%, 80%, 80%, and 63.1% in smear-negative pulmonary TB patients group. Our result has pointed out serology could help for assessment of TB activity in smear-negative pulmonary patients.

To replace the "gold standard" culture, a serological test should possess sensitivities of over 80% and test specificities of over 95%, according to the recommendations of the World Health Organization [27]. In Turkey, TST values could not help precise diagnosis of TB due to moderate prevalence of TB and BCG vaccination. Studies pointed out that TST positivity were detected 24-77% in general population and similarly, 56-69% in TB patients in the country [28, 29]. Therefore, TST is a valuable but nonspecific test for assessment TB patients in Turkey. Our results demonstrated the IgG tests based on recombinant mycobacterial antigens (38-kDa and 16-kDa) present very good specificity, but lower than the acceptable level of sensitivity for the diagnosis of TB in concordance with literature. It can be concluded that 38-kDa and 16-kDa based tests could be used in combination with other methods to increase diagnostic accuracy in this disease. In inactive TB cases, as sensitivity was significantly lower than pulmonary TB group statistically, serological response to combined 38 kDa and 16 kDa antigens seemed to be a hint for assessing pulmonary TB activation. In spite of low sensitivity in our cases, serological methods may be valuable in the diagnosis of

extrapulmonary TB that usually represents an especially difficult form to be confirmed bacteriological and patients from whom it is difficult to obtain specimens.

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Figure legends

Figure 1. Antibody values of test groups.

Sm(+): Smear positive pulmonary tuberculosis, Sm(-): Smear negative pulmonary tuberculosis, TM lymph: TB lymphadenitis, TB pl: TB pleuritis.

Figure 2. Maximum, minimum and mean values (serounits/ml) of study groups.

Figure 1- Antibody values of test groups.

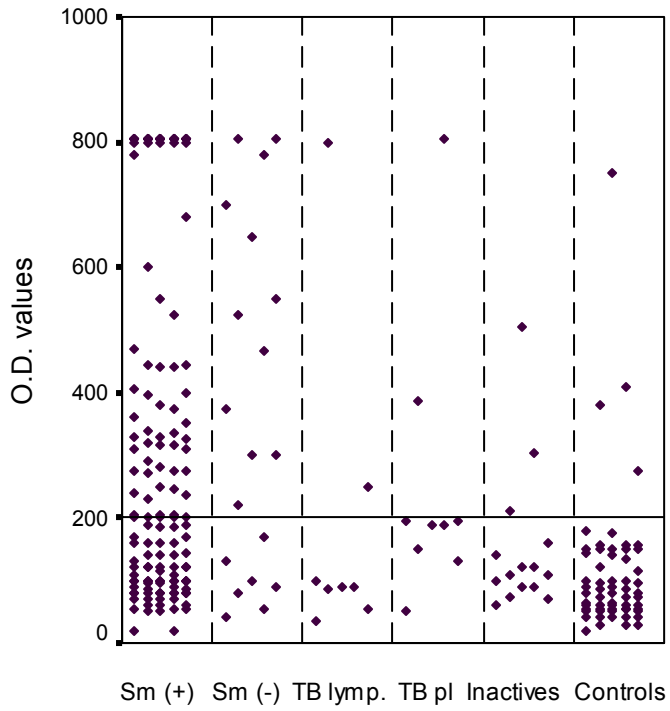


Figure 2- Maximum, minimum and mean values (serounits/ml) of study groups.

