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Humoral immune response against 38-kDa and 16-kDa mycobacterial antigens in tuberculosis

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ABSTRACT: Several ELISA tests based on mycobacterial antigens have been used for the rapid diagnosis of tuberculosis (TB), although demonstration of *Mycobacterium tuberculosis* in a smear or culture is the most reliable method. In the present study, the diagnostic value of 16-kDa and 38-kDa mycobacterial antigens was investigated in patients who were diagnosed with tuberculosis by clinical and/or bacteriological findings in Turkey.

The PATHOZYME-TB Complex Plus commercial ELISA kit was used for measuring immunoglobulin G against 38-kDa and 16-kDa recombinant antigens. Humoral immune response was analysed in a group of 179 TB patients (143 smear-positive, 19 smear-negative, eight lymphadenitis and nine pleuritis), 15 inactive TB cases and in control groups consisting of 40 healthy volunteers and 20 subjects with pulmonary diseases other than TB.

The sensitivity, specifity, positive predictive value and negative predictive value of the test were determined at 52.5%, 93.3%, 95.9% and 39.7%, respectively in TB cases. Antibodies were detected at above cut-off level in three (20%) out of 15 subjects with inactive TB.

In conclusion, the ELISA test has a very good specifity and an acceptable sensitivity and positive predictive value. It is thought that it could be used in combination with other methods to increase diagnostic accuracy, especially for culture-negative tuberculosis cases, which are difficult to diagnose.

KEYWORDS: Immune response, mycobacterial antigens, tuberculosis

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

The standard diagnosis of tuberculosis (TB) is still made by clinical examination, direct sputum microscopy and mycobacterial culture. To improve the diagnosis of TB, more rapid diagnostic techniques have been investigated in recent years. Despite the increasing development of techniques for the rapid identification of mycobacteria, there is still a 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 view of a global increase in the number of cases of active TB, which primarily affects developing countries [2–4].

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

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

ELISA is a highly sensitive and reproducible serological technique. The method does not require sophisticated instrumentation, is simple, and employs inexpensive reagents, meaning that

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ELISA can be easily performed in the laboratory. The use of the ELISA method with highly specific antigens is a good option for TB serodiagnosis [2, 8, 9].

Turkey is a country with moderate prevalence (40 cases per 100,000 people) and incidence (26 cases per 100,000 people per year) of TB [10]. The objective of the present study was to assess the clinical usefulness of detecting specific immunoglobulin (Ig)G using a commercially available immunoassay test in the diagnosis of TB in Turkey.

METHODS AND MATERIALS

Setting

The present study was conducted in a tertiary reference centre of pulmonary diseases and TB in western Turkey and was approved by the hospital ethical committee.

Study subjects

Serum samples were collected from consecutive patients treated by one ward in the hospital between September 2003 and May 2005. All patients were immunocompetent, HIV negative and none of them were drug abusers. All patients were aged $\geqslant$16 yrs.

Tuberculosis group

In total, 179 samples from HIV seronegative individuals were studied. The inclusion criteria were defined according to World Health Organization (WHO) criteria for TB patients [10].

Inactive tuberculosis group

Patients in this group had been treated either with a complete course of antituberculosis or had presented with chest radiographs showing a fibrocalcified pulmonary TB lesion. All chest radiographs of the patients were stable for $\geqslant$6 months, and patients were sputum smear and culture negative for TB. Fifteen serum samples were tested in this group.

Control group

The control group consisted of 60 patients from two main populations. The smaller of the two subgroups (n=20) comprised patients who were admitted to the hospital with pulmonary diseases other than TB. The diseases included pneumonia, chronic obstructive pulmonary disease, bronchiectasis and lung cancer. The other group consisted of 40 healthy blood donors, 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 cut-off controls included in the kit were tested in duplicate.

Bacille Calmette–Guerin vaccination and tuberculin skin test status

Bacille Calmette–Guerin (BCG) vaccination is mandatory in Turkey. Tuberculin skin test (TST) is not a certain diagnostic tool, owing to BCG vaccination programmes and the endemicity of TB in the country. In the present study, TSTs were not performed on all patients as comparative data for the serological status of patients. However, TST values were noted for the patients who underwent TST during their hospitalisation. Numbers of BCG scars were recorded for all patients.

Serological test

A commercially available ELISA kit, the PATHOZYME-TB Complex Plus (Omega Diagnostics, Alloa, UK) was used. In the PATHOZYME-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 microtitre 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-labelled 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. Absorbance values at 450 nm were recorded. Four standards (with 2, 4, 8 and 16 serounits·mL^{-1}) were provided to generate a semi-log 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^{-1}.

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

Statistical analysis

The Chi-squared test was used to calculate the significance of difference of positivity percentages (in comparison with TB groups). Fisher's exact test was used for significance of difference of positivity percentages of patients 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. Differences were accepted as significant when the p-value was <0.05.

RESULTS

In the present study, 179 TB patients were enrolled as the TB group. Serum samples from 15 inactive TB patients and 60 serum samples from controls were also studied. Numbers of cases, demographic characteristics and differences in ages of the subject groups with controls can be seen in table 1. There was no significant difference between the mean age of the TB group and that of the control group ($p>0.05$). The TB subgroups' mean ages did not differ significantly from each other ($p>0.05$). The pulmonary diseases subgroup of the control cases had a mean age significantly older than the healthy controls ($p<0.05$).

Mean$\pm$SD values of the antibody titres of 38-kDa and 16-kDa mycobacterial antigens in smear-positive and smear-negative patients were determined as 337.94$\pm$285.12 and 375.79$\pm$276.10 serounits·mL^{-1}, respectively. Maximum, minimum and mean values of antibody titres for each examined group are shown in figures 1 and 2. The mean value of antibody titres for the TB lymphadenitis patients was lower than those of other TB groups ($p<0.05$). The difference

TABLE 1 Demographic characteristics for tuberculosis (TB) and control groups

Study groups	Subjects n	Age yrs	p-value#
TB group	179	42.03±16.7 (16–74)	>0.05
Sm+	143	39.25±16.63 (16–74)	>0.05
Sm-	19	40.3±16.96 (28–58)	>0.05
TB pleuritis	9	28.42±3.09 (22–38)	<0.05
TB lymphadenitis	8	48.87±17.68 (25–72)	>0.05
Inactive TB group	15	63±10.09 (46–73)	<0.05
Control groups	60	46.06±14 (24–78)	
Non-TB pulmonary disease	20	55.95±11.52 (25–78)	
Healthy controls	40	41.12±7.6 (24–56)	
Total		44.58±16.2 (16–78)	

Data are presented as mean±SD (range), unless otherwise stated. Sm+: smear-positive pulmonary TB; Sm-: smear-negative pulmonary TB. #: p-values were calculated for difference between ages of TB patients and controls.

between mean values for smear-positive and smear-negative pulmonary TB cases was not significant (p>0.05). Pulmonary TB and TB pleuritis patients' mean values were significantly higher than inactive TB and control cases. The difference between mean values for inactive cases and controls was not statistically significant (p>0.05).

Sensitivity was found to be 52.5% in TB patients. Antibody levels were detected higher than the cut-off level in the serum of three (20%) out of 15 inactive cases. The serum of one (5%) patient in the pulmonary disease control group and three (7.5%) of those in the healthy control group were found to be positive. Sensitivities, specificities, NPVs, PPVs and diagnostic accuracy rates of tests in TB cases are shown in table 2. The difference between sensitivities of smear-positive and -negative pulmonary TB groups is not significant (p>0.05); neither is

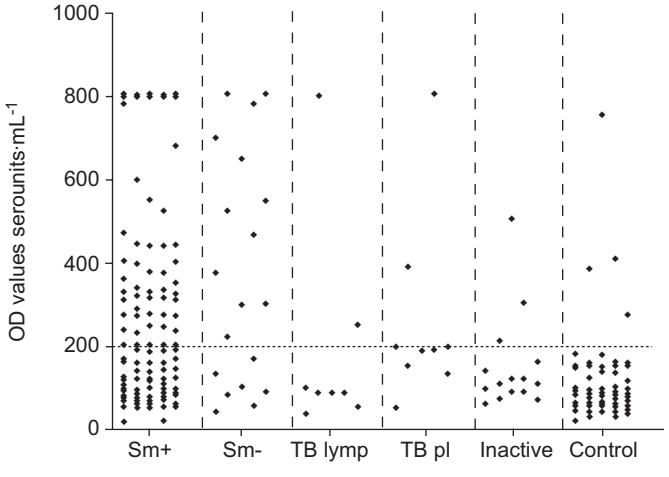

FIGURE 1. Values of antibody titres for 38-kDa and 16-kDa mycobacterial antigens in the test groups. OD: optical density; Sm+: smear-positive pulmonary tuberculosis (TB); Sm-: smear-negative pulmonary TB; TB lymp: TB lymphadenitis; TB pl: TB pleuritis.

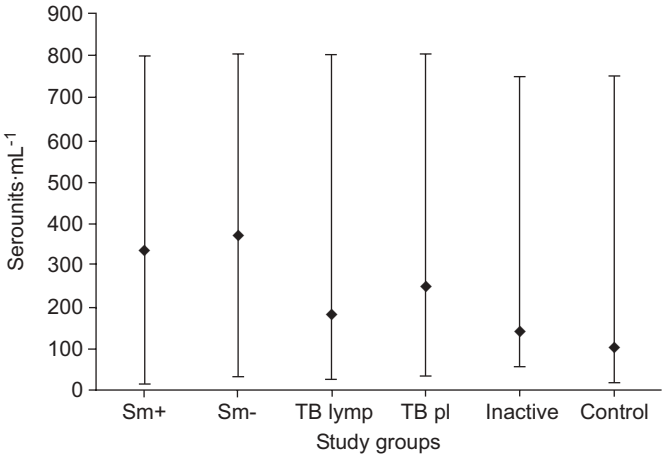

FIGURE 2. Maximum, minimum and mean values of antibody titres for 38-kDa and 16-kDa mycobacterial antigens of study groups. Sm+: smear-positive pulmonary tuberculosis (TB); Sm-: smear-negative pulmonary TB; TB lymp: TB lymphadenitis; TB pl: TB pleuritis. ♦: mean values.

that between pulmonary and extrapulmonary TB groups (p>0.05). However, significant differences between TB and both inactive and control groups were found (p<0.05).

In the two subpopulations of control cases (healthy subjects and those with pulmonary diseases other than TB), there were no differences in optical density means and serologic positivity rate (p>0.05).

TST results were observed in 53 patients (45 smear-positive and eight smear-negative; 27 seropositive and 26 seronegative). Mean TST values were found to be 14.5±6.5 mm and 17±3.9 mm in smear-positive and -negative patients, respectively (p>0.05). Five (9.4%) patients' TST diameters were <10 mm. Four out of these five patients were seropositive. The mean diameters of TST of seropositive and seronegative patients were determined as 13.8±7.3 mm and 16±4.6 mm, respectively (p>0.05).

No BCG scars were observed in 11 patients. All BCG-negative patients were found to be TST positive. Five (45.4%) BCG-negative patients were seropositive and six (54.6%) BCG-negative patients were seronegative (p>0.05).

DISCUSSION

The present study shows that humoral response to mycobacterial antigens could be a significant indicator for active pulmonary TB and could help in the diagnosis of TB.

According to the literature, serological tests have high specificity and a much lower sensitivity in the adult population. The sensitivity of the tests also depends 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

TABLE 2 Sensitivities, specificities and negative and positive predictive values of the PATHOZYME-TB Complex Plus test in tuberculosis (TB) cases

	Cases n	Seropositive cases n	Sensitivity	Specificity	PPV	NPV	Diagnostic accuracy
Sm+	143	78	54.55	93.33	95.12	46.28	66.01
Sm-	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	#	#	#

Data are presented as %, unless otherwise stated. Sm+: smear-positive pulmonary TB; Sm-: smear-negative pulmonary TB; PPV: positive predictive value, NPV: negative predictive value. #: statistical values did not present owing to the low number of samples.

reported for the 38-kDa antigen varies greatly (16–94%), largely depending on the smear status and disease manifestation. The specificities of the test reported previously coinciding (from 88–100%), but the reported sensitivities of the test vary 33–89% for smear-positive TB patients and 16–54% for smear-negative TB patients [5, 13–17]. Some of the values from the literature are shown in table 3 for convenient comparisons between main past investigations. Previous studies have reported lower sensitivities with the recombinant version of the 38-kDa antigen, with sensitivities of the ELISA technique ranging 16–36% for smear-negative patients and 36–67% for smear-positive patients [5, 7, 19]. CHIANG *et al.* [8] reported that the 38-kDa antigen had a sensitivity in diagnosis of 64%. In receiver operating curve analysis, when the specificity was set to 100%, the 38-kDa antigen had the highest sensitivity of the antigens studied. SAMANICH *et al.* [19] detected antibodies to the 38-kDa antigen in 57.8% of pulmonary cavitary TB patients. In a recent study in Turkey, ERER *et al.* [18] reported 46.5% sensitivity with 100% specificity in pulmonary TB.

Although antibody response to the 38-kDa antigen in pulmonary TB has been extensively studied, there are only a few reports about the utility of the 38 kDa-based serological tests in extrapulmonary TB. Antibodies were detected in

TABLE 3 Different literature results for sensitivities of antibody responses against 38-kDa antigen and 16-kDa antigen in various tuberculosis (TB) patient populations

Study [Ref.]	38-kDa antigen	16-kDa antigen	38-kDa and 16-kDa antigen
MEENA [16]	39 (79)		
CHIANG [8]	312 (64.1)		
WILKINSON [17]	292 (38)		
WELDINGH [11]	48 (19)		
ERER [18]	43 (46.5)		
UMA [12]	(61)		
BOTHAMLEY [14]	25 (84)#		
	27 (82)¶		
	41 (90)+		
SAMANICH [19]	19 (57.8)		
WILKINS [4]	122 (73)§		
	122 (70)¶		
LYASHCHENKO [20]	59 (25)	59 (44)	
SILVA [5]	42 (45)#	42 (33)#	
	15 (33)¶	15 (13)¶	
BECK [7]	51 (59)	51 (50.9)	
DEMKOW [2]	24 (61)#		24 (59)#
	243 (27)¶		243 (23)¶
	52 (42)§		52 (42)§
IMAZ [21]			17 (58.8)#
			41 (29.3)¶
JULIAN [3]			29 (31)#
			11 (0)¶
DEMKOW [6]			30 (56)f

Data are presented as n (%). #: smear-positive patients; ¶: smear-negative patients; +: TB without bacteriological confirmation; §: extrapulmonary TB; f: bone and joint tuberculosis.

12–56% of extrapulmonary TB patients [12]. WILKINS and IVANYI [4] described competition ELISA assays based on the high sensitivity and specifity of the 38-kDa monoclonal antibody; antibodies to the 38-kDa antigen were detected in 73% of cases of extrapulmonary TB at a chosen specifity of 98%.

A combination of antigens was also found to be more useful in serodiagnosis [22]. This combined use of antigens maximises the effectiveness of serodiagnosis, but it is not possible to detect all antibodies as well, as this could cause low specifity. POTTUMARTHY *et al.* [23] indicated that maximum sensitivity was obtained when seven tests were combined, but also that specifity fell to 55% in controls. The combinatory use of the 38-kDa and 16-kDa antigens may increase the test sensitivity compared with the 38-kDa antigen alone [24]. Seropositivity for the 38-kDa and 16-kDa antigens together was found by JULIAN *et al.* [3] to be 31% in smear-positive, 0% in smear-negative and 8% in extrapulmonary TB, and by IMAZ *et al.* [21] to be 58.8% in smear-positive and 29.3% in smear-negative TB. BECK *et al.* [7] have detected antibodies against 16-kDa and 38-kDa antigens in 50.9 and 59% of samples of TB patients, respectively. DEMKOW *et al.* [2] have found 59, 23, 40 and 43% rates for culture-positive, culture-negative, TB nodes and TB pleuritis, respectively. For inactive TB cases, some studies have found positive rates 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.* [20] 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 [25]. However, significant variance in the serological 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 human leukocyte antigen (HLA) types [9]. HOUGHTON *et al.* [26] pointed out that the frequency of recognition using the same recombinant 38-kDa antigen preparation ranged 35–82% with samples from smear-positive patients from four different geographical areas. WELDINGH *et al.* [11] have observed a large variation in sensitivity when testing for the same antigens with sera from TB patients living in Denmark and sera from TB patients living in Uganda. In accordance with earlier results [27], it was found that the highest proportion of positive antibody responses was in 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 patients from areas of high and low endemicity are admitted to hospital, and this factor may also influence the magnitude of the antibody response mounted by the patients.

In most studies, responses to the mycobacterial antigens were more common in patients with active TB who were smear positive than with smear-negative disease [5]. In the present study, response to the 38-kDa and 16-kDa antigens was higher in smear-negative than -positive patients, in contrast to results published in the aforementioned literature. According to the literature, serological response depends on dissemination of pulmonary lesions, in addition to phase of disease, duration of antigenic stimulation and the HLA types of patients [2, 3, 9, 20, 21]. In the TB groups presently studied, dissemination of lesions was not assessed and classified. It is probable that higher serological reactivity could result from patients in the smear-negative pulmonary TB group having disseminated lesions. Smear-negative TB diagnosis takes much more time than in bacteriologically confirmed cases, so antigenic stimulation could be presented for longer and humoral response can be stronger.

Sensitivity for extrapulmonary TB cases was found to be concordant with 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 of the PATHOZYME-TB Complex Plus test appeared to be weak.

It is difficult to assess TB activity in patients presenting with pulmonary disease symptoms and in whom chest radiography shows fibrocalcified tuberculosis lesions. Inactive pulmonary TB and smear-negative pulmonary TB cases are difficult to differentiate from each other. As part of the present study, serological responses to smear-negative cases were compared with inactive cases (certain negative cases for TB) and found helpful for differentiation. The present results indicate that serology could help in the assessment of TB activity in smear-negative pulmonary patients.

To replace the ''gold standard'' culture test, a serological test should possess sensitivities of >80% and test specificities of >95%, according to the recommendations of the WHO [28]. In Turkey, the TST could not help greatly in the diagnosis of TB, owing to the moderate prevalence of TB and BCG vaccination. Studies pointed out that TST positivity was detected in 24–77% of the general population and similarly in 56–69% of the TB patients in the country [29, 30]. Therefore, TST is a valuable but nonspecific test for the assessment of TB patients in Turkey. The present results demonstrate that the IgG tests based on recombinant mycobacterial antigens (38-kDa and 16-kDa) present very good specificity but a lower than acceptable level of sensitivity for the diagnosis of TB in concordance with the published literature.

It can be concluded that 38 kDa- and 16 kDa-antigen-based tests could be used in combination with other methods to increase diagnostic accuracy in tuberculosis. In inactive tuberculosis cases, as sensitivity was statistically significantly lower than in the pulmonary tuberculosis group, serological response to combined 38-kDa and 16-kDa antigens seemed to be an indicator for assessing pulmonary tuberculosis activation. In spite of low sensitivity in the current cases, serological methods may be valuable in the diagnosis of extrapulmonary tuberculosis, which is often an especially difficult form to be confirmed bacteriologically in patients from whom it is difficult to obtain specimens.

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