MYCOBACTERIUM TUBERCULOSIS 38 kDa ANTIGEN AND ITS ENCODING GENE—EXPERIENCE IN DIAGNOSTIC APPLICATIONS

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INTRODUCTION

Tuberculosis continues to be a major public health problem in developed as well as developing countries. The incidence of the disease has been increasing since 1985 worldwide. The reasons for the increase include the emergence of multiple drug resistant strains and complications caused by the pandemic Acquired Immuno Deficiency Syndrome (AIDS). It is estimated that 1/4th of the World's population is infected, 12-16 million have the disease, 6-8 million develop tuberculosis every year and 2-3 million die from it. Among infectious diseases tuberculosis is a leading cause of death. The World Health Organisation took the extreme step of declaring tuberculosis as a Global Emergency in 1993 (1).

Tubercle bacilli was identified by Robert Koch more than a century ago but the tests used for the diagnosis of tuberculosis are not adequate. Diagnosis is based on detection of AFB by microscopy and culture. Microscopy is quite insensitive, requires more than 50,000-1,00,000 organisms for a positive result. More than 70% of cases with pulmonary tuberculosis are negative by microscopy. Culture, although sensitive, takes a long time of 6-8 weeks for a positive result. Patients with extrapulmonary tuberculosis such as tuberculous meningitis do not give positive cultures.

Thus a need for sensitive and specific tests for diagnosis of tuberculosis exists for a long time. Many attempts have been made using various antigens of mycobacteria in serodiagnosis. The 38 kDa antigen of *M.tuberculosis* has been shown to be highly immunodominant in B and T cell responses and is known to be sero specific to *M. tuberculosis* complex group of organisms (2,3). We have extensively used the native and recombinant 38

kDa antigen in immunodiagnosis as well as the gene encoding this protein in PCR. We describe here our experience in diagnostic applications.

Rapid Dot-ELISA for the qualitative detection of the 38 kDa antigen of *M.tuberculosis* in sputum samples in pulmonary tuberculosis (4,5).

A Dot-ELISA for the detection of the specific 38 kDa antigen of *M.tuberculosis* was developed. This involves the spotting of sputum samples (10ul) after initial processing on a nitrocellulose paper, drying, blocking and reaction with the specific monoclonal antibody, HGT3a which reacts to the 38 kDa antigen. The paper is then reacted with an anti mouse Ig-HRP conjugate and color developed using appropriate substrates. Sputum samples that contain the antigen give a clear green spot against white background while the samples that do not contain antigen are colorless. Three clinical trials have been conducted including samples from a TB detection camp and the test gives high sensitivity and specificity (Table 1).

Also a large number of samples can be processed in a day without any fatigue and the results scored easily by visual examination. The Dot-ELISA developed to detect the 38 kDa antigen in pulmonary tuberculosis has many advantages.

—Test is rapid and simple.

—Easy visualisation of positive results.

—No sophisticated instruments required.

—Can be performed at small health care centres.

—Spotted samples are stable at room temperatures for upto six weeks.

Thus this can be used for diagnosis of pulmonary tuberculosis and also in large scale studies and epidemiological surveys.

Detection of anti 38 kDa antibody in pulmonary tuberculosis and tuberculous meningitis (6).

While the Dot-ELISA can be used as a qualitative test in screening large number of patients

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it is necessary to evaluate a quantitative test for the detection of anti 38 kDa antibody levels in pulmonary and extra pulmonary tuberculosis. Tuberculous meningitis is a disease which affects children the most and is known to be fatal if not treated in time and also causes CNS damage. Early diagnosis and initiation of treatment can arrest the complications and help patients in recovery. Diagnosis of tuberculous meningitis is based on empirical tests as AFB detection and culture are almost always negative. As the 38 kDa antigen is serospecific to the *M.tuberculosis* complex it was felt that anti body responses to this antigen in CSF might be a good indicator of the disease process. A simple ELISA to determine the amount of antibody in CSF was developed. It was shown using this assay that 60-80% of the samples could be classified as TBM with certainty with 95% specificity. Patients studied included those where diagnosis was confirmed only on postmortem examination, culture for 6-8 weeks or on empirical tests and follow up. Antibody levels tended to decrease on treatment indicating that the test could be used in monitoring the course of the disease. The following Table 2 gives the sensitivity and specificity in different diseases.

The sensitivity of this PCR was 10fg of template DNA which is equivalent to 2-3 mycobacteria. Specificity of the test is very good. Template DNA from 13 other mycobacteria, 11 bacterial species, human and rat DNA showed no amplification indicating the high specificity. Sputum samples of patients with tuberculosis gave positive PCR (7,8).

Production of 38 kDa antigen using recombinant DNA technology

The *E.coli* strains used were JM83 and BL21 and plasmid pET3a was used as cloning vector (9)

The genes encoding the 38 kDa antigen was prepared by PCR using *M.tuberculosis* H37Rv DNA as template, standard PCR conditions with annealing temperature of 42°C and the following primers.

Upstream Primer :

5'TATGGGGCCATATGAAAATTCGTTTGCATA3'

Downstream primer:

5'TATAGGGCGGATCCTAGCTGGAAATCGTCG3'

The purified PCR product was restricted with NdeI and BamHI and ligated with the expression

Table 1 : Sensitivity and specificity of Dot-ELISA pulmonary tuberculosis

Disease	% Positive
AFB Positive	
Culture Positive	100
AFB Negative	
Culture Positive	80
AFB Negative	
Culture Negative	36
Controls	5

Polymerase Chain Reaction in Diagnosis

With the advent of the recombinant DNA technology many techniques such as dot blot DNA hybridisation with labelled probes and PCR became available. PCR has the added advantage of amplifying DNA sequences of interest million fold and thus aid in easy detection leading to early diagnosis. As the 38 kDa antigen is specific it was decided to use a specific sequence of the 38 kDa gene as template for amplification. Primers KD1 and KD2 were synthesized. These primers flanked a 340 bp fragment of the 38 kDa gene.

vector pET3a, which was restricted earlier with these two enzymes and the resulting construct was then introduced into *E.coli* expression host BL21(DE3) plys S. For production of the recombinant polypeptide, BL21 (DE3) strain of *E.coli* containing recombinant plasmid was grown in LB medium containing 100 µg/ml ampicillin, at 37°C to an A590 of 0.3, induced by the addition of IPTG to 100 µM and growth continued for another 2h. The induced culture was chilled quickly on ice and cells were harvested by centrifugation. The induced recombinant cells were resuspended in lysis buffer containing 0.2% lysozyme, 2 mM PMSF,

Table 2 : Sensitivity and specificity of anti 38kDa antibody detection in pulmonary tuberculosis and tuberculous meningitis

	Disease	% Positive
Pulm. TB	AFB Positive	
	Culture Positive	75
	AFB Negative	
	Culture Positive	92.3
	AFB Negative	
	Culture Negative	66.6
TBM	Controls	10
	Post Mortem Proved	60
	Culture Positive	80
	Clin Proven	62.5
	Tuberculoma	0
	Controls	5

50 mM Tris-HCl, pH 8.0, 1mM DTT and 2mM EDTA. They were incubated on ice for 30-45 mins and NaCl and Triton X-100 added to 10 mM and 0.2%, respectively. The suspension was homogenised and sonicated briefly to decrease the viscosity. The lysate was centrifuged briefly at low speed (5000 x g for 10 mins). The protein profiles of the pellet and the supernatant were analysed by 12.5% SDS-PAGE. Immunoblot analyses was done with the MoAb antibody HGT3a and polyclonal anti BCG rabbit immunoglobulins. The recombinant proteins were purified by SDS-PAGE followed by elution from the gel. The eluted protein was then filtered through a 0.45 µm filter and dialysed extensively against normal saline. The protein concentration was determined aliquoted and lyophilised.

Expression and purification

Since it is known that proteins synthesised to a high level in *E.coli* accumulate in insoluble forms as inclusion bodies low speed centrifugation was used to sediment the inclusion bodies and avoid other soluble proteins in the lysate. A protein band of 38kDa was observed only in the induced incombant lysate pellet fraction along with a large protein aggregate of slightly higher molecular weight which is absent in its superna-

tant and as well as the pellet fractions of other non-recombinant cell lysates on staining with Coomassie blue stain.

Immunogenicity and Immunoreactivity of r38Ag

To ascertain that the protein overexpressed by this recombinant clone is indeed *M.tuberculosis* 38 kDa antigen, the proteins were transblotted on to nitrocellulose membranes, and probed with the specific MoAb HGT3a. The antibody reacted specifically with overexpressed recombinant antigen (data not shown). In order to determine the immunogenicity of the purified recombinant protein, antiserum from the rabbit immunised with this protein, was used in Western blot analyses. The rabbit antiserum reacts with the r38Ag and irradiated *M tuberculosis* sonicate antigen indicating that the antibody generated reacts not only with r38Ag, but also the native antigen present in *M.tuberculosis* sonicates (10).

Having assessed the purity, and immunogenicity of the recombinant protein, serological reactivity of the antigen with sera from patients with pulmonary tuberculosis and healthy individuals was assessed by western blot. The 38 kDa band was seen with sera from patients with tuberculosis but not healthy individuals.

Detection of antibody in CSF samples in Tuberculous Meningitis using r38Ag.

In order to study whether the recombinant 38 kDa antigen gives the same degree of sensitivity and specificity in diagnosis in TBM as the native antigen a similar ELISA replacing the native antigen with r38Ag was standardised and 43 CSF samples from patients with TBM and 48 controls were studied. It was observed that the r38Ag gave high antibody titres in CSF samples but there was no discrimination between TBM and controls.

CONCLUSION

Our experience has revealed that the detection of 38 kDa antigen or antibody to the antigen in various fluids is useful in diagnosis of various

manifestations of tuberculosis. The PCR developed for 340bp sequence of its encoding gene also shows a high degree of sensitivity and specificity. Thus the 38 kDa antigen/antibody combination or the PCR are ideal for development of kits for diagnosis. These immunoassays to be successful, isolation of the 38 kDa antigen in large quantities is essential. Using recombinant DNA technology and expression in *E.coli* this has been achieved. However, such recombinant antigen does not have the same immunological properties as the native antigen and hence not suitable in immunodiagnosis. To fully realise the potential of the MoAb defined antigens such as the 38 kDa antigen, 19 kDa antigen and others it is essential to devise alternative vector-host systems that help in glycosylation, do not accumulate as inclusion bodies and can be isolated with less damage.

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