Evaluation of a Serological Test for the Diagnosis of Tuberculosis Using the 38KDa Antigen

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Objective: **To evaluate an enzyme immunoassay utilising the 38 KDa Mycobacterial antigen as a possible test for the laboratory diagnosis of tuberculosis.**
Method: The sera of 37 patients with confirmed tuberculosis, 37 tuberculin-negative, apparently healthy individuals and 27 tuberculin-positive, apparently healthy individuals were tested. Moreover, seropositive, apparently healthy individuals were tested by a peripheral-blood-based PCR assay using a broad range bacterial primer and one that is specific for the *M. tuberculosis* complex.
Results: **Four of 37 patients with confirmed tuberculosis, 10 of 37 tuberculin-negative, apparently healthy individuals and none (0 of 27) of the tuberculin-positive, apparently healthy individuals were seropositive. All seropositive, apparently healthy individuals were negative by PCR.**
Conclusion: **The sensitivity of the enzyme immunoassay utilising the 38 KDa antigen in this study was 10.8% and the specificity 84.4%. Thus, it appears that this test may not be used for the diagnosis of tuberculosis in the Lebanese.**

Key words: *Tuberculosis, enzyme immunoassay, 38 KDa antigen, PCR.*

Approximately 1.7 billion individuals in the world harbor *Mycobacterium tuberculosis* and 1 out of 30 of these develop the disease. About 95% of the cases have been reported from developing countries. In industrialised countries, tuberculosis is on the rise mainly because of AIDS and the emergence of multi-drug resistant strains. Between 1985 aod 1991 tuberculosis cases increased by 33% in Switzerland, 30% in Denmark, 20% in Norway, 18% in Ireland and 18% in the U.S.A. (l).

The laboratory diagnosis of tuberculos is based on an acid fast smear, culture and a tuberculin skin test. Each of these tests have some drawbacks. Culture is specific but time-consuming. Moreover, suspected cases might be missed. An acid fast smear is less specific than culture and more cases are missed using this test. The tuberculin skin test can not be used to distinguish those who had been vaccinated from those with the disease. More recently, PCR as a diagnostic and confirmatory test for tuberculosis has been introduced. This test is specific and sensitive but its use in surveillance and epidemiological studies might not be practical.

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In a number of studies, the use of serological tests to detect anti-*Mycobacterium tuberculosis* antibodies for the diagnosis of tuberculosis were assessed. Different antigens were used in these tests such as the 38KDa glycoprotein (2,3), A60 antigen complex (4,5), heat shock proteins (6), diacyl trehalose (7), phenolic glycolipids (8), lipoarabinomannan (9), excretory-secretory antigens (10) and purified protein derivatives (9).

In Lebanon, epidemiological studies have not been done but unpublished scattered reports claim that tuberculosis is on the rise. A number of the above-mentioned reports indicated that the sensitivity and specificity of the serological test used was 80% or more. Based on this, a serological test might be used for an epidemiological study. However, before doing so, it was thought to evaluate one of the serological tests using patients and controls in Lebanon.

Material and Method

Subjects:

The targeted population consisted of thirty seven patients with a confirmed diagnosis of pulmonary tuberculosis (acid fast smear, culture, tuberculin skin test, X-ray). Twenty seven apparently normal individuals who were tuberculin-positive and 37 apparently normal individuals who were tuberculin-negative, were included in this study. None of the subjects were HIV-positive.

Enzyme immunoassay utilising the 38 KDa antigen:

The Pathozyme-TB complex enzyme immunoassay for the detection of IgG antibodies to *M. tuberculosis* in human serum was used (*Omega Diagnostics Limited*). This test utilises the 38 KDa antigen. The procedure described by the manufacturer was followed, and is stated hereafter.

One hundred ml of each, diluted serum (1:50) to be tested and controls (undiluted, high and low positive, in duplicates) were dispensed into the appropriate wells of a plate containing the 38 KDa antigen. The plate was gently shaken and incubated in a moist chamber at 37^{0}C for 1 hour. The wells were then washed with the buffer and 100 ml of conjugate was added to each. The plate was gently shaken and incubated in a moist chamber at 37^{0}C for 30 minutes. The wells were than washed with the buffer, followed by the addition of 100 ml of enzyme substrate to each, and incubation at 37°C in a moist chamber for 15 minutes. One hundred ml of stop solution was then added

to each well and the optical density was read at a wavelength of 450 nm. The cut off level was calculated by dividing the average optical density obtained with the low positive control by 1.5.

Peripheral-blood-based PCR assay:

White blood cells were separated from blood of individuals that were serologically positive for *M. tuberculosis* but were tuberculin-negative and apparently healthy. Separation was done by CPT cell preparation tubes (Becton Dickinson). Cells were washed twice with phosphate buffered saline (*PBS*), pH 7.2. Total DNA was extracted by using a lysis buffer containing Triton X-100 detergent (0.5 %) and proteinase K enzyme (2.5mg/ml). The positive control consisted of genomic DNA obtained from *M. tuberculosis* strain H37-RV (obtained from *CDC, Atlanta*). Additional controls consisted of total DNA extracted from blood of a seropositive patient with active tuberculosis (seropositive) and a seronegative, apparently healthy individual.

Two sets of oligonucleotides were used. The first set targets a 1037 base pair (bp) sequence on the 16srRNA gene and consists of primer 264 (specific for Mycobacteria) and primer 285 (a broad range bacterial primer). The second *set* targets the IS6110 insertion sequence specific for the *M. tuberculosis* complex amplifying a 123 bp fragment. PCR reactions and cycling conditions were set according to Kirshna et. al. (11) and Eisenach et. al. (12). respectively.

A minicycler (*MJ Research, Watertown, Mass.)* was used for PCR-amplification. Amplicons were electrophoresed on a 2% agarose (*FMC, Rochland, USA*) gel, stained with ethidium bromide (1 mg/ml), observed under UV light and photographed with type 667 Polaroid film.

Results

Enzyme immunoassay utilising the 38 KDa antigen:

The cut of point was 0.331. The average optical density of the low positive control was 0.496 (which *is* greater than 0. 3 indicating validation of the assay). All negative results had an optical density equal or less than 0.331. Positive results were those that had an optical density greater than 0.496 (optical density of low positive control).

Four of 37 patients with confirmed tuberculosis, 10 of 37 tuberculin-negative, apparently healthy individuals and none (0 of 27) of the tuberculin-positive, apparently healthy individuals were seropositive. The sensitivity of this immunoassay was 10.8% and the specificity 84.4% (Table I).

Peripheral-blood-based PCR assay:

The apparently healthy individuals were negative by PCR using two sets of primers. The DNA bands observed in Figures 1 and 2 are non-specific amplicons of the genomic human DNA. None showed the expected amplicon sizes as seen in *M. tuberculosis* control strain.

Table I. Determination of the sensitivity and specificity of the immunoassay.

	TBC	No TBC	Total
Test positive	4	10	14
	(True positive)	(False negative)	(Test positive)
Test negative	33	54	87
	(False positive)	(True negative)	(Test negative
Total	37	64	101
	(TBC)	(No TBC)	

Sensitivity = [4/(4+33)] x 100 = 10.8%

Specificity = [54/ (54+10)] x 100 = 84.4%

Healthy subjects were all classified together regardless of their tuberculin reactivity.

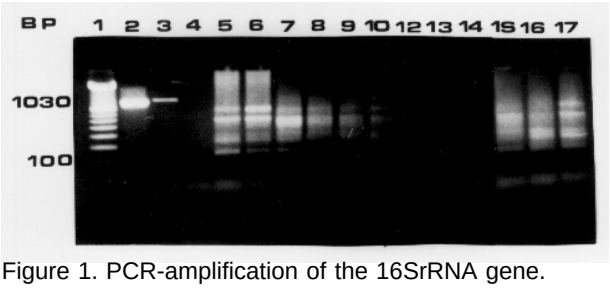

Figure 1. PCR-amplification of the 16SrRNA gene. Lane 1: lOObp ladder, lane *2&3 :M. tuberculosis* positive control, lane 4: negative control, lane 5: seronegative healthy individual, lanes 6-3 & 15-16 seropositive individuals, lane 17: seropositive TB patient.

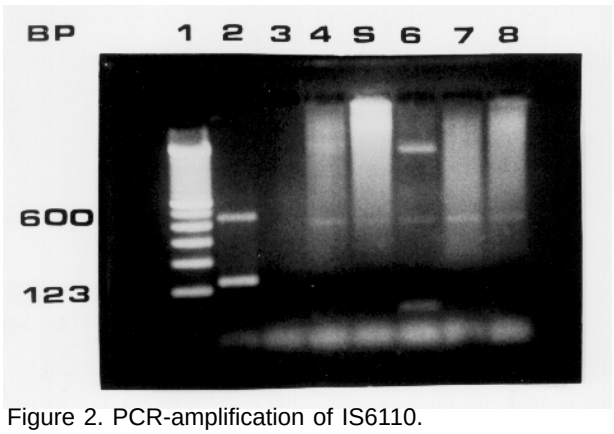

Figure 2. PCR-amplification of IS6110. Lane 1: 100 bp ladder, lane *2: M. tuberculosis* positive control, lane 4: seronegative healthy individual, lanes 5-7: seropositive healthy individuals, lane 8: seropositive TB patient.

Discussion

It has been reported that the 38 KDa antigen was a potential reagent for use in the serological diagnosis of tuberculosis. A sensitivity of 80% and specificity of 100 % have been reported using this antigen (2,3). However, in this present study, a sensitivity of 10.8 % and specificity of 84.4 % were obtained. This discrepancy in sensitivity may be due to the difference in the test procedure used.

Where as Bothamley et al. (2) used a solid phase antibody competition assay, we used the direct enzyme immunoassay.

The detection of seropositive individuals in the apparently healthy group led us to believe that these individuals were exposed but were not detected using conventional tests for tuberculosis. In an attempt to test this hypothesis peripheral-blood-based PCR assay was done. This test was reported to have a sensitivity of 95% and specificity of 89% (13). All the individuals tested were PCR-negative suggesting that they were not exposed.

The nature of the environmental Mycobacteria may infleunce the host's anti-*M. tuberculosis* response (14, 15). The environmental Mycobacteria in Lebanon may differ from that in other countries, and these differences may account for the sensitivity and specificity obtained in this study. Moreover, human genetic variation is an important determinant of the outcome of infection with *M. tuberculosis* (16). Such variation may also effect the humoral immune response. However, these suggestions are speculative and require further investigation.

In conclusion, the enzyme immunoassay used in this study probably can not be used for diagnostic purposes or for epidemiological studies in Lebanon.

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