DIAGNOSIS OF TUBERCULOSIS BY USING ELISA TO DETECT 38 KDA MYCOBACTERIAL ANTIGEN IN THE PATIENTS

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SUMMARY: ***Tuberculosis (TB)****, caused mainly by Mycobacterium tuberculosis, is a major health problem. It has been found in Neolithic remains, and is still the largest cause of death from a single infectious disease. There are about 20 million new active cases of TB each year and there are between 3-8 million deaths per year from this infection. This represents greater than 25% of all avoidable deaths worldwide.*

The importance of mycobacterial secreted proteins in infection has been suggested by many investigators. Three important antigens, the 38 kDa, 30/31 kDa and SOD molecules, have been found to be secreted by M. tuberculosis. The importance of the 38 kDa in TB has been shown by many investigators. We have developed different tests for the detection of these antigens in the TB patients sera. The performance of both a Direct and Sandwich ELISA using FF11 (anti-38 kDa m-Ab) for the detection of the 38 kDa M. tuberculosis antigen has been evaluated in our study.

This test been found to have a specificity of 84-88% with positive predictive values of 80-84% which, although not ideal, is quite helpful in developing some new specific diagnostic test. Our test has the advantage that antigens may be detected in the early stages of infection and we have shown that it can be used for monitoring effective chemotherapy by detecting the decline in the 38 kDa antigen in previously positive patients. However, a detailed study is needed on larger numbers of confirmed TB cases and normals.

Key Words: M. tuberculosis, mycobacterial antigen, ELISA test.

INTRODUCTION

Tuberculosis, the largest cause of death from a single infectious disease, is still a major health problem worldwide (13,10). About one-third of the world's population is infected with *M. tuberculosis.* However, of those who become infected, only 10% develop clinically apparent disease. In infected individuals the organisms persist and retain the potential to become reactivated and cause progressive disease (7).

The introduction of mass BCG vaccination programmes had only a small effect, but the introduction of case finding and the isolation of patients, particularly of infectious cases, together with the application of new

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drugs accelerated a decline in cases by 7-8% annually (16). In developing countries, by contrast, the rate of infection has remained constant or is only declining very slowly (2). Such countries are still confronted with a major tuberculosis problem due to socio-economic under-development. In such countries, control programmes are very difficult to sustain. Thus even today, despite effective drugs, tuberculosis remains a global health problem of major importance. Skin test surveys in poorer countries suggest that in some regions more than 50% of the adult population have been exposed to *M. tuberculosis* at some time. In the developed countries, by contrast, the incidence of this infection has been in steadily decline during the last two decades. However, the AIDS pandemic has interrupted this trend in Europe and the United States and it has allowed the disease to reach epidemic proportions in Africa. AIDS patients have been calculated to be 100 times more likely to contract TB compared with normal individuals.

Primary pulmonary tuberculosis is usually symptomless in adults, and is discovered only on routine screening of contacts. It is a mild illness which may resolve spontaneously without sequelae (9,15). In infants and children under 5, however, the infection may have serious immediate consequences. Children under four year of age have a high risk of mortality and morbidity, since tubercular meningitis and miliary tuberculosis are the most common in this age group. Positive bacterial isolation in children is difficult because the disease is frequently asymptomatic and paucibacillary (19).

The risk of increasing spread of tuberculosis and development of drug resistance make early diagnosis a matter of utmost concern, and improved rapid methods for laboratory confirmation are urgently required.

Very few reports are available for diagnosis of tuberculosis based on antigen detection. The detection of mycobacterial antigens in clinical specimens may offer good diagnostic value and can be used for monitoring the effect of therapy in previously positive patients.

The 38 kDa protein is one of the major secreted antigen of *M. tuberculosis*. This protein has been found to be homologous to *E. coli* PhoS gene product which is involved in phosphate metabolism (1). The importance of the 38 kDa in TB has been shown by many investigators (18). The performance of both a Direct and Sandwich ELISA using FF11 (anti-38 kDa m-Ab) for the detection of 38 kDa *M. tuberculosis* antigen has been evaluated in our study.

MATERIALS AND METHODS

Patients: 25 confirmed positive pulmonary TB patients samples were selected for the test. Patients were diagnosed on the basis of the presence of AAFB in sputum, and/or culture of *M. tuberculosis*.

Normals: 25 normal sera were provided by the Regional Blood Transfusion Centre for the study.

FF11: This is a monoclonal antibody which was raised in our laboratory by immunizing mice with MTSE. It reacts with the 38 kDa antigen of *M. tuberculosis*.

Sandwich ELISA: Immune complexes are precipitated with 2% (w/v) polyethyleneglycol 6000 (PEG) overnight and washed in PEG buffer at 4°C and then dissolved in veranol buffer. Immune complexes were applied onto 20 μg/ml anti-38 kDa *M. tuberculosis* mouse monoclonal antibody-coated plates. After addition of the patients and control immune complexes, 100 μl of 5 μg/ml of sheep anti-human C_3d (to detect C_3d- a degraded product of the C_3 subunit of complement) in 2% normal human serum + 1% BSA-PBS Tween-20, was applied to each well. The test was developed with Donkey anti-sheep IgG-HRP, unreactive with either mouse or human immunoglobulins.

Direct ELISA: In a second set of assays, plates were coated with 25 μl of the immune complexes to which was then applied the anti-38 kDa m-Ab for 1 hour, and then, after washing, sheep anti-mouse IgG HRP was applied.

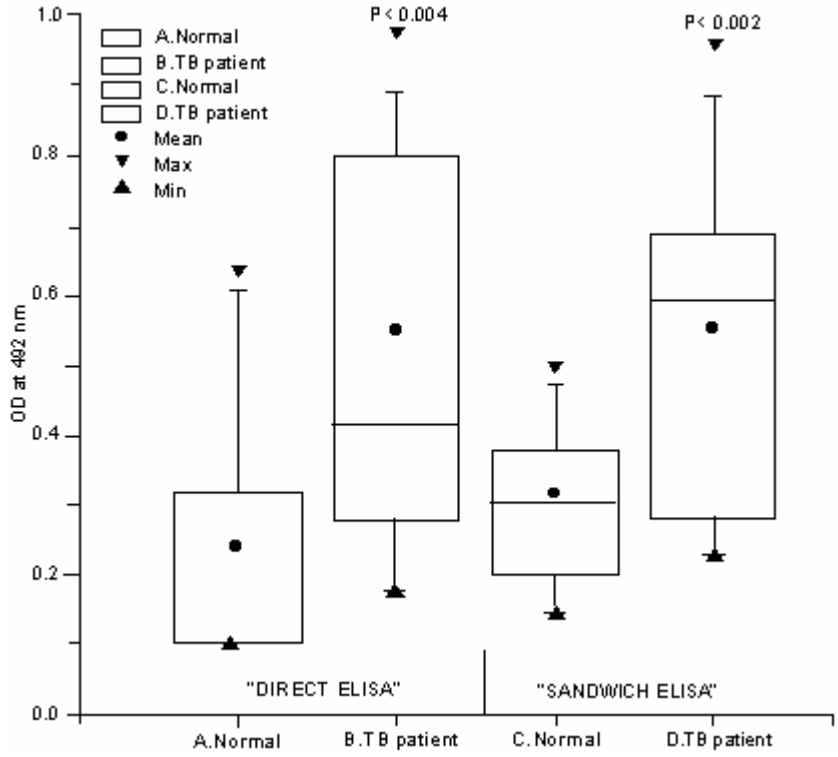
Results are recorded on the basis of OD levels. A value above the mean of the normal ± 2SD was considered as positive (Table 1).

Direct ELISA= Immune complexes + anti-38 kDa m-Ab (FF11) + sheep anti-mouse IgG-HRP.

Table 1: Categories of OD results.

Categories	Direct ELISA	Sandwich ELISA
Normal	0-0.25	0-0.35
Positive	>0.25	>0.35

Figure 1: Distribution of the 38 kDa antigen in normal and TB patients' serum immune complexes.



Sandwich ELISA= Anti-38 kDa m-Ab (FF11) + immune complexes + sheep anti-C3d + Donkey anti-sheep IgG-HRP.

In Figure 1, the single or upper box represents the distribution of OD values in which 50-75% of the subjects lie. The lower box is the 25-50% distribution. The p values represent differences between tuberculosis patients results of normal subjects in the two types of test.

RESULTS AND DISCUSSION

The presence of a high level of 38 kDa antigen was detected in most cases of tuberculosis (Figure 1) using both the Sandwich ELISA and the Direct ELISA. Although some overlap is seen with the results of normal subjects immune complexes, a highly significant difference is seen between normals and tuberculosis patients ($p<0.004$).

On the basis of the OD results, the distribution of the 38 kDa antigen levels (normal or positive) in the serum immune complexes of TB patients and controls were recorded and this is shown in Table 2.

The performance of both a Direct and Sandwich ELISA using FF11 (anti-38 kDa m-Ab) for the detection of the 38 kDa *M. tuberculosis* antigen has been evaluated in our study. These tests detect the 38 kDa antigen, which is more specific to tuberculosis, in the immune complexes of tuberculosis patients. This test been found to have a specificity of 84-88 %, which is higher than the antigen-capture ELISA using polyclonal anti-*M. tuberculosis* IgG antibodies. These tests have a positive predictive values of 80-84 % which, although not ideal is quite helpful in developing some new specific diagnostic tests (Table 3).

Table 2: Distribution of the 38 kDa antigen level in TB patients and normal controls.

Group	Normal OD Value		Positive	
	DE	SE	DE	SE
TB patients (25)	9	9	16	16
Normal (25)	22	23	3	4

Table 3: Data Analysis of the test for the 38 kDa antigen in TB patients and normal controls.

	TB patients		Normal	
	DE	SE	DE	SE
Total numbers	25	25	25	25
True positives	16	16	0	0
False positives	0	0	3	4
True negatives	0	0	22	21
False negatives *(including borderlines)*	9	9	0	0
	DE		SE	
Test sensitivity	64 %		64 %	
Test specificity	88 %		84 %	
Test positive predictive value	84 %		80 %	
Test negative predictive value	70 %		70 %	

DE= Direct ELISA SE= Sandwich ELISA

Harboe and Wiker (11) in a review illustrated that the 38 kDa secretory protein is one of the most important specific antigens of *M. tuberculosis*. This antigen induces B and T cell responses with high specificity for tuberculosis and is considered as a prime candidate for development of new diagnostic reagents for tuberculosis.

Other studies have also indicated that the 38 kDa molecule may be a valuable antigen for serodiagnosis of tuberculosis based on detection of antibody to this molecule. Antibodies to the 38 kDa antigen occur in a high percentage of TB patients and have a high specificity for tuberculosis when tested at the epitope level by antibody competition ELISA with the purified antigen. However in this test insufficient sensitivity represents an important problem e.g. 20-30% of tuberculosis patients are negative for antibodies, and on the other hand antibodies to the 38 kDa are detected in infections with mycobacteria other than *M. tuberculosis*. The antibody response to the 38 kDa antigen is immunodominant in smear positive pulmonary TB where a sensitivity of up to 85% can be achieved with a specificity of 97% (5,13). In addition, in those initially antibody-negative, 38 kDa directed antibodies are usually the first to appear in the first month after commencing treatment.

Ivanyi *et. al.* (12) found that 74% of all, or 55% of untreated active, TB patients had antibodies to the TB72 epitope of the 38 kDa antigen. Among 42 control patients without TB, only one was positive and three were marginally positive. The sensitivity was found to be almost equal when antibodies were measured to a single epitope or to the whole molecule. There have been very few reports on detection of mycobacterial antigens in serum from patients with mycobacterial diseases. Several studies are available based on antigen detection in CSF for diagnosis of tuberculous meningitis (14). Antigen-detection test has certain advantages over the antibody-based test particularly in the context of secretory proteins. As secretory proteins appear earlier in infection, detection of such proteins indicate active infection through the presence of actively-proliferating mycobacteria. It is also reported that the levels of antigens in the circulation declines with effective chemotherapy, while some anti-mycobacterial antibodies are absent in early active infection; they only start appearing after chemotherapy and then only gradually decline. It is also possible that high levels of antibody

could persist due to cross stimulation. Our 38 kDa antigen-detection ELISA test could provide useful information for monitoring the efficacy of chemotherapy, and could differentiate between active and treated infection.

We have evaluated a monoclonal antibody to the 38 kDa antigen of *M. tuberculosis* which may, on the one hand, provide a test which is more specific to tuberculosis, and on the other reveal the significance of finding antigens related to this disease in other conditions. In an analysis of 25 confirmed tuberculosis cases for the 38 kDa antigen in immune complexes, the test has a positive predictive value of 89% with a specificity of 92%.

The importance of the 38 kDa antigen in the diagnosis of tuberculosis has recently been studied by Bothamley and Rudd (4) using the TB72 anti-mycobacterial 38 kDa m-Ab. They used an ELISA competition assay for detection of the TB72 epitope present on the 38 kDa of *M. tuberculosis*. They claim that the sensitivity of the test (66%) in patients with smear negative; culture positive pulmonary TB was similar to that in patients with smear positive disease. A higher sensitivity of 70% in smear negative and culture positive had been obtained by Wilkins and Ivanyi (18). Our test for the detection of the 38 kDa antigen was found to have a similar sensitivity (64%). However, our test has slightly lower specificity (88%) compared to Bothamley and Rudd 97% (14). Although our m-Ab is specific for the 38 kDa antigen of mycobacteria, it may not be as species-specific as the TB72 m-Ab which recognizes a specific epitope present only on the *M. tuberculosis* 38 kDa antigen. Therefore to improve our antigen-detection test we need to use a similar sort of species-specific antibody.

Unfortunately we could not acquire a sufficiently detailed clinical history of the patients. Probably some of the patients were on chemotherapy, which reduces the antigen concentration in the circulation. In the antibody detection test, antibody persist and also stars appearing after treatment in previously negative patients. Our test has the advantage that antigens may be detected in the early stages of infection.

We have shown that it can be used for monitoring effective chemotherapy by detecting the decline in the 38 kDa antigen in previously positive patients (data not shown). However, for this, a detailed study is needed on a larger number of confirmed TB cases.

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