Rapid Sero Diagnosis of Pulmonary Tuberculosis through Humoral Immune Response Against 38-kDa and 16-kDa Mycobacterial Antigens

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Abstract: Tuberculosis still constitutes a major health problem through the world in spite of major advances in diagnosis, treatment and prevention. Annually, 8 million people become ill with tuberculosis, and 2 million people die from the disease worldwide. Several ELISA tests 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. The present study aimed to measure the diagnostic value of 16-kDa and 38-kDa mycobacterial antigens in patients who were diagnosed with Tuberculosis by clinical and, or bacteriological findings. This study was carried out in Chest Department of Benha University Hospital on 5 groups which were (40) patients smear positive pulmonary tuberculosis, (20) patients smear negative pulmonary tuberculosis, (20) patients inactive pulmonary tuberculosis and control group of (20) patients with disease other than tuberculosis. Eg. COPD-Broncheactasis, and (20) normal healthy subjects. Medical history, clinical examinations, complete blood picture, ESR, Liver Function Tests, Kidney Function Tests, Fasting Blood Sugar, Tuberculine Skin Test by Montoux Method, sputum for Acid Fast Bacilli by Z.N. stain, pathozyme-TB Complex plus commercial ELISA kit for measuring Immunoglobulin G against 38-kDa and 16-kDa recombinant antigens of all tested groups of TB and control groups. Our findings demonstrated that specificity, sensitivity, positive predictive value, negative predictive value for smear positive group were 97%, 75%, 98.4% and 30% respectively. And for smear negative pulmonary tuberculosis were 95%, 65.2%, 95%, 81% and 80% as regarding specificity, sensitivity, positive predictive value, negative predictive value respectively. Antibodies were detected at above cut-off level in (2) 10% out of (20) subjects with inactive TB. In conclusion, the ELISA Test has a very good specificity and an acceptable sensitivity and positive predictive value for diagnosis and follow-up of Pulmonary Tuberculosis. **Recommendation:** It is recommended that it could be used in combination with other method to increase the diagnostic accuracy especially for smear negative Tuberculosis cases.

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1. Introduction:

Tuberculosis still constitutes a major health problem throughout the world in spite of major advances in diagnosis, treatment, and prevention of Tuberculosis. (***Trollip, et al 2001***). In 2004, around 14.6 million people had active TB disease with 9 million new cases. The annual incidence rate varies from 356 per 100,000 in Africa to 41 per 100,000 in the America. (***WHO 2006***).The rise in HIV infections and the neglect of TB control programs have enabled a resurgence of tuberculosis. (***Lade Marco and Castro 2003***). The emergence of drug resistant strains has also contributed to this new epidemic with, from 2000 to 2004, 20% of TB cases being resistant to standard treatments and 2% resistant to second line drugs. (***MMWR et al., 2006***).The rate at which TB new cases occurs varies widely, even in neighboring countries, apparently because of differences in Health Care System. (***Sobero and Reobody 2006***). Several Enzyme –Linked Immuno Sorbent Assays (ELISAs) have been tried to achieve the rapid, early and easy diagnosis of Pulmonary Tuberculosis. (***Chan et al.,2000***).However, a large variability in their accuracy has been reported depending on the antigen employed, the Immunoglobulin (Ig) class measured, the strain variation, and the deviation in the antibody response among different ethnic groups (***Bothamley, 1995***). The evaluation of different serological kits at the same time with the same group of sera has been rarely carried out. (***Chiang et al.,1997***). Immunological methods use the specific humoral or cellular responses of the host to guide the presence of infections or disease. They do not require a specimen from the site of infection.Numerous serological tests that use various antigens, such as secreted and heat shock proteins, lipopolysaccharides, and peptides, have been

developed. (***Bothamley, 1995***). These tests use various modifications of Enzyme-linked Immunosorbent Assay (ELISA) or Immunochromatograpic methods to detect different antibody classes. (***Verbon et al., 1993)***.

Aim of the Work:

The present study was carried out to measure the diagnostic value of 16-kD and 38- kD mycobacterial antigens in patients who were diagnosed with tuberculosis by clinical and bacteriological findings.

2. Subjects and Methods:

120 subjects were included in this study, they were chosen from out patient clinic of Benha Faculty of Medicine. They were classified into five major groups:

Group I: Included 40 patients of smear positive active pulmonary tuberculosis.

Group II: Included 20 patients of smear negative active pulmonary tuberculosis.

Group III: Included 20 patients of Inactive pulmonary tuberculosis.

Group IV: (Healthy Control) Included 20 healthy subjects free from diseases and all were non-smokers.**Group V:** Included 20 patients with diseases other than tuberculosis. Eg. COPD-Broncheactasis and Pneumonia. All patients were selected from Out Patients clinics of Benha University Hospital

.**Smear Positive Pulmonary TB:**

A patients with at least two sputum specimens positive for Acid Fast Bacilli by microscopy or a patient with at least one sputum specimen positive for Acid Fast Bacilli by microscopy and radiographic abnormalities consistent with Pulmonary TB, and decision by physician to treat with a full course of anti-TB chemotherapy or patients with at least one sputum specimen positive for Acid Fast bacilli by microscopy, which is culture positive for M. tuberculosis.

Smear Negative Pulmonary Tuberculosis:

A patients with two sets (taken at least two weeks apart) of at least three sputum specimens that are negative for AFB, radiological abnormalities consistent with pulmonary tuberculosis and lack of clinical response of at least two weeks of broad spectrum antibiotics.

Inactive Pulmonary Tuberculosis:

Patient who have been treated either with complete course of anti-tuberculosis or had presented with chest radiography, showing a fibro calcified pulmonary TB lesion. All chest radiographs of the patients were stable for > 6 months and patients were sputum smear and culture negative for TB. Patients were not on corticosteroids or other Immunosuppressive agents, had no clinical evidence of AIDS and HIV negative.

All Subjects were subjected to the followings:

Medical history and clinical examination. Radiological examination by plain chest X-ray postero-anterior and lateral views.Complete blood picture, ESR (Erythrocyte Sedimentation Rate), Liver Function Tests, Kidney Function Tests, Fasting Blood Sugar (to exclude the presence of other concomitant diseases such as liver disease and diabetes mellitus. Tuberculin Skin Testing by Mantoux Method. Sputum for Acid Fast Bacilli by Z.N. stain. Measurement of Immunoglobulin G against 38-kDa and 16-kDa recombinant antigens by using The PATHOZYME-T.B. Complex Plus Commercial ELISA kit. Bacille Calmette-Guerin (BCG) vaccination is mandatory in Egypt. Tuberculin Skin Test (TST) is not a certain diagnostic tool, owing to BCG vaccination programmes and endemicity of TB in Egypt. Serological Test**:** A commercially available ELISA kit, the PATHOZYME-TB complex plus (*Omega diagnostics, Alloa, UK*) 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. Diluted (1:50) serum was distributed in micro titre wells and incubated for 60 mins at 37°C. Unbound serum was removed by washing with a buffer solution. The wells were subsequently incubated with peroxidase-labelled anti-Human conjugate at 37°C for 30 mins. After another wash cycle, peroxidase substrate tetramethylbenzidine containing hydrogen peroxide was added to the wells and the colorimetric reaction was prolonged for 15 mins in dark at 37°C until stop reagent was added. Absorbance values at 450 nm were recorded. Four standards (with 2, 4, 8 and 16 sero unit/ml^{-1}) were provided to generate a semi-lag reference curve. Because the sera were diluted 1:50, the units extrapolated from the curve were multiplied by 50 to obtain sero units for result interpretation. According to manufacturer's instructions, a result was considered positive when the level of antibodies in a sample was higher than 200 sero units/ ml^{-1}.The serum samples and positive and 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. (***Omega Diagnostics, Alloa, UK)***.

Statistical Analysis:

The chi-squared test was used to calculate the significance of difference of positivity percentages of patients in comparison with the other 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.

3. Results:

Table I: Age Distribution of Studied Group

Age (Years)	Group 1 No=40	Group 2 No=20	Group 3 No=20	Group 4 No=20	Group 5 No=20
Age	40 – 65	30 – 50	30 – 60	22 – 55	25 - 60
Mean	48.6	44	47.6	46.5	45.4
SD±	25.2	30.3	15.6	20.5	25.4
T. Test	0.002	0.002	0.12	0.17	0.15
P Value	>0.05 Non-significant	>0.05 Non-significant	>0.05 Non-significant	>0.05 Non-significant	>0.05 Non-significant

Table 2: Sex Distribution of Studied Groups

	Patients	**Control**
Male	64 (80%)	28 (70%)
Female	16 (20%)	12 (30%)
Total	80	40

Table 3: Specificity, Sensitivity-PPV, NPV and diagnostic accuracy of smear positive and smear negative TB cases

Group	**No. of Positive Cases**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Diagnostic Assay**
Smear (+) No=40	30	75%	97%	98%	30%	80.5%
Smear (-) N=20	13	65.2%	95%	80.1%	80%	86%

PPV ie Positive Predictive Value NPV ie Negative Predictive Value

Table 4: Statistical Comparison between number of Positive Test of Smear Positive Group, Control Groups and Inactive TB Group

Group	**No.**	**(+) Test**	**%**	**PValue**	**Significant**
Smear (+)	40	30	75%		Highly
Healthy Control	20	2	10%	<0.001	significant
Smear (+)	40	30	75%	< 0.001	Highly
diseases other than TB	20	1	5%		significant
Smear (+)	40	30	75%	<0.001	Highly
Inactive TB	20	4	20%		significant

Table 5: Statistical Comparison between number of positive cases (%) (Smear negative group), (control groups) and inactive TB group

Group	**No:**	**(+) Test**	**Percent of Positive Test**	**P value**	
Smear (-)	20	13	65%	< 0.001	Highly
Healthy Control	20	2	10%		Significant
Smear (-)	20	13	65%	<0.001	Highly
Diseases other than TB	20	1	5%		Significant
Smear (-)	20	13	65%	< 0.001	Highly
Inactive Pulmonary TB	20	4	20%		Significant

Table 6:Statistical Comparison between number of positive cases of Inactive TB group and control groups (Healthy and diseases other than TB)

Group	**No.**	**Positive Test**	**%**	**PValue**	
Inactive TB	20	4	20%	> 0.05	Non-Significant
Healthy Control Group	20	2	10%		
Inactive TB	20	4	20%	> 0.05	Non-Significant
Diseases other than TB	20	1	5%		

Table 7:Statistical comparison between maximum, minimum and mean values ± SD of antibodies titre for smear positive TB and Inactive and control groups

Group	**Maximum**	**Minimum**	**Mean**	**SD±**	**PValue**
Smear Positive	900	10	400	20.11	<0.001
Inactive	500	5	120	2.8	Highly Significant
Smear Positive	900	10	400	20.11	< 0.001
Healthy Control	500	5	90	1.3	Highly Significant
Smear Positive Diseases Other than TB	900 300	10 5	400 80	20.11 4.7	< 0.001 Highly Significant

Table 8: Statistical comparison between maximum, minimum, mean values ±SD of antibodies titre for smear negative TB and inactive and control groups

Group	Maximum	Minimum	Mean	SD±	PValue
Smear Negative Inactive	850 500	15 5	350 120	21.2 2.8	<0.001 Highly Significant
Smear Negative Healthy Control	850 400	15 5	350 90	21.2 1.3	< 0.001 Highly Significant
Smear Negative Diseases Other than TB	850 300	15 5	350 80	21.2 4.7	< 0.001 Highly Significant

Table 9:Statistical comparison between maximum, minimum, mean values ±SD of antibodies titre of inactive TB group and control groups

Group	Maximum	Minimum	Mean	SD ±	PValue
Inactive Healthy Control	500 400	5 5	120 90	2.8 1.3	> 0.05 Non- Significant
Inactive Diseases Other than TB	500 300	5 5	120 80	2.8 4.7	> 0.05 Non- Significant

Table 10: Statistical comparison between maximum, minimum, mean values ± SD of antibodies titre of smear positive and smear negative TB

d	Maximum	Minimum	Mean	SD ±	PValue
Smear Positive	900	10	400	20.11	0.05
Smear Negative	850	15	350	21.2	Insignificant

4.Discussion

Tuberculosis is diagnosed definitively by identifying the causative organism (Mycobacterium tuberculosis) in clinical sample (for example sputum or pus). When this is not possible, a probable diagnosis maybe made by using imaging (X-ray or scans) and/or Tuberculine Skin Test.The main problem with Tuberculosis diagnosis is the difficulty in culturing this slow-growing organism in the laboratory (It may take 4-12 week for Blood or Sputum Culture).

A complete medical evaluation for TB must include medical history, physical examinations, chest X-ray, microbiological smears and cultures. Tuberculine Skin Test and even serological tests.

The interpretation of the Tuberculine Skin Test depends upon the person's risk factors for infection and progression for TB disease, such as exposure to other cases of TB or Immunosuppresed. (***CDC 2000)***.The past decade has been dramatic developments in serological tests for tuberculosis. The long history of serological tests for Tuberculosis is indicator to the need for sensitive diagnostic test, especially when sputum smear is unhelpful. (***Bathmaly et al., 1995)***. Several Enzyme-linked Immunosorbent Assays (ELISA's) have been tried to achieve the rapid, early and easy diagnosis of pulmonary tuberculosis. (***Chan et al., 2000)***.However, a large variability in their accuracy has been reported depending on the antigen employed, the Immunoglobulin (Ig) class measured, the strain variation, and the deviation in the antibody response among different ethnic groups. (***Bothmly et al., 1995)***. Many Mycobacterial antigens have been identified, such as 71, 65, 38, 23, 19, 16, 14 and 12-kDa proteins.

The 38-kD 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. The 16-kDa antigen is an immuno dominant 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. (***Beck et al., 2005)***. The present study was designed to elucidate and to study the humoral immune response against 38-kDa and 16-kDa Mycobacterial antigens in tuberculosis and to compare by healthy subjects and by persons with diseases other than TB. E.g. Broncheactasis, Pneumonia. This study showed that there's no significant results as regarding the age between the studied groups. (**Table 1**). On the other hand our results demonstrated that there is higher sensitivity (75%), higher specificity (97%), positive predictive value of (98%) and negative predictive value (30%) for smear positive pulmonary tuberculosis. (**Table 3**). There was also higher specificity (95%), acceptable sensitivity (65.2%), PPV (80%) and negative NPP (80%) for ELISA test as smear negative pulmonary tuberculosis (**Table 3**).

The diagnostic accuracy for smear positive TB was (80.5%) and for smear negative TB was (86%). (**Table 3**). Our results demonstrated also that there is highly significant differences between numbers of positive cases for the test (ELISA-T Pathozyme TB complex plus) as regarding smear positive pulmonary TB, inactive pulmonary TB, healthy control and

diseases other than tuberculosis group. (**Table 4**).There was also that , there is highly significant differences between number of positive cases for the test as regarding smear negative pulmonary TB, inactive pulmonary TB, healthy control group and diseases other than tuberculosis group. (**Table 5**).

Our study is in agreement with that done by (***Senol et al., 2007***) who studied 143 smear positive pulmonary tuberculosis and 19 smear negative pulmonary tuberculosis for their Humoral Immune response against 38-kDa and 16-kDa mycobacterial antigens by pathozyme-TB complex plus commercial ELISA kit. He said that there is higher specificity for the test (93.33%), accepted sensitivity (54.5%) and diagnostic accuracy (66.1%) between smear positive pulmonary tuberculosis and also for smear negative pulmonary tuberculosis. He found that there is higher specificity (93.33%) and high sensitivity (63.16%) and higher diagnostic accuracy (86.8%). (***Chiang et al., 1997)*** reported that 38-kDa antigen had a sensitivity in diagnosis of 64% when the specificity was set to 100%, the 38-kDa antigen had the highest sensitivity of the antigen studied.

Our results also in agreement with the results of (***Bathmaley el at., 1995***) who found that a monoclonal antibody competition assay anti-38 kDa antibody is the most sensitive serological test for smear negative tuberculosis so far. (***Cole et al., 1996***) did evaluationof previous version of ICT (Immunochromatography Test) for tuberculosis which contained only the 38 kDa antigen with sera from 152 patients with active pulmonary tuberculosis and showed a sensitivity of 89% for smear positive patients and sensitivity of 74% for smear negative patients, with specificity of 93%. Our results demonstrated also that there is highly significant difference in antibodies level between smear positive pulmonary tuberculosis, inactive tuberculosis, healthy control group and patients with diseases other than tuberculosis. (**Table 7**).

There is also highly significant differences in antibody levels between smear negative pulmonary tuberculosis, inactive tuberculosis, helthy control group and patients with diseases other than tuberculosis. (**Table 8**).The difference between antibody level between smear positive and negative pulmonary TB group is not significant. (**Table 10**).

These results are in agreement with that done by (***Senol et al.,2007***) who found that there is no significant difference between sensitivities of smear positive and negative pulmonary TB groups. He found also that there is a significant differences in antibody level between TB and both inactive and control groups.In contrast to a study done by (***Abdel Noor et al 1999***) in Lebanon, that he study the sensitivity and specificity of 38-kDa antigen in 37 patients with confirmed tuberculosis, he found that the sensitivity of the immunoassay for 38 kDa was 10.8% and the specificity 84.4%. This contrast could be explained by the nature of the environmental mycobacteria that may influence the host's anti M. tuberculosis response. (***Bahr et al., 1986)***.

Human genetic variation is an important determinant of the outcome of infection with M. tuberculosis. Such variation may also affect the humoral immune response. However, these suggestions are speculative and require further investigation. (***Hill et al.,1999)***. The 38 kDa antigen is a serological antigen and is also a core component in different commercial TB serological tests. (***Weldingh et al.,2005)***. The recognition frequency 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. (***Silva et al., 2003)***.

Our study lacking demonstration of antibody in extra pulmonary tuberculosis so in the future we need to study antibody response in extra-pulmonary tuberculosis. **and Ivanyj.,1990** described competition ELISA assays based in the high sensitivity and specificity of the 38-kDa monoclonal antibody; antibodies to 38 kDa antigen were detected in 73% of cases of extra pulmonary TB at a chosen specificity of 98%. A combination of antigens was also found to be more useful in serodiagnosis. (***RAJa.,2004)***.

We combined the use of 38 – kDa and 16 kDa antigens to increase the test specificity and sensitivity, this also with agreement with that done by (***Chan et al., 2000***) who reported that the combined use of 38 kDa and 16 kDa antigens may increase the test sensitivity compared with the 38 kDa antigen alone. It can be concluded that 38 kDa and 16 kDa antigen based test could be used in combination with other methods to increase the diagnostic accuracy of 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. The combined uses of both 38 kDa and 16 kDa antigen raises the diagnostic accuracy of the test rather than to use one antigen alone. So this test could be used in diagnosis and follow-up and assessment of activity of both smear positive, smear negative and inactive pulmonary tuberculosis. Further studies need to assess the effectiveness of the test to help in assessment of diagnosis of extra pulmonary 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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