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INFLUENCE OF SERUM 55 KDA- TUBERCULOSIS ANTIGEN ON LEVELS OF TUMOR NECROSIS FACTOR IN PATIENTS WITH PULMONARY TUBERCULOSIS

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ABSTRACT

One-third of the world's population is infected with tuberculosis (TB). Our aim was to determine performance characteristics of dot-ELISA in detection of TB infection and to determine the influence of TB infection on serum tumor necrosis factor-alpha (TNF-α) level. Sputum and serum samples of patients with pulmonary TB (n=112), non-tuberculous diseases (n=38) and healthy volunteers (n=12) were examined for TB infection. The performance characteristics of Ziehl-Neelsen stain for the detection of *Mycobacterium tuberculosis* (MTB) in sputum of pulmonary TB patients were evaluated. The sensitivity, specificity, efficiency of Ziehl-Neelsen stain was 48 %, 100 % and 64 %; respectively. The Western blot and dot-ELISA were used to identify the target TB antigen using the TB-55 mAb. The target antigen was identified at 55-kDa in serum samples from individuals with pulmonary TB using western blot techniques. The dot-ELISA detected the 55-kDa TB antigen in serum samples with 85 % sensitivity, 94 % specificity and 88 % efficiency. The levels of TNF-α were measured in 40 selected serum samples (20 positive TB antigen patients and 20 negative TB antigen individuals) using ELISA. The serum levels of TNF-α were (83.4 ± 12.3 pg/ml) in patients positive for 55 kDa-TB circulating antigen, with a statistical significant difference ($P < 0.0001$) from the levels in negative individuals for 55 kDa-TB (19±1.73 pg/ml).

INTRODUCTION

One third of the world's population is exposed to MTB infection **[David et al., (2004) and Gagneux et al., (2006)]**. Conventional TB diagnosis continues to rely on smear microscopy, culture and chest radiography. These tests have known limitations **[Pai et al., (2006)]**. Conventional tests for detection of drug resistance are slow, tedious and difficult to perform in field conditions. New tools include newer versions of nucleic acid amplification tests, immune-based assays, skin patch test and rapid culture systems were used **[Ravn et al., (2005)]**. Although the ideal test for TB is still not in sight, substantial progress has been made in the past decade. With the resurgence of interest in the development of new tools for TB control, it is likely that the next decade will see greater progress and tangible benefits **[Dinnes et al., (2007)]**. **Attallah et al., (2003,2005)** developed a simple and rapid dot-ELISA based on the detection of a 55-kDa TB antigen for field diagnosis of pulmonary and extra-pulmonary tuberculosis using monoclonal antibody (TB 55 mAb). The dot-ELISA detected the TB-55 kDa antigen in 90% sera of individuals with extra-pulmonary TB and in 87% sera of individuals with pulmonary TB with a specificity of 97% among control individuals. Infection with MTB is accompanied by an intense local inflammatory response which may be critical to the pathogenesis of TB **[Bothamley (2008)]**. Activation of components of the innate immune response, such as recruitment of polymorphonuclear and mononuclear phagocytes and induction of pro-inflammatory cytokines, such as TNF-α, by MTB occurs early after MTB infection, however, may persist as the organism establishes itself within granulomas **[Lin et al., (2008)]**. MTB and its protein and non-protein components are potent in induction of cytokines and chemokines from polymorphonuclear and monocytes **[Toossi (2000)]**. In the present study, serum 55-kDa TB antigen was identified using western blot and its diagnostic accuracy using dot- ELISA for discriminating patients those with TB infection from controls, including non-tuberculous patients and healthy volunteers were assessed.

2. MATERIALS AND METHODS

2.1. Sputum and serum samples

Sputum and serum samples of 162 individuals (126 males, 36 females; aged 26-56 year) were obtained from Chest Hospital, Damietta,

Egypt. Sputum smear were examined using Ziehl Neelsen Stain for detection acid fast bacilli (AFB). Blood samples were allowed to clot for separation of sera. Tubes were centrifuged at 4000 rpm for 10 minutes serum were separated and stored at – 20°C. Patients with pulmonary TB (n = 112) were diagnosed by sputum smear for acid-fast bacilli, radiographic evidence or clinical symptoms. In addition, sputum and serum samples of patients admitted to the hospital for a defined acute or chronic non-tuberculous diseases (n=38) including; chronic obstructive pulmonary disease (n =18), asthma (n =5), ischemic heart disease (n =6), pneumonia (n =3), bronchitis (n =2), lung cancer (n =1) and lung infection (n =3) as well as sputum and serum samples of healthy volunteers (n = 12) with no signs of clinical impairment and normal chest radiographs were included as controls.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western Blot

Serum samples at 30 µg lane were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunostained using the TB-55 mAb as previously described by **Attallah et al (2003)**.

Dot-enzyme linked immunosorbent assay (dot-ELISA)

The dot-ELISA as a simple and rapid assay was used for screening of TB antigen in serum using specific IgG TB-55 mAb according to **Attallah et al., (2003)**. In brief, all the assay steps run out on the surface of nitrocellulose membrane filter fixed in a plastic cartilage. Tested samples (500 µg/dot) were added to the membrane surface. After washing, the TB-55 mAb antibody diluted in 0.01M PBS was added. Alkaline phosphatase conjugated goat antibody diluted in 0.05 M Tris buffer was added to the membrane surface. The NBT/BCIP substrate solution was used to develop the color within 2 min. The reaction was then stopped and the result was evaluated.

Detection of Tumor Necrosis Factor using ELISA Method:

The levels of TNF-α were measured in 40 selected serum samples (20 positive TB antigen patients and 20 negative TB antigen individuals) using ELISA. Human TNF-α, was quantitated by commercially available ELISA kit (Quantikine, R&D Systems, Inc., 614 McKinley Place N.E., Minneapolis, USA), according to the manufacturer's instructions. A monoclonal antibody specific for TNF-α has been pre-coated on a

microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After 2 hrs of serum sample incubation, plates were washed 3 times, and then 200 µl of an enzyme linked monoclonal antibody were added to each well. Following a wash to remove any unbound antibody, enzyme reagent substrate solution (100 µl) is added to the wells and color develops in proportion to the amount of cytokines. The reaction stopped and absorbance was read at 450 nm using ELISA reader (Σ960 Metretech, Germany).

Statistical analyses

All statistical analyses were done by a statistical software package "SPSS 12.0 for Microsoft Windows, SPSS Inc.) and considered statistically significant at a two-sided $P < 0.05$. Numerical data were expressed as mean ± SD. The Mann-Whitney U-test was used for comparisons between independent groups. The diagnostic sensitivity, specificity, efficiency, and positive predictive (PPV) and negative predictive (NPV) values were calculated **[Gahen, Gambino (1977)].**

RESULTS

Evaluation of efficiency of Ziehl-Neelsen stain.

Sputum samples from 112 clinically diagnosed as TB patients were screened for the detection of MTB using Ziehl-Neelsen stain. 54 of the 112 cases with pulmonary TB, were positive for MTB (48 %) and 58 cases (52 %) were smear negative. So the sensitivity of Ziehl-Neelsen stain was 48 % using clinical examination as gold standard method. All sputum samples from 38 clinically diagnosed as respiratory diseases other than TB (n= 38) and healthy controls (n= 12) were smear negative for MTB. So the specificity and efficiency of Ziehl-Neelsen stain were 100 % and 64 %; respectively.

Identification of the TB-55 mAb target antigen in serum samples:

Serum samples from TB patients and non infected individuals were analyzed by 12% one-dimensional SDS-PAGE under reducing conditions and stained with Coomassie blue. TB – 55 mAb was used as a probe in western blot assay. An intense sharp band was present in serum samples of pulmonary TB patients at 55-kDa but no reaction with non infected samples was observed. (figure 1).

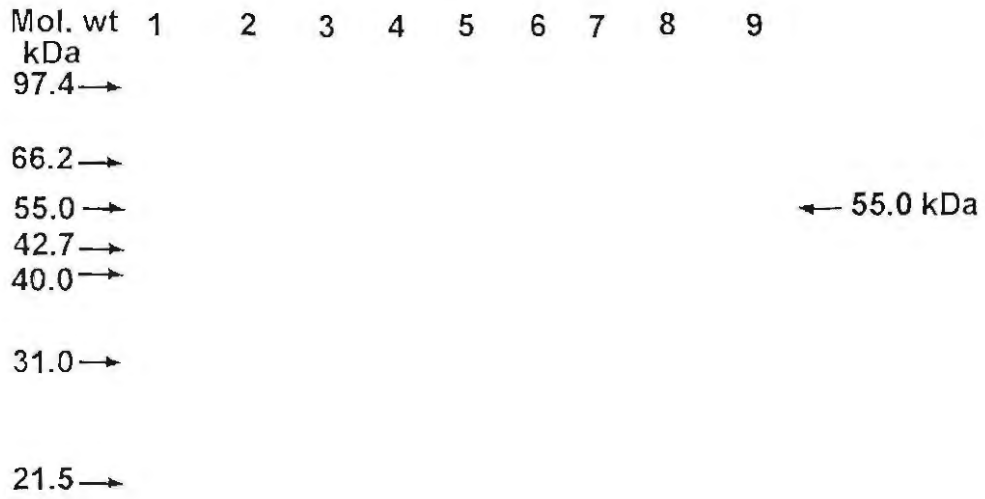

Fig. (1): Western blot analysis of sera from controls individuals and pulmonary TB patients. Lanes (1, 3, 5, 7 and 9): serum samples from TB patients demonstrating TB 55 kDa antigen band. Lanes (2, 4, 6, and 8): serum sample from controls individuals with no antigen band. Molecular weight markers (Mr.) include: Phosphorylase B (97.4 kDa), Bovine serum albumin (66.2 kDa), Glutamate dehydrogenase (55.0 kDa), ovalbumin (42.7 Da), aldolase (40.0 kDa), Carbonic anhydrase (31.0 kDa) and Soybean trypsin inhibitor (21.5 kDa).

Rapid detection of the 55-kDa circulating antigen in sera using dot-ELISA:

TB-55 mAb antibody was used as a probe in dot-ELISA to detect a target TB antigen in serum according to **Attallah et al., (2003)**. Serum samples of patients with pulmonary TB (n= 112) were tested for circulating TB antigen 55- kDa using dot-ELISA. Of 112 pulmonary TB cases, 95 (85 %) were positive for circulating tuberculosis antigen 55-kDa. So the sensitivity of dot-ELISA for the detection of TB antigen was 85 % using clinical examination as gold standard method. 36 samples out of 38 patients with respiratory diseases other than TB and 11 samples out of 12 healthy individuals were negative for TB antigen with 94 % specificity. The TB antigen 55 kDa was detected in 3 out of 50 serum samples of controls (6 %; false positive). So the specificity and efficiency of dot-ELISA for the detection of TB antigen were 94 % and 88 %; respectively. (table 1).

Table (1): Validation of dot-ELISA test for diagnosis of pulmonary TB.

Group	No.	Dot-ELISA*		% positive
		Positive	Negative	
Pulmonary TB	112	95	17	85
Controls	50	3	47	6
Non – tuberculous diseases	38	2	36	5
Healthy individuals	12	1	11	8

* Rapid detection of the 55-kDa TB antigen in serum.

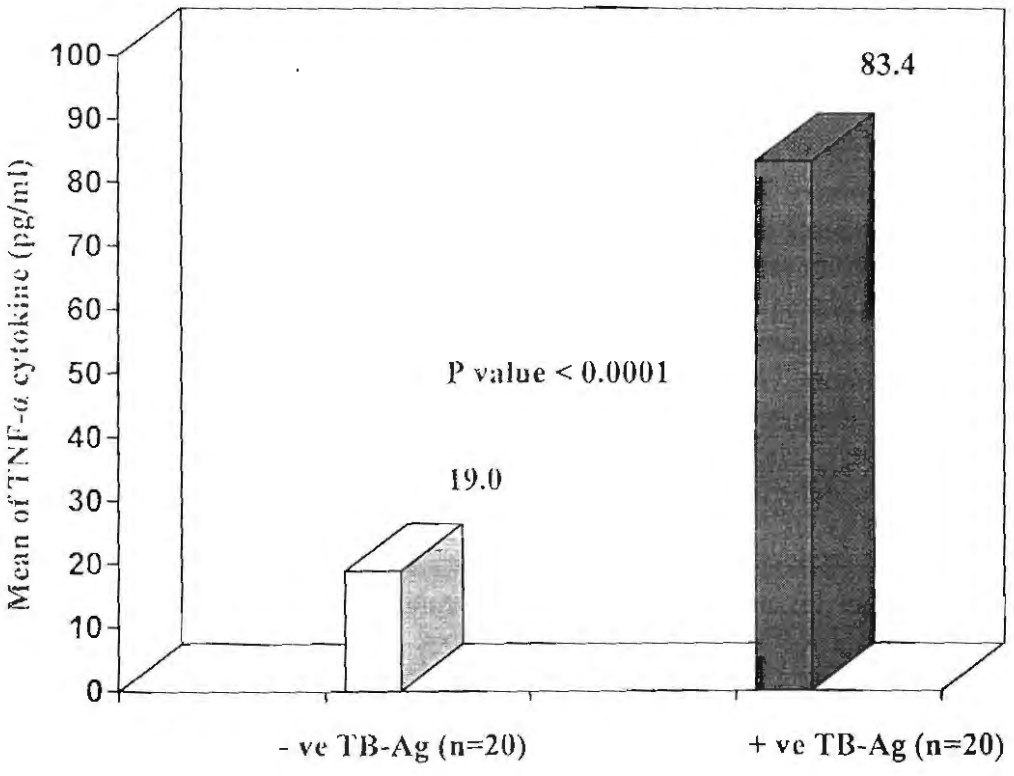

Fig. (2): Mean levels of TNF-α in 40 selected serum samples (20 positive TB antigen patients and 20 negative TB antigen individuals). A significance difference ($p < 0.0001$) was shown between positive and negative TB antigen individuals.

Quantitative determination of TNF-α in serum samples using ELISA.

The standard curve was constructed by plotting the means of absorbance's at 450 nm for each standard (0, 15.6, 31.2, 62.5, 125, 250, 500, 1000 pg/ml) on the X axis against the concentration on the Y axis. The concentration of unknown samples can be determined from the standard curve of TNF-α. The levels of TNF-α were measured in 40 selected serum samples (20 positive TB antigen patients and 20 negative TB antigen individuals) using ELISA. The mean serum level of TNF-α was found to be (83.4 ± 12.3 pg/ml) in patients positive for 55 kDa-TB circulating antigen, with a statistical significant difference ($P < 0.0001$) compared to the levels in negative individuals for 55 kDa-TB (19 ± 1.73 pg/ml); figure 2.

DISCUSSION

TB is one of the biggest global health problems. One-third of the world's population is latently infected with TB **[Hansted et al., (2007)]**. It is assumed that about 10% of individuals infected with MTB develop TB, and the remaining 90% suppress contain MTB through their immune systems, but have a latent TB infection. An early accurate diagnosis in-patients with active disease is essential to reduce morbidity and mortality **[Harada (2006)]**. Laboratory diagnosis of MTB currently depends on acid-fast staining **[Kennedy et al., (1994)]**. While this technique had been continuously refined and improved, they still have several limitations **[Benneson (1995)]**. In the present study, microscopic examination of AFB smears has sensitivity and efficiency values low enough to be useful only as a presumptive screening test. AFB on stained smears are not very sensitive because between $5x10^3$ and $1x10^4$ AFB per ml of sputum must be present to be detected under the microscope **[Hleifets & Good (1994)]**. In the present study, a 55-kDa TB antigen was identified in sera of individuals with pulmonary TB using specific monoclonal antibody designated TB-55 mAb. Several MTB antigens were detected in serum e.g., 30-kDa antigen and 31- kDa antigen **[Ng et al., (1995) and Nair et al., (2001)]**. Although the ELISA system is very practical and sensitive, the testing equipment required is not always available in areas where TB is endemic. An alternative to ELISA could be the dot method, which uses only a paper matrix to which the antigen is spotted, and the development of the antigen– antibody reaction is done by an enzyme or the use of a colloidal gold conjugate **[Stott (1989)]**.

The technical aspects of the dot-ELISA can be performed very simply and the staff of a single laboratory can easily handle large number of serum specimens. In the present study, the efficiency of the dot-ELISA in patients with pulmonary TB was 88 %. Moreover, the diagnostic potential of TB antigen detection has been evaluated in different body fluids [**Ashok et al., (2002) and Lenka et al., (2000)**] with sensitivity rates of 41–93% and specificity rate of 86–100%. There is an urgent need to identify reliable correlates of immunity against TB for the evaluation of TB vaccines currently under development [**Doherty (2005)**]. In TB, T cells are responsible for protection but also the pathology caused by inflammatory responses. Most T cells are divided into Th1 and Th2 subsets depending on the type of cytokines produced [**Mustafa & Oftung (1995)**]. TNF-α is required for appropriate chemokine expression by MTB infected macrophages, both in vitro and in vivo [**Roach et al., (2002) and Algood et al., (2005)**]. In the present study, the levels of TNF-α were measured in 40 selected serum samples (20 positive TB antigen patients and 20 negative TB antigen individuals) using ELISA. The serum levels of TNF-α were higher in patients positive for 55 kDa-TB antigen, with an extremely statistical significant difference ($P< 0.0001$) from the levels in negative individuals for 55 kDa TB antigen. Previous studies have shown higher serum levels of TNF-α in pulmonary TB patients compared to the control subjects [**Kart et al., (2003)**]. Also, serum TNF-α level was significantly higher in both active and inactive TB patients than healthy controls, and significantly higher in active than inactive TB patients [**Tang et al., (2003) and Olobo et al., (2001)**]. Our results agree with above reports in that serum TNF-α level are elevated in pulmonary TB patients compared to control individuals. The present data demonstrate that the 55-kDa antigen has highly diagnostic potential and its has ability to discriminate TB patients from controls, including non-tuberculous patients. However, TNF-α production in response to this antigen may serve as a marker of disease activity of TB in an area of TB endemic.

CONCLUSION

Compared with the Ziehl-Neelsen stain, the detection of 55 kDa-TB antigen appears to be as sensitive and specific for diagnosis of TB and its presence causes elevated serum TNF-α level.

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تأثير وجود أنتيجين الدرن ٥٥ كيلو دالتون على مستوى عامل النخر السرطانى فى المرضى المصابين بالدرن الرئوى

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مرض الدرن من الامراض البكتيرية المزمنة التي تصيب ثلث سكان العالم وفى هذه الدراسة تم فحص ١١٢ عينة بصاق وسيرم للأشخاص مصابين بالدرن الرئوى بالإضافة إلى استخدام ٣٨ عينة بصاق وسيرم للأشخاص مصابين بأمراض صدرية غير الدرن ، ١٢ عينه بصاق وسيرم لأشخاص أصحاء كمجموعة ضابطة. تم تحديد كفاءة الفحص المجهرى لعينات البصاق لمرضى الدرن الرئوي والمجموعة الضابطة في الكشف عن بكتيريا الدرن فوجد أنه ذو حساسية (٤٨%) وخصوصية (١٠٠ %) وكفاءة (٦٤%). وباستخدام تقنية النقل المناعى وجسم مضاد وحيد النسيلة (TB-55 mAb) تم التعرف على أنتيجين الدرن عند ٥٥ كيلو دالتون فى عينات سيرم لأشخاص مصابين بالدرن الرئوى وكذلك استخدمت طريقة الاليزا النقطية كطريقة سريعة للكشف عن انتيجين الدرن فكانت حساسية هذه الطريقة (٨٥%) وخصوصيتها (٩٤ %) وكفاءتها (٨٨%). تم تحديد تركيزعامل النخر السرطانى الفا فى ٤٠ عينة سيرم مختاره باستخدام طريقة اليزا فاوضحت النتائج أن٢٠ عينة من عينات السيرم التى يوجد بها انتيجين الدرن تحتوى على تركيزأعلى من عامل النخر السرطاني الفا (٨٣,٤ ±١٢,٣ بيكوجرام/ميللى) بينما ٢٠ عينة من عينات السيرم التى لا يوجد بها انتيجين الدرن تحتوى على تركيز(١٩,٠ ± ١,٧٣ بيكوجرام/ميللى) بالاضافة الى وجود فرق احصائى ($P < 0.0001$) بينهم.

الخلاصة: أظهرت طريقة الاليزا النقطية للكشف عن انتيجين الدرن فى السيرم أنها طريقة ذات حساسية وخصوصية عالية بالمقارنة بالفحص المجهرى لعينات البصاق كما وجود أن انتيجين الدرن يسبب ارتفاع فى مستوى عامل النخر السرطاني الفا.