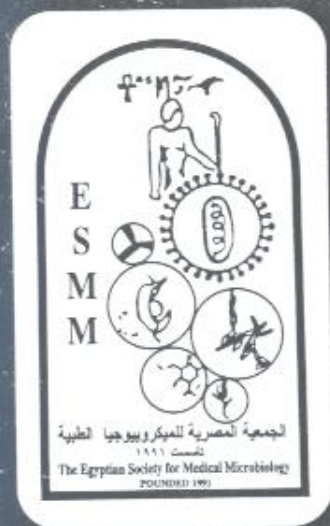


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A60 Antigen as a Rapid Serodiagnosis Versus Clinical and Conventional Diagnostic Approaches of Active Pulmonary Tuberculosis in Al-Medina, Saudi Arabia

Ali A. Abdelrahman¹, Mohamed Y. Abdul-Aziz², Khalid Hussein³, Haider Abdelsalam³,
and Abdulalla Raffai³

¹Department of Medical Microbiology and Immunology, and ²Department of Internal Medicine, ^{1,2}Faculty of Medicine, Taibah University, ³King Fahd Hospital, Medina, Saudi Arabia

Problems involved in diagnosis of tuberculosis with sputum smear, culture and radiological examination have necessitated exploring the utility of immunodiagnosis of tuberculosis as a convenient and rapid test to supplement clinical information for definite diagnosis. We have evaluated the serodiagnosis by Enzyme-Linked Immunosorbent Assay (ELISA) for detection of immunoglobulin G (IgG) antibodies against A60 antigen complex in parallel with other familiar diagnostic methods. Serum specimens from 70 patients with active pulmonary tuberculosis (group I) along with 70 healthy controls (group II) were examined. Sputum smears stained with Ziehl-Neelsen (Z-N) for acid-fast bacilli in 60 patients (85.7%) were positive. In 48 patients (68.6%) the bacilli grew on Löwenstien-Jensen medium. The overall positivity of tuberculin skin test was 50 (71.4%) and Chest X-Ray (CXR) was consistent with pulmonary tuberculosis among the 53 (75.7%) patients. Group I showed significantly higher titers of IgG antibodies against A60 antigen complex than group II ($P < 0.001$). ELISA result 61(87%) were positive. The overall sensitivity and specificity obtained using ELISA was 90% and 95.7% respectively in active pulmonary tuberculosis. A60 immunodiagnosis is rapid, inexpensive, highly sensitive and specific method for diagnosis of active *M. tuberculosis* infection. It will help in vague, unclear or even uncertain diagnosis to start patients on medication.

INTRODUCTION

Tuberculosis (TB) remains a pressing global public health problem. Millions die of TB annually in spite of the availability of highly effective therapy. New advances in the diagnosis of TB, however, offer the promise of improved TB control⁽¹⁾. Early identification and diagnosis of cases of active TB are keys to the effectiveness of control programs. However, maintaining a high index of suspicion for TB is crucial and diagnostic testing remains problematic. Culture of appropriate specimens for isolation of *M. tuberculosis* (MTB) and susceptibility testing is the cornerstone for TB diagnosis. Traditional diagnostic techniques based on acid fast bacilli (AFB) direct smear microscopy lack sensitivity and the isolation of the tuberculosis bacillus on conventional Löwenstein-Jensen (LJ) culture

media are time consuming and require 4 to 8 weeks to obtain a result⁽²⁾. The approach is to diagnose pulmonary and extra-pulmonary TB, including applicability of newer rapid diagnostic tests. The methods of diagnosis of pulmonary tuberculosis mainly depend upon initial clinical suspicion and radiographic findings with subsequent bacteriological confirmation by sputum direct smear microscopy examination and culture⁽³⁾. Recent molecular biology techniques have made it possible to diagnose tuberculosis (TB) in a few hours. However, it is expensive and not a readily available facility in most of the hospitals in the developing countries⁽²⁾.

ELISA based on serological test to detect specific antibodies against *M. tuberculosis* is simple, inexpensive and

potentially practical method for the diagnosis of active pulmonary TB. Another rapid diagnostic method would be the detection of specific antibodies in tuberculosis patients. However, almost all of the assays are limited by sensitivity, especially in smear negative TB patients⁽³⁾. Many antigens have been evaluated in order to develop a rapid TB diagnostic test⁽⁴⁾, using culture filtrates, and extracts either from *M. tuberculosis* or from *M. bovis*. Antigen 60 complex is a major mycobacterial cell wall antigen and composed of proteins, carbohydrates and lipids in roughly equal amounts⁽⁵⁾. The antigen shows cross reactions with other species as *M. lepra*, *Nocardia* and *Corynebacterium*⁽⁶⁾. Serology can help in demonstrating the existence of an infectious reservoir that favors the spread of the disease^(7, 8). Many studies explored the use of A60 antigen in detection of TB antibody from pulmonary and extra-pulmonary sera with varying success (32.1%-88.5%)^(9,10). It is a thermostable complex present in the cytoplasm of exponentially growing mycobacteria, accumulated within the cell wall of stationary cells and released during the active disease^(11, 12). It has been seen that TB patients were usually positive for IgG antibodies more often than IgM⁽³⁾. In this study we evaluated ELISA for A60 antigen for the serodiagnosis among other familiar diagnostic methods of active pulmonary tuberculosis and in healthy controls with no evidence of latent *M. tuberculosis* infection.

PATIENTS AND METHODS

Study population: Group I: Seventy patients of the present work were recruited from those who are regularly attending outpatient clinic and who were admitted as a case of active pulmonary tuberculosis to the Chest Hospital, Medinah Menawarah, Saudi Arabia. The study was continued for two years and included all the cases attended to the hospital. The facility will only allow Z-N stain in the laboratory. Other

investigations of staining, culture and ELISA were done at King Fahd Hospital. Patients were referred mostly as pulmonary TB. All patients were subjected to full clinical examination and a tuberculin skin test (TST), CXR, sputum for Z-N stains for AFB and *M. tuberculosis* culture were requested.

Group II: Seventy normal healthy volunteers, of matched age and sex were taken as a control group. Healthy blood donors and persons, attending for routine medical check-up for employment, who were HIV negative, negative TST, negative family history for TB and normal CXR were included. All cases were subjected to thorough medical history taking and examination with stress upon symptoms and signs of active tuberculosis features of chronic diseases such as renal, hepatic gastrointestinal or endocrine diseases. In addition to routine investigations such as complete blood count, serum albumin, ALT levels, blood sugar and serum creatinine. All patients and controls were HIV negative

Diagnosis of Active Pulmonary Tuberculosis: T.B patients were defined as two criteria out of three: Positive of AFB, radiographic and culture evidence of MTB. Clinically, chronic cough is the most common symptom of pulmonary tuberculosis; early dry then productive, hemoptysis, pleuritic pain and dyspnea including fever, night sweats, chills, anorexia, weight loss and malaise. Upper-zone lung disease on the chest radiograph may be presenting symptoms. Physical findings in pulmonary tuberculosis are not generally helpful in defining the disease. Rales heard in the area of involvement as well as bronchial breath sounds if there is lung consolidation. The identification of persons who have active pulmonary tuberculosis includes history of exposure and classic symptoms of pulmonary tuberculosis.

Ziehl-Neelsen stain: Bacteriological evaluation is generally required to confirm the diagnosis of pulmonary tuberculosis. Positive AFB smears were included. In all patients with suspected active disease, three successive morning sputum samples for mycobacterium acid-fast stain examination. The sputum samples were subjected to decontamination, liquefaction using N-acetyl-L-cystine and NaOH 4% methods using BBL Mycoprep (Specimen Digestion/ Decontamination Kit) from Becton Dickinson (Cokeysville Maryland USA) for staining with Ziehl Neelsen stain⁽¹³⁾.

Culture of tuberculosis: *M. tuberculosis* cultures were collected. All clinical specimens suspected of containing mycobacteria were inoculated (after appropriate digestion and decontamination, if required) onto Löwenstein-Jensen culture media which is much more sensitive than microscopy, being able to detect as few as 10 bacteria/mL of material. The growth of the organisms is necessary for precise species identification⁽¹³⁾. A positive culture for MTB is generally required to evaluate the diagnosis.

Tuberculin skin test (TST): Skin purified protein derivatives (PPD) testing was performed for all patients and control⁽¹⁴⁾.

Chest radiography: CXR was done for all patients and control subjects. It is frequently used to determine whether or not the patient is likely to have active pulmonary TB. A number of patterns are suggestive on chest radiography, but none is diagnostic; including, parenchymal consolidation, cavitary lesions, right upper lobe infiltrate, fibronodular infiltrate, hilar lymphadenopathy, pleural effusion and a miliary pattern in symptomatic patients⁽¹⁵⁾.

ELISA (TB IgG) test: Each costs 11 Saudi Riyals and takes less than 2 hours to be performed. One

hundred and forty serum samples were obtained from the two groups. The test was performed to detect IgG antibodies to A60 antigen using commercially available kits (Anda Biological, Strausberg, France) according to manufacturer's instructions. Serum dilution of 1:100 was used in the assay. Positive and negative reference sera were included in run along with test sera. For determining IgG units, the curve was constructed by plotting the values of different reference sera. Thereafter, concentration of IgG antibodies in test serum in terms of units/ml were determined by extrapolating the value of serum against the reference curve⁽⁶⁾. For the ELISA technique, the diluted samples (serum) are incubated with the Antigen-60 sensitized wells of a microtitre plate for an hour at 37° C. After washing, the wells are filled with a solution containing anti-human IgG conjugated to peroxidase. After incubation for 30 minutes at 37° C, the wells are washed and incubated in the presence of an enzymatic substrate and H₂O₂. The enzyme turns the colourless substrate into a blue colour. The reaction is stopped with H₂SO₄, with the production of a yellow colour. The intensity of the colouration is read in a spectrophotometer and is proportional to the specific antibodies present in the analyzed sample. The IgG results are quantitative. In order to cope with the unavoidable daily variations observed with ELISA determinations, calibrators are included in the kit. A value of 2 IgG serounits has been empirically attributed to the calibrator that gives an absorbance of 0.4-0.5 under optimal conditions. The cut-off point of positive ELISA test was more than or equal to 125 IU/ml in the control group, and 225 or more IU/ml in group I patients with suspected active pulmonary TB⁽¹⁶⁾.

The reaction (colour) was quantitated using an enzyme-linked immunosorbent assay (ELISA) reader. The sensitivity of a test is the percentage of people with the condition

who have a positive test. If false-negative results are uncommon, the sensitivity is high.

Sensitivity is the proportion of true positive that are correctly identified by the test while, specificity is the proportion of true negative that are correctly identified by the test.

The discriminatory value of a given level serum IgG antibodies to A60 antigen detecting active tuberculosis was calculated according to the following formulae⁽¹⁷⁾:

$$\text{Sensitivity} = \frac{\text{True-positive} \times 100}{\text{True-positive} + \text{False-negative}} \quad \text{Specificity} = \frac{\text{True-negative} \times 100}{\text{True-negative} + \text{False-positive}}$$

Positive predictive value (PPV) is the proportion of patients with positive test results who are correctly diagnosed. Negative predictive value (NPV) is the proportion of patients with negative test results who are correctly diagnosed.

Statistical analysis: Data were collected, tabulated and analyzed on Microsoft package using statistical program for social science (SPSS) version 10. The difference in immunoglobulin (IgG) titer between the active pulmonary tuberculosis and healthy controls in serum were analyzed using Mann Whitney Willcoxon test. It was used to compare nonparametric quantitative variables between two groups ($SD > 25\%$ mean). Screening tests were used to identify sensitivity, specificity, positive and negative predictive values (PPV & NPV) of Amsel criteria⁽¹⁷⁾.

RESULTS

Total number of sera studied by ELISA was one hundred and forty, including seventy patients

in group I and 70 healthy individuals in group II. Table (1): Summarizes demographic and clinical features of the studied group. Patients age ranged from 14-63 years. Persistent cough was present in all patients (100%), expectoration in 53 patients (76%), hemoptysis in 49 patients (70%) and fever present in 59 patients (84%). Thirty-five patients (50%) were cigarette smokers and 22 patients (31%) had positive family history of tuberculosis. Seven patients (10%) had associated extra-pulmonary tuberculosis at onset of active pulmonary disease including 1 spinal tuberculosis, 1 tuberculous meningitis, and 5 cervical lymphadenitis. Diabetes mellitus was the most frequently associated disease in 10 (14.3%). CXR indicate 50% of parenchymal consolidation, 20% hilar lymphadenopathy, 24.3% cavitory lesions, 5% pleural effusion, 15.7% fibrosis, 11.4% miliary pattern and 24.3% no signs with active disease.

Table (1): Demographic, clinical and radiographic findings of studied population (Active pulmonary TB patients & healthy controls).

Variables	Group I	Group II
	NO (%)	NO (%)
Male	51 (73%)	44 (63%)
Female	19 (27%)	26 (37%)
Age years (mean \pm SD)	49.6 \pm 13.5	46.9 \pm 10.8
Country of origin		
Saudi	41 (59%)	39 (56%)
Non Saudi	29 (41%)	31 (44%)
Presenting Symptoms		
Cough	70 (100%)	
Expectoration	53 (76%)	
Fever	59 (84%)	
Hemoptysis	49 (70%)	
Pleuritic pain	31 (44%)	
Dyspnea	21 (30%)	
No symptoms	16 (23%)	
Smoking	35 (50%)	
Family history of TB	22 (31%)	
Extra pulmonary TB	7 (10%)	
Associated Diseases		
DM	10 (14.3%)	Non
ESRD, HD	1 (1.4%)	
CLD	1 (1.4%)	
Chest X-Ray (CXR)		
Parenchymal consolidation	35 (50%)	All (Normal)
Hilar lymphadenopathy	14 (20%)	
Cavitary lesions	17 (24.3%)	
Pleural effusion	4 (5.7%)	
Fibrosis	11 (15.7%)	
Miliary pattern	8 (11.4%)	
No signs with active disease	17 (24.3%)	

DM = Diabetes mellitus, ESRD = End stage renal disease, HD = Hemodialysis, CLD = Chronic liver disease.

The present study indicated that Z-N. stain for AFB was negative in 10 (14.3%) patients, and positive in 60 (85.7%). Culture technique results: Forty-eight patients (68.6%) showed positive growth indicative of *M. tuberculosis* in conventional L.J. medium. Tuberculin skin test (TST) was found to be positive in 50 patients (71.4%). CXR was consistent with active pulmonary tuberculosis in 53 patients (75.7%). Figure (1) summarizes the positivity rate of all tests in active pulmonary TB.

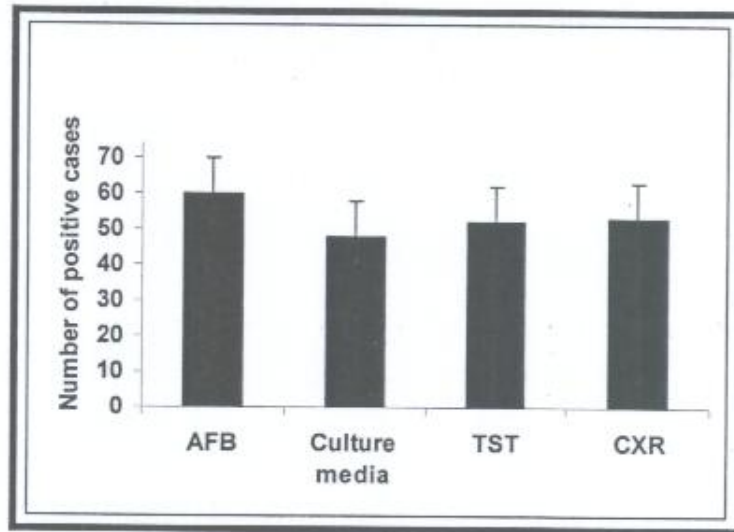


Figure 1: Comparison of different diagnostic results

It is necessary to compare different diagnostic parameters in TB patients and healthy control with the A60 IgG concentration. Results of ELISA test in active TB versus the healthy controls (Group II) including direct Z-N stain, culture method and CXR are summarized in Table (2). IgG antibodies against A60 antigen titer was significantly higher in group I than in group II.

Table (2): Conventional diagnostic methods and ELISA test in active TB (Group I) versus the healthy controls (Group II).

Diagnostic method	TB patients No. (%)	Healthy controls No. (%)
Smear positive (Z-N)	60 (85.7%)	0 (0%)
Culture positive	48 (68.6%)	0 (0%)
CXR consistent with TB	53 (75.7%)	0 (0%)
PPD test positive	50 57 (75%)	0 (0%)
Positive A60 IgG Test	61 (87%)	3 (4%)
A60 IgG Titer	411±329 IU/ml	111±79 IU/ml

ELISA for IgG antibodies against A60 antigen complex showed positive titers if more than 225 IU/ml and negative if less than 125 IU/ml. Group I was positive in 61 (87%) patients and 3 (4%) in group II (control) ($P < 0.001$). IgG antibodies against A60 antigen titer were significantly higher in group I than in group II. Group I had significantly higher titers of IgG antibodies against A60 antigen complex.

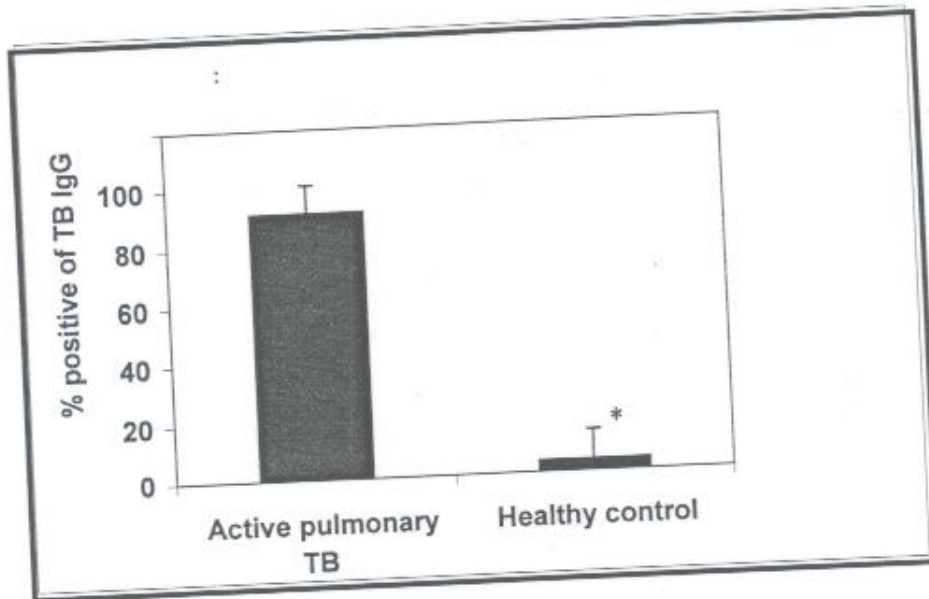


Figure 2: ELISA results from patients and healthy individuals

Regarding comparison between patients of active pulmonary and patients with both pulmonary and extra-pulmonary results, the positivity rate of ELISA among the positive other conventional methods for diagnosing are illustrated in Table (3). Active pulmonary with extra-pulmonary patients had higher titers of IgG antibodies (569 ± 379 IU/ml) than those with only active pulmonary (411 ± 329 IU/ml).

Table (3): Conventional diagnostic methods and ELISA test in patients with pulmonary versus the extra pulmonary TB.

Diagnostic method	Active pulmonary patients No. (%)	Active extra-pulmonary patients No. (%)
CXR consistent with TB	53 (75.7%)	3 (42%)
PPD test positive	50 57 (75%)	5 (71%)
Positive ELISA Test	61 (87%)	7 (100%)
ELISA Titer	411 ± 329 IU/ml	569 ± 379 IU/ml

Table (4) summarizes the positivity rate of ELISA among the positive other conventional methods for diagnosing active TB including direct Z-N stain, culture method and CXR.

Table (4): Positive rate of ELISA among different diagnostic methods in active pulmonary TB patients.

Variable	All tuberculosis patients	Patients with positivity of A60 IgG (61)
	(No. / %)	(No. / %)
Smear positive (Z-N)	60 (86%)	60 (98%)
Smear negative	10 (14%)	1 (2%)
Culture positive	48 (69%)	48 (79%)
Culture negative	22 (31%)	13 (21%)
CXR consistent with TB	53 (76%)	53 (87%)
CXR not consistent with TB	17 (24%)	8 (13%)
TST positive	57 (75%)	53 (87%)
TST negative	13 (25%)	8 (13%)

Screening tests to identify sensitivity, specificity, positive and negative predicative value (PPV & NPV) showed higher values compared to other methods. Our results indicated that the overall sensitivity and specificity obtained using ELISA was 90% and 95.7% respectively in active pulmonary tuberculosis (Table 5).

Table (5): Diagnostic potential of ELISA as compared to positive conventional methods (Sensitivity and specificity).

Diagnostic Method (in active pulmonary TB)	Sensitivity %	Specificity %	PPV*	NPV**
AFB (Z-N) stain compared culture	100	45.5	80	100
ELISA compared AFB (Z-N) stain	100	90	98	100
ELISA compared to culture	100	95.5	98	100
ELISA compared to CXR consistent with TB	100	94.1	98.1	100
Overall ELISA in active pulmonary TB	90	95.7	95.5	90.5

* PPV = Positive predicative value.

** NPV = Negative predicative value.

DISCUSSION

Tuberculosis has a devastating impact on developing countries. A number of rapid diagnostic tests have been developed in an effort to improve the diagnostic accuracy for TB and to speed presumptive identification prior to diagnosis by cultures⁽¹⁸⁾. Thus, a positive rapid diagnostic test can be helpful since the specificity is quite high, but a negative test, especially on a smear-negative specimen, does not exclude the diagnosis of TB⁽¹⁹⁾.

The study revealed that cough (100%), expectoration (76%), fever (84%) and hemoptysis (70%) respectively are the most clinical presentation of active tuberculosis. Tuberculous lymphomenengitis is the most common extra-pulmonary complication of T.B.(5 out of 7). The present study revealed that CXR was consistent with pulmonary tuberculosis among 53 (75.7%) patients. The most common radiological features suggestive of active TB are parenchymal consolidation (50%), hilar lymphadenopathy (20%) and cavitary lesions (24.3%) respectively (Table 1). Chest

radiography is frequently used to gauge whether or not the patient is likely to have active pulmonary TB, but the sensitivity and specificity are low⁽²⁰⁾. Sputum culture was more likely to be positive in patients with features suggestive of active TB on chest radiography than from those patients without features more characteristic of inactive disease⁽²¹⁾.

Data showed that the tuberculin test (PPD) allergy is not an absolute criterion for the diagnosis of active TB. The overall positivity of tuberculin skin test in figure (1) was 50 (71.4%). There might be a relation between humoral anergy and PPD (tuberculin) tolerance observed among the cases. Skin testing for TB is an epidemiologic tool to assess exposures and should not be performed as a test to diagnose active TB. It is neither sensitive nor specific⁽²²⁾. TST known to be negative due to suppressed T cell response in the phase of active disease and not useful in subjects with previous history of active TB or BCG vaccination. The PPD skin test has a reported false-negative rate of 25 percent during the initial evaluation of persons with active tuberculosis⁽²³⁾. This high false-negative rate appears to be due to poor nutrition and general health, overwhelming acute illness, or immunosuppression.

Many studies demonstrated that the gold standard for the diagnosis of TB is the demonstration of *M. tuberculosis* on smear or culture^(2, 13). Sputum smears for acid-fast bacilli in 60 patients (85.7%) were positive and 48 patients (68.6%) the bacilli grew on Löwenstein-Jensen medium. The major limitation of smear microscopy is its low sensitivity. Traditional culture techniques on solid media are more sensitive than smear microscopy but require several weeks to demonstrate the presence of *M. tuberculosis*⁽²⁾. In this study, the standard culture method was negative in one third of our patients. This is possibly due to few bacilli in smear positive for 22 patients or due to technical facilities in collecting and transporting the

samples from chest hospital to King Fahd hospital laboratory.

Regarding serological comparison, our patients showed significantly higher titers of IgG antibodies against A60 antigen complex than control ($P < 0.001$). ELISA results were positive in 61 (87%). The majority of apparent healthy controls of our study were negative in ELISA but 4% were positive. This might be due to possible contact to TB infected persons. The serological titer may become significant. Data showed that 50% of the employees in contact with T.B patients were A60 seropositive⁽²⁴⁾. *Nocardia* and *leishmania* infections give false positive results which are expected to occur at random found that hemodialysis and renal transplant were also seropositive⁽²⁵⁾. These patients were discovered to be colonized by atypical mycobacteria. The positive cases observed may be due to inapparent infection gained by contact with an infections focus.

The specificity of the ELISA test based on an antigen 60 is high in comparison to other methods. The overall sensitivity and specificity obtained using ELISA was 90% and 95.7% respectively in active tuberculosis (Table 5). Other studies have demonstrated similar results of specificity (96 to 99%)⁽²⁶⁾. The wish to observe a complete correlation between the serological data with clinical, radiological and bacteriological diagnosis. Evaluation pulmonary infection through radiology showed (75.7%) positivity, a positive AFB (85.7%) and positive culture (68.6%) while IgG seropositivities increase to (87%). In our setting the specificity for the serodiagnosis of tuberculosis maximizes the effectiveness of the test and considered to be an additional benefit being rapid and inexpensive. The possibility of false positive test as a result of latent tuberculosis was eliminated by choosing healthy controls with no evidence of previous *M. tuberculosis* infection. The false positive test results among the control individuals could be due to cross-reaction with

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المستضد (أ ٦٠) كطريقة تشخيص مصلية سريعة مقارنة بطرق التشخيص الإكلينيكية والعادية

لمرضى الدرن الرئوى النشط فى المدينة المنورة بالمملكة العربية السعودية

على عبد اللاه عبد الرحمن^١ ، محمد ياقوت عبدالعزيز^٢ ، خالد حسين^٣ حيدر عبد السلام^٣ ،
عبد الله رفيعى^٣

قسم الميكروبيولوجيا والمناعة^١ ، قسم الأمراض الباطنية^٢ ، كلية الطب^١ ، جامعة طيبة ، مستشفى الملك فهد^٣
بالمدينة المنورة - المملكة العربية السعودية

يعترض تشخيص مرض الدرن (السل الرئوى) عن طريق فحص عينات البصاق الممسوحة أو المزروعة أو عن طريق الأشعة السينية على الصدر بعض المشاكل، مما استدعى البحث عن طرق أخرى سهلة وسريعة لدعم المعلومات السريرية للمساعدة فى الوصول إلى التشخيص السليم. فى هذه الدراسة تم تقييم اختبار (إليزا) باستخدام المستضد أ ٦٠ لتقدير نسبة الأجسام المناعية (المستضدات) من نوع جى (آى جى جى) فى مصل الدم مقارنة بالطرق التشخيصية الأخرى فى مرض الدرن الرئوى النشط فى سبعون مريض بالدرن الرئوى النشط (المجموعة المرضية) وسبعون شخصاً من مرتادى المستشفيات لا يعانون من أى شكوى أو أعراض لأمراض صدرية (مجموعة ضابطة). ووجدت نتائج إيجابية فى ستين مريض (٨٥,٧%) بفحص البصاق بصبغة (زيل نلسن) وثمانية وأربعون مريض (٦٨,٦%) عن طريق زرع البصاق على وسط (لوفينشتين جنسن) وخمسون مريضاً (٧١,٤%) باستخدام اختبار التيوبركلين وثلاثة وخمسون مريض (٧٥,٧%) باستخدام الأشعة السينية على الصدر وواحد وستون مريض (٨٧%) باستخدام طريقة إليزا وقد وجد أن المستضد أ ٦٠ ذو دلالة إحصائية عالية ($P < 0.001$) وأن نسبة الحساسية والخصوصية كانت ٩٥,٧%، ٩٠% بالترتيب.

ولهذا يمكن استخدام هذه الطريقة كأحد الوسائل السهلة والسريعة والرخيصة وعالية الحساسية والتخصص لتشخيص مرض الدرن الرئوى النشط الذى يصعب تشخيصه النهائى بالطرق العادية وذلك للمساعدة فى بدء العلاج.