**The Leprosy Mission**



**Anandaban Leprosy Hospital**

**Mycobacterial Research Laboratory**

**Report of Research Project**

**to**

**Nepal Health Research Council**

# *"Rapid & simple immunodiagnostic tests for the detection of tuberculosis"*

**by**

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## Summary

Two new immuno-diagnostics for tuberculosis were tested in Nepali TB patients and their contacts. A rapid method for detection of antibodies to tuberculosis (Amrad/ICT) was used in 303 individuals (50 endemic controls, 50 TB contacts, 153 TB patients and 50 leprosy patients). Antibodies were detected in 54/153 (35%) of TB patients, 0% of endemic controls and TB contacts, but in 12/50 (24%) of leprosy sera. Antibodies were found largely among smear positive pulmonary TB patients, with very low rates of antibodies in smear negative and non-pulmonary patients. The TB kit in its present form is clearly not sensitive or specific enough for use in Nepal. The sera will be re-tested in a new rapid assay kit containing new proteins, which the manufacturer hopes will be more sensitive and specific for detecting TB.

Blood was also tested in a simple overnight assay for the production of interferon -gamma (IFN-$\gamma$) to TB proteins with the aim of detecting latent TB infection among Nepali contacts of TB patients. 50 non-exposed Nepalis, 63 TB contacts and 114 TB patients were tested against 5 antigens. The results expressed as group means show stronger IFN-$\gamma$ response to PPD and the 38kD protein of *M. tuberculosis* among both contacts and TB patients compared with endemic controls. We plan to further investigate this significant difference in the more sensitive ELISPOT assay and to assess the utility of these findings for the detection of latent TB infection.

## Introduction

The incidence of tuberculosis (TB) is increasing world wide and every year large numbers of people are affected directly or indirectly from this disease in both developing and industrial countries. According to the WHO 1.7 billion population of world are infected with the tubercle bacilli. In Nepal, every year 45,000 people die from TB. The early diagnosis of patients with active disease is essential for starting timely treatment and to reduce mortality and morbidity, however, detection in early stages by conventional methods may be difficult. The definitive diagnosis of tuberculosis is based upon the isolation of organisms by culture in clinical specimens or tissues. However, these conventional methods for detection of *M. tuberculosis* are not widely available and are time consuming. The increasing rates of co-infection with HIV and TB in many countries sharpens the need for rapid diagnosis as early treatment of TB in HIV+ individuals is associated with a better prognosis for the individual as well as a reduction in the risk to the community of TB transmission. Some advocate the screening of all HIV+ individuals for latent tuberculosis, which may only be detectable by immune-based diagnostics, such as antibody or activated T-cell detection and the chemoprophylaxis of latently infected individuals.

Two rapid and simple immuno-diagnostics have recently been developed. Amrad-ICT, an Australian biotech company has developed a rapid method to detect tuberculosis antibodies. In early studies in China 95% of sputum positive and 85% of sputum negative pulmonary TB patients had detectable antibodies by this method. CSL, another Australian company have developed a simple and rapid assay for the diagnosis of tuberculosis based on the detection of gamma interferon (IFN-γ) liberated in the blood incubated *in vitro* with PPD (Purified Protein Derivative) from *Mycobacterium tuberculosis, M. bovis* and *M. avium.* With this assay in an Australian population, which was not vaccinated with BCG, the assay had a specificity of 98% and a sensitivity compared to tuberculin skin testing of 90%. While these early results are encouraging, the real challenge is to apply these types of rapid diagnoses is in TB-endemic countries like Nepal. Here the healthy population is usually BCG-vaccinated

and exposed to tuberculosis as well as other mycobacteria in the environment and hence have a higher rate of immune activation to tuberculosis.

In the present study, we have tested a new version of the rapid antibody detection kit which uses 5 tuberculosis antigens to improve the sensitivity of the assay. We have also tested subjects in a modification of the IFN-γ assay, using protein antigens specific to *M. tuberculosis*, which would have the potential to distinguish between healthy BCG vaccinees and individuals latently infected with TB.

## Study Population and Methods:

*1. Study Groups*

Blood samples from TB Patients and TB contacts, were collected from the TB Clinic, Patan Hospital, Lalitpur, Nepal and the Yala Urban Health Project. Patients having active tuberculosis were divided into pulmonary or extra-pulmonary groups and pulmonary disease smear positive or negative. Healthy controls who were not exposed to TB ( by questioning whether they or any of their family had previously had TB), were staff members of United Mission to Nepal (UMN) head office. In all groups, BCG vaccination status was checked by examining individuals for a vaccination scar. All subjects were Nepali and signed a consent form after the tests were explained to them in their own language and before the blood was drawn. The study protocol was approved by the Nepal Health Research Council.

**Table 1: Study Population.**

| Group | | Total | Male | Female | Age Range |
|---|---|---|---|---|---|
| TB Patients | All | 152 | 72 | 80 | 12-67 |
| | PTB +ve Sputum | 77 | | | |
| | PTB -ve Sputum | 18 | | | |
| | Non-Pulmonary TB | 58 | | | |
| Healthy Contacts | | 63 | 30 | 33 | 15-43 |
| Control | | 50 | 34 | 16 | 20-57 |
| Leprosy Patients | | 50 | 35 | 15 | 15-70 |

*2. Method for Antibody detection*

Anti-TB antibody test cards were supplied by Amrad ICT Diagnostics (Figure 1). This consisted of a unique cardboard folder . The right hand face contained a nitro-cellulose (NC) strip (6mm x 22mm) on which the 4 antigen lines were applied (T). These were antigens of MW 38 Kda, 19 Kda, 6 Kda, and mixture of 2 unknown non-protein antigens were arranged from top to bottom of the card. A control antigen (C), anti-human IgG, was also applied as a positive control. At the base and top of the nitro-cellulose strip are thin absorbent pads (1 & 2). The left hand face of the device has a window cut into it (5). A conjugate pad (4) was situated above the window on which 5 µl of goat anti-human IgG conjugate linked to colloidal gold was dried. There was a thick absorbent pad below the window. Two drops of buffer reagent are added to lower pad (1) followed by application of 30 µl of serum to the upper pad above the nitro-cellulose (NC) strip (2). Serum was allowed to diffuse down the NC to the marked line (L), at which point 1 drop of buffer was added to the transfer pad (3). At this point the folder was closed with an adhesive strip. The final result of the test was read after 15 minutes. The intensity of the reaction to each antigen line was categorised as follows:

\+ positive, a very faint pink red line
++ positive, easily visible pink red line
+++ strong positive, pronounced reddish line
++++ strong positive, a deep purple red line

For every test the control line had to become positive for the test to be accepted. Negative subjects had control line positive but with all antigen lines negative.

*2. Whole blood assay*

*2.1 Antigens*

The antigens used in the whole blood assay are shown in table 2

**Table 2. Antigens used forin whole blood assay**

| Antigens | Origin | Source | MW | Provided By |
|---|---|---|---|---|
| PPD | *M.bovis, M. tuberculosis* | Crude culture filtrate | Various | Statens Seruminstitut, Denmark. |
| ESAT6 | *M.tuberculosis* | Native, Culture filtrate | 6KDa | Statens Seruminstitut, Denmark. |
| MPT64 | *M. tuberculosis Some M. bovis* strains | Recombinant *M.smegmatis* | 23KDa | Centenary Institute of Cancer Medicine and Cell Biology, Australia. |
| 38KDa | *M.tuberculosis, M.bovis.* | Native, Culture filtrate | 38KDa | Colorado State University, Colorado, USA. |

PPD (Purified Protein Derivative), is culture filtrate from *M.bovis* and widely used as skin test antigen in the Mantoux test for tuberculosis.

ESAT6 which is 6kDa antigen of *M.tuberculosis*. The low mass secreted ESAT6 protein was recently identified as a key molecule recognised by memory effector T cells in a mouse model of long -lived immunity to TB. The gene coding for ESAT6 is lacking in all the strains of BCG tested.

MPT64 is a 23kDa secreted protein restricted to members of the *Mycobacterium tuberculosis* which elicits T cell response and cutaneous DTH reactions in *M.tuberculosis* infected animals. The gene coding for MPT64 is lacking in some but not all BCG strains and it elicits immune response by DTH in guinea pigs sensitised with *M.tuberculosis.*

The 38kD is a lipoprotein which though expressed in both *M. tuberculosis* and *M. bovis* appears to elicit a serological and cell-mediated immune response in both TB patients and their contacts. Some regions of the protein, appear to define a TB-specific immune response.

*Method for Whole Blood Assay*

1ml of blood was added to wells of 24 well tissue culture plate was stimulated by each of antigens PPD, ESAT6, MPT64 and 38KDa and mitogen PHA at final concentration of 10μg/ml. The plates were incubated at 37 °C with 5% $CO_2$ for 24 hours. The supernatants were collected from each well and interferon-gamma (IFN-γ) was measured by using capture ELISA.

IFN-γ measurement was performed using a standard PharMingen protocol (PharMingen International, USA). Purified mouse anti-human IFN-γ mAB (2ug/ml) in 0.1 M Sodium-bicarbonate coating buffer (pH 8.2) was coated into the wells of the ELISA plate Dynatech Immulon 4 ELISA plates (Dynatech, Chantilly, USA) and incubated overnight at 4°C. Plates were washed twice with PBS/T buffer. The wells were then blocked with 200 μl of 5% BSA in PBS/T, for 2 hours at room temperature and washed again as before. Antigen stimulated supernants were added to the plate in duplicate wells at a dilution of 1:2 and PHA

stimulated supernatant were diluted 1:50 with RPMI media containing 5% heat inactivated human AB serum. Serial dilutions of pure recombinant human IFN-γ (from 1-2500 U/ml) were added to other wells. Plates were incubated from overnight at 4°C, then washed and 100 µl of mouse anti-human IFN-γ mAB biotin labelled (1µg/ml) was added in the plates and incubated for 1 hour at room temperature. The plates were again washed and 100 µl of avidin peroxidase conjugate (1mg/ml Sigma) diluted in 1:800, was added to the plate and incubated for a further 30-40 minutes in room temperature. The plates were again washed and 100ul of substrate solution (O-phenylenediamine 0.4mg/ml, plus 0.4 µl/ml hydrogen peroxide in citrate phosphate buffer pH 5.0) was added. The reaction was developed in dark for 10 minutes and was stopped with 2.5 M sulphuric acid. Plate wer read in a Dynatech MRX microplate plate reader at 492nm.

Results were automatically converted from mean OD measurement to IFN-γ in (U/ml) according to the standard curve using the curve fit program . A positive IFN-γ measurement in the negative control (medium alone) culture supernatants, if detected, was subtracted from measurements in test wells.

**Results.**

*1. Serology*

54 (35%) of TB patients had anti-tuberculosis antibodies as shown by positive reactions on the card while 12 (24%) of leprosy patient also had antibodies. When results were analysed on the basis of types of TB, 56% of sputum positive pulmonary tuberculosis, 17% of non-pulmonary tuberculosis and 5.5% of sputum negative pulmonary tuberculosis had detectable antibodies.

Antibody response to each of the four different antigens was analysed (Table 3). None of the 100 control (unexposed) or TB contacts had antibodies to any of three antigens, making the specificity for TB disease very high (100%). Sensitivity however was poor and cross-reactivity with the other major mycobacterial disease, leprosy was significant. Antibodies to the first antigen line (38 kDa) was higher in smear positive PTB patients than to any other antigen (36%). In addition, the responses appeared to be TB specific with only a small minority of leprosy patients (5%) responding. Line 2 was detected by antibodies in a small minority of TB patients only. Likewise line 3 was recognised by a minority of TB patients with a small number of leprosy sera also reactive. Line 4 showed the most cross-reactivity with leprosy sera.

**Table 3: Antibody detection by using TB test card for different groups.**

| Patient Group | No. +ve | Line1+ve | Line2+ve | Line3+ve | Line 4+ve |
|---|---|---|---|---|---|
| PTB+ve (n=77) | 43 (55.8%) | 28 (36.3%) | 3 (4%) | 10 (12.9%) | 19 (24.6%) |
| PTB -ve (n=18) | 1 (5.5%) | 0 | 0 | 1 (5.5%) | 0 |
| NPTB (n=58) | 10 (17.2%) | 3 (5%) | 1 (1.7%) | 1 (1.7%) | 7 (12%) |
| Contacts (n=50) | 0 | 0 | 0 | 0 | 0 |
| Controls (n=50) | 0 | 0 | 0 | 0 | 0 |
| Leprosy (n=50) | 12(24%) | 2 (4%) | 0 | 2 (4%) | 8 (16%) |

*2. Whole blood assay*

The results of the whole blood assay is shown in Table 4. IFN-γ responses to PPD were higher in TB contacts and TB patients than in endemic controls. Further responses to the 38kD protein were significantly increased in contacts and PTB -ve

and non PTB patients. Responses to ESAT-6 were not significantly increased in any groups compared with controls, while responses to MPT64 were significantly lower in contacts and patients compared with controls.

**Table 4** : IFN-γ produced in overnight whole blood assay (geometric means (SD)).

| Antigen | Control (n=50) | Contacts (n=63) | PTB +ve (n=55) | PTB -ve (n=15) | NPTB (n=44) |
|---|---|---|---|---|---|
| PPD | 18.4 (7.5) | 26.2 (5.9)*** | 38.1 (4.3)*** | 52.1 (3.7)*** | 34.6 (6.0)*** |
| ESAT6 | 1.44 (2.5) | 1.9 (2.33) | 1.8 (2.8) | 3.18 (3.5) | 3.0 (4.3) |
| MPT64 | 7.8 (4.6) | 2.01 (2.56)*** | 1.97 (2.9)*** | 3.3 (4.8)** | 2.54 (3.8)*** |
| 38KDa | 3.75 (4.17) | 5.8 (4.8)* | 4.0 (5.6) | 7.4 (4.3)** | 10.2 (8.2)*** |
| PHA | 4.1 (4.1) | 1.95 (2.8)** | 1.4 (2.3)*** | 1.45 (4.0)* | 1.58 (2.6)** |

Significant difference compared with healthy controls *$p<0.05$, **$p<0.01$, ***$p<0.001$

**Conclusions**

The serological responses in Nepali TB patients to tuberculosis antigens in the rapid test kit were confined largely to the PTB smear positive group, with very low responses in other patient groups and no responses in endemic controls or TB contacts. There was a variable specificity for tuberculosis in different bands as judged by the reactivity of the leprosy sera. A calculated overall specificity of 76% and a sensitivity for any form of TB of 35% mean that this test in its present form is unacceptable for the diagnosis of TB.

The IFN-γ responses of contacts and patients to TB antigens is of interest. However as a diagnostic aid, the test in its present form is also inadequately specific or sensitive. There is interest in examining the numbers of IFN-γ producing cells in the 'ELISPOT' test particularly to PPD and the 38kD to see if this more sensitive test can detect differences between contacts and controls and between controls and patients with smear negative pulmonary TB and non-pulmonary TB.

**Future plans**

We have been requested by Amrad ICT to re-test the sera we have collected in an improved antibody detection kit. We have agreed to do this and will complete this study on the sera already collected in the coming months and will forward the results to you. It may be however, that the antibody response to TB in a Nepalese population is not an adequately sensitive method for detecting TB disease.

We hope to examine a small group of TB patients by ELISPOT to quantitate the numbers of TB antigen specific IFN-γ producing cells to PPD and the 38kD antigen. This test which has been shown to be significantly more sensitive than measuring free IFN-γ in culture, is at present really of research interest. However, simplifications in the methodology may mean in the future that this technique may detect latent TB infection in BCG-vaccinated endemic populations.

**Thanks**

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Figure 1: The Amrad ICT TB antibody detection card used in this study