

In-vitro assessment of cell-mediated immunity by demonstrating effector-t cells for diagnosis of tuberculosis in Nepalese subjects

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ABSTRACT

The immune response against *Mycobacterium tuberculosis* (MTB) is cell mediated. T-cells become sensitized when they encounter MTB antigens and subsequently activated effector T-cells produce a number of cytokines including interferon- γ (INF- γ) to fight the infecting organisms. Demonstration of either production of INF- γ or presence of effector T-cells sensitized to MTB specific antigens *in vitro* can be diagnostic for TB infection. Aim of this study was to determine the efficacy of commercially available T-SPOT.TB kit which is used for the *in vitro* diagnosis of TB infection and to determine if this test has any cross reactivity in leprosy patients. Blood sample was taken from 30 sputum AFB positive, 30 sputum AFB negative healthy controls and 10 cases of paucibacillary leprosy patients. The blood samples were processed to separate peripheral blood mononuclear cells. The final cell suspensions were cultured along with MTB specific antigens namely- Early Secretory Antigenic Target (ESAT-6) and Culture Filtrate Protein (CFP 10) along with negative and positive controls. The production of INF- γ was demonstrated by enzyme linked immunospot (ELISPOT) assay technique. All AFB positive samples produced INF- γ after exposure to MTB specific antigens. 4 (16.6%) of healthy controls were also found reactive for INF- γ . The sensitivity and "specificity" for active disease of the ELISPOT (T-SPOT.TB) in respect to AFB microscopy was 100% and 85.7% respectively. Assessment of CMI against tuberculosis, by demonstrating effector T-cell sensitized to MTB antigens can be use to aid the diagnosis of tuberculosis. T-SPOT.TB has no cross reactivity with leprosy patients.

Keywords: Tuberculosis, CMI, ELISPOT, Interferon - γ .

INTRODUCTION

Tuberculosis (TB) is an infectious disease which affects one-third of the world's population, killing approximately 2 million annually.¹ TB remains a major public health problem in Nepal killing about 5000-7000 per year.² Though numerous assays are available, accurate diagnosis of TB continues to be a major issue in laboratory medicine, due to poor sensitivity, time to result and cost of testing.^{3,4}

The immune response to infection with *Mycobacterium tuberculosis* (MTB) is predominantly a cell mediated immunity (CMI) response, in which T-cells are sensitized with MTB antigens. Activated effector-T cells produce different cytokines including interferon- γ (INF- γ) to fight the infecting organism.⁵ Due to the short life of effector T-cells, their continuing presence indicates the cellular immune response is currently encountering and fighting a pathogen somewhere in the body.⁶ Measuring the presence of effector T-cells in a sample therefore diagnoses an ongoing infection.

Recently, *in vitro* tests based on detection of CMI for the diagnosis of TB have become commercially available. These tests use the MTB - specific antigens namely- Early

Secretory Antigenic Target- 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP-10) peptides to stimulate *M. tuberculosis*- sensitized T-cells for the production of INF- γ .^{7,8} ESAT-6 and CFP-10 antigens are specific for MTB and are produced from genomic area called Region of Difference 1 [RD1]. RD 1 was lost from the *M. bovis* genome when the organism was passaged during the development of the BCG vaccine. If the patient sample contains *M. tuberculosis*- sensitized T-cells, after exposure to these antigens *in vitro*, will secrete INF- γ .⁹⁻¹² Therefore demonstration of the production of INF- γ from the effector T- cells *in vitro* can be used to diagnose TB.

Diagnosis of TB by *in vitro* demonstration of CMI is now commercially available as QuantiFERONTB Gold (Cellestis Ltd, Victoria, Australia) and T-SPOT.TB assay (Oxford Immunotec; Oxford, UK). These assays have been approved for diagnostic use in Europe and the USA and are being used to diagnose both latent TB infection and active TB diseases.¹³⁻¹⁵

The aim of this study was to evaluate the T-SPOT.TB kit in smear positive TB patient, healthy controls and identify any cross reactivity in leprosy patients.

Table-1: Comparison of result among smear positive group, healthy control group and PB leprosy group

	Reactive	Non- Reactive	Indeterminate
Smear Positive Group (n=30)	27	0	3
Healthy Control Group (n=30)	4	24	2
PB Leprosy Group (n=10)	0	10	0

MATERIALS AND METHODS

Participants: Seventy participants were selected in this study, 30 were sputum AFB positive patients, 30 were sputum AFB negative healthy controls and 10 were cases of paucibacillary (PB) leprosy. 10 ml of heparinized venous blood was collected from each subject.

T-SPOT.TB: The T SPOT.TB test was performed according to the manufacturer’s instructions. Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Hypaque density gradient centrifugation from 10 mL of the heparinized blood (Fig. 1). After centrifugation the white, cloudy band of PBMCs was removed and washed in sterile cell culture medium (GIBCO AIM-V) to ensure complete removal of endogenous IFN-γ. Then a viable cell count was performed using trypan blue dye in a haemocytometer. PBMCs were diluted with the AIM-V medium so that the final solution contained 2.5x10⁵ cells / 100μL. Four wells of a 96-well microtitre plate, pre-coated with monoclonal antibodies directed against IFN-γ were used for each sample. 50μL AIM-V medium was added in first well as negative control, 50iL of ESAT-6 and CFP 10 was added for second and third wells respectively and 50μL of phytohaemagglutinin was added in fourth well as positive control. Then 100μL of the patient’s final cell suspension was added to all wells and incubated in a humidified incubator at 37°C with 5% CO₂ for 16-20 hours. During this period MTB- sensitized T-cells were stimulated by these antigens producing IFN-γ. Secreted IFN-γ is captured by specific antibodies on the surface of the well. The plate was then washed with 200μL Phosphate buffered saline. 50μL of second antibody, conjugated to alkaline phosphatase, directed to a different epitope of the IFN- γ was added and incubated at 2-8°C for 1 hour. After washing again, 50μL substrate solution was added to each well and incubated at room temperature for 7 min. In this period the substrate was cleaved by bound enzyme to form a spot of

insoluble precipitate at the site of the reaction. Finally the plate was thoroughly washed with distilled water to stop the reaction and the number of spots formed in each well was counted under 10X microscope lens. Each spot represents the footprint of an individual cytokine-secreting T cell (Fig. 2). The number of spots obtained provides a measurement of the abundance of *M. tuberculosis* -sensitized effector T- cells in the peripheral blood.

Test wells were scored as reactive if either or both ESAT-6 and CFP-10 contained at least five spot forming cells more than the negative control well. But when negative control well contained >5 spot, the test was considered reactive, if test well contain at least twice the spot number than negative control well.^{16,17}

Statistical analysis: The sensitivity, "specificity" for active disease, positive predictive value (PPV) and negative predictive value (NPV) were calculated by comparing results of T-SPOT.TB in Smear positive group and healthy controls.

RESULTS

Three samples from smear positive patients and two from healthy controls were indeterminate due to insufficient responses to the positive control. All the remaining samples from smear positive patients were reactive. Four (16.6%) of healthy control were also reactive. All 10 PB leprosy cases were non reactive (Table-1).

Table-2 shows comparison of T-SPOT.TB results in smear positive subjects and healthy controls. Sensitivity of the T-SPOT.TB is 100% and the "specificity" for active disease is 85.7%.

DISCUSSION

Diagnosis of TB is often achieved through the identification of AFB in sputum microscopy. Although microscopy is simple, specific and rapid it lacks sensitivity, requiring > 10³- 10⁴ bacilli per mL of specimen.³ Definitive diagnosis is based on culture of the *Mycobacterium tuberculosis* complex. However, culture of MTB takes up to 6 to 8 weeks. Molecular tests, such as PCR, and phage based assays are available, but are subjected to possible contamination and subsequent false positive results.^{4,18}

In light of these diagnostic problems, clinicians often rely on tuberculin skin testing (TST) to demonstrate an infection with MTB. TST is based on determination of CMI against MTB *in vivo*. However, the diagnostic value of TST is

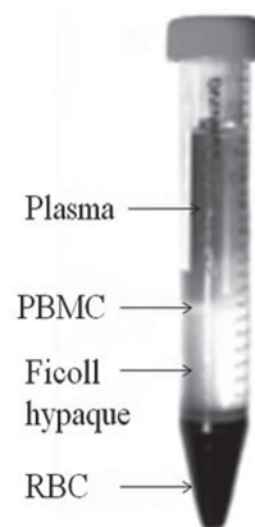


Fig. 1. Separation of PBMC by Ficoll-Hypaque density gradient centrifugation

Table-2: Comparison in AFB smears Positive Subjects and Healthy Controls

	Reactive	Non-Reactive
Smear Positive Patients (n=27)	27	0
Healthy Controls (n=28)	4	24

Sensitivity- 100%, "Specificity" for active disease-85.7%, PPV- 87.1%, NPV- 100%

Table-3: comparison of sensitivity in T-SPOT.TB studies

Study, Year (Reference)	Sensitivity (%)
Meier <i>et al.</i> , 2005 (13)	96
Lee <i>et al.</i> , 2006 (26)	95
Goletti <i>et al.</i> , 2006 (27)	91
Jafari <i>et al.</i> , 2006 (28)	100
Kang <i>et al.</i> , 2007 (24)	87
Detjen <i>et al.</i> , 2007 (29)	93
Wang <i>et al.</i> , 2007 (30)	87
Janssens <i>et al.</i> , 2007 (31)	98
Ozekinci <i>et al.</i> , 2007 (32)	93

limited. It shows high rates of false-positive results due to antigenic cross-reactivity with BCG vaccine and also with environmental mycobacteria.^{16,19,20} Further, false-negative results among immunocompromised patients are not uncommon leading to poor sensitivity. Variability when placing the TST and interpreting the results is common and potential disadvantage of an anamnestic response with successive tests. Further the test requires two patient visits, one to give the test and one to have it read and approximately one-third of those who are given the TST never return to have the result read.²¹⁻²³

Different studies have found that the sensitivity and specificity of the demonstration of effector T-cells producing IFN- γ on exposure to the MTB specific antigen is much better than traditional TST.^{15,24,25} Therefore, the *in vitro* assessment of CMI using MTB specific antigen is preferred to TST.

In our results T-SPOT.TB has an excellent sensitivity of 100%. All of the patients with active disease were T-SPOT.TB positive. Based on the high sensitivity in active disease, a negative test makes active TB infection unlikely. Previous studies have also found the test to have a high sensitivity, ranging from 87 to 100% (Table-3). Therefore, this test can be very valuable in screening for tuberculosis.

On the other hand previous studies have also indicated a high specificity, ranging from 92 – 100%.^{26,29,33} We obtained a "specificity" of only 85.7%. All of these studies were conducted in countries with low prevalence of TB in control

groups selected to have no identifiable exposure to TB. The lower specificity value in this study is probably due to the high prevalence of TB in our country so latent TB infection cannot be excluded in the control group. This will produce a positive T-SPOT.TB result.²⁵ To determine the utility of the T-SPOT.TB test to rule out active TB, the background prevalence of positive T-SPOT.TB results in a population must be known.³⁴ This is probably the first study in Nepal, demonstrating IFN- γ producing sensitized T-cells for diagnosis of TB. No data are available to compare specificity of this assay in high prevalence area like Nepal. Therefore further studies in healthy populations should be done to find background prevalence of positive T-SPOT.TB in Nepal.

On the other hand, we found all PB leprosy patients to be negative by T-SPOT.TB suggesting that this test has no cross-reaction with leprosy even though L-ESAT, a *M. leprae* antigen is very homologous to the T-ESAT-6 used in this test. Therefore, this test can be used in our country even though leprosy is endemic. However, further studies with larger sample size are required to verify this.

The major limitation of T-SPOT.TB is that it is several times more expensive than TST making it not feasible for universal use in developing countries. On the other hand there were five (7.1%) indeterminate results due to insufficient responses to the positive control. This may be patient specific, or caused by the unfamiliar protocol or poor viability of cells in our hands. Further, use of T-SPOT.TB could be limited in routine laboratory due to relatively short time-frame allowed between blood sampling and processing, error in separating, counting and incubation of PBMCs and counting the spot-forming units after completion of the test.

T-SPOT.TB for the assessment of CMI for tuberculosis, by demonstration of effector T-cell sensitized to MTB antigens can be an alternative to aid the diagnosis of tuberculosis. Compared to the TST, the test is more sensitive and specific and does not require patients to return for a second visit. Further, it can also used in detecting latent TB infection and continues to perform well in immunocompromised subjects. This test does not appear to have cross reactivity with leprosy. Cost and technical limitations may prevent the replacement of the TST in developing countries. However this test can provide valuable information about the disease and aid in diagnosing TB.

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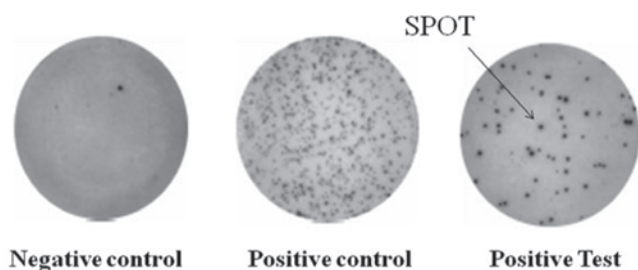


Fig. 2. Visualization of spot under 10x microscope

REFERENCES

1. Global tuberculosis control: surveillance, planning, financing. WHO report 2006. Geneva, World Health Organization (WHO/HTM/TB/2006.362).
2. National Tuberculosis Control Programme, Annual Report (2004/2005) NTC, Thimi, Bhaktapur, Nepal.
3. Hobby GL, Holman AP, Iseman MD, Jones JM. Enumeration of tubercle bacilli in sputum of patients with pulmonary tuberculosis. *Antimicrobiol Agent Chemother* 1973; 4: 94-104.
4. Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989; 339: 237-8.
5. Goldsby RA, Kindt TJ, Osborne BA. Immune response to infectious disease. Kuby Immunology. 4th ed. New York: W.H. Freeman and company, 2000: 425-48.
6. Pathan AA, Wilkinson KA, Klenerman P *et al*. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in *Mycobacterium tuberculosis*-infected individuals: associations with clinical disease state and effect of treatment. *J Immunol* 2001; 167: 5217-25.
7. Brandt L, Elhay M, Rosenkrands I, Lindblad EB, Andersen P. ESAT-6 subunit vaccination against *Mycobacterium tuberculosis*. *Infect Immun* 2000; 68: 791-5.
8. Arend SM, Andersen P, van Meijgaarden KE *et al*. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. *J Infect Dis* 2000; 181: 1850-4.
9. Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J Bacteriol* 1996; 178:1274-82.
10. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, Small PM. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Sci* 1999; 284: 1520-3.
11. Berthet FX, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). *Microbiol* 1998; 144: 3195-3203.
12. Ravn P, Demissie A, Egualé T *et al*. Human T cell responses to the ESAT-6 antigen from *Mycobacterium tuberculosis*. *J Infect Dis* 1999; 179: 637-45.
13. Meier T, Eulenbruch HP, Wrighton-Smith P, Enders G, Regnath T. Sensitivity of a new commercial enzyme-linked immunospot assay (T SPOT-TB) for diagnosis of tuberculosis in clinical practice. *Eur J Clin Microbiol Infect Dis* 2005; 24: 529-36.
14. Liebeschuetz S, Bamber S, Ewer K, Deeks J, Pathan AA, Lalvani A. Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. *Lancet* 2004; 364: 2196-2203.
15. Lalvani A, Pathan AA, McShane H *et al*. Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. *Amer J Respir Crit Care Med* 2001;163:824-8.
16. Ewer K, Deeks J, Alvarez L *et al*. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of *Mycobacterium tuberculosis* infection in a school tuberculosis outbreak. *Lancet* 2003; 361: 1168-73.
17. Lalvani A, Nagvenkar P, Udwadia Z *et al*. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent *Mycobacterium tuberculosis* infection in healthy urban Indians. *J Infect Dis* 2001; 183: 469-77.
18. Shrestha R, Neupane K, Gyawali P, Macdonald M. Evaluation of mycobacteriophage assay for rapid detection of *M. tuberculosis* in clinical specimen. *J Nepal Med Lab Sci* 2008; 9: 13-7.
19. Chapman AL, Munkanta M, Wilkinson KA *et al*. Rapid detection of active and latent tuberculosis infection in HIV-positive individuals by enumeration of *Mycobacterium tuberculosis*-specific T cells. *AIDS* 2002; 16: 2285-93.
20. Kwamanga DO, Swai OB, Agwanda R, Githui W. Effect of nontuberculous *Mycobacteria* infection on tuberculin results among primary school children in Kenya. *East African Med J* 1995; 72: 222-7.
21. Chaparas SD, Vandiviere HM, Melvin I, Koch G, Becker C. Tuberculin test: Variability with the Mantoux procedure. *Amer Rev Respir Dis* 1985; 132: 175-7.
22. Hansted E, Sitkauskienė B, Kevalas R, Tattersall A, Day T. Research for practice: a new in vitro test for identification of tuberculosis infection. *Medicina (Kaunas)* 2007; 43: 519-22.
23. Mazurek GH, Jereb J, Lobue P, Iademarco MF, Metchock B, Vernon A; Division of Tuberculosis Elimination, National Center for HIV, STD, and TB Prevention, Centers for Disease Control and Prevention (CDC). Guidelines for using the QuantiFERON-TB Gold test for detecting *Mycobacterium tuberculosis* infection, United States. *MMWR Recomm Rep* 2005; 54: 49-55.
24. Kang YA, Lee HW, Hwang SS *et al*. Usefulness of whole-blood interferon-gamma assay and interferon-gamma enzyme-linked immunospot assay in the diagnosis of active pulmonary tuberculosis. *Chest* 2007; 132: 959-65.
25. Pai M, Zwerling A, Menzies D. Systematic review: T-Cell-based assays for the diagnosis of latent Tuberculosis infection: An update. *Ann Intern Med* 2008; 149: 177-84.
26. Lee JY, Choi HJ, Park IN *et al*. Comparison of two commercial interferon-gamma assays for diagnosing *Mycobacterium tuberculosis* infection. *Eur Respir J* 2006; 28: 24-30.
27. Goletti D, Carrara S, Vincenti D *et al*. Accuracy of an immune diagnostic assay based on RD1 selected epitopes for active tuberculosis in a clinical setting: a pilot study. *Clin Microbiol Infect* 2006; 12: 544-50.
28. Jafari C, Ernst M, Kalsdorf B *et al*. Rapid diagnosis of smear-negative tuberculosis by bronchoalveolar lavage enzyme-linked immunospot. *Amer J Respir Crit Care Med* 2006; 174: 1048-54.
29. Detjen AK, Keil T, Roll S *et al*. Interferon gamma release assays improve the diagnosis of tuberculosis and nontuberculous mycobacterial disease in children in a country with a low incidence of tuberculosis. *Clin Infect Dis* 2007; 45: 322-8.
30. Wang JY, Chou CH, Lee LN *et al*. Diagnosis of tuberculosis by an enzyme-linked immunospot assay for interferon-gamma. *Emerg Infect Dis* 2007; 13: 553-8.
31. Janssens JP, Roux-Lombard P, Perneger T, Metzger M, Vivien R, Rochat T. Quantitative scoring of an interferon-gamma assay for differentiating active from latent tuberculosis. *Eur Respir J* 2007; 30: 722-8.
32. Ozekinci T, Ozbek E, Celik Y. Comparison of tuberculin skin test and a specific T cell-based test, T-Spot.TB, for the diagnosis of latent tuberculosis infection. *J Int'l Med Res* 2007; 35: 696-703.
33. Kang YA, Lee HW, Yoon HI *et al*. Discrepancy between the tuberculin skin test and the whole-blood interferon gamma assay for the diagnosis of latent tuberculosis infection in an intermediate tuberculosis-burden country. *J Amer Med Assoc* 2005; 293: 2756-61.
34. Van Leeuwen RML, Bossink AWJ, Thijsen SFT. Exclusion of active *Mycobacterium tuberculosis* complex infection with the T-SPOT™.TB assay. *Eur Respir J* 2007; 29: 605-7.

Cell-mediated response is the predominant form of immune response to tubercular infection, while both cell-mediated and humoral immune responses are elicited by most non-tubercular infections in human body. Thus, ADA activity, a marker of T-cell activation and cell-mediated immune response can help differentiate tubercular aetiology from non-tubercular. Piras et al were first to report high ADA in tubercular pleural effusion (27). Present study showed that levels of pleural fluid ADA were significantly higher than serum ADA. In the present study, a few cases with pulmonary tuberculosis showed elevated ADA activity in spite of sputum negative for tuberculosis.

Macrophages and Dendritic Cells in the Early Phase of Tuberculosis Infection. Alveolar macrophages are considered the first cells infected by inhaled Mtb. They actively produce antimicrobial factors and they initiate the immune response against Mtb itself^{36,37}. It is generally agreed that Mtb proliferates within alveolar macrophages and DC and induces release of cytokines such as TNF- α , IL-1, IL-6, and IL-12, which in turn activate macrophages to induce Mtb killing⁴². IL-12 and IL-18 produced by macrophages and DC induce the production of IFN- γ by many T cell subsets^{37,43,44,45}. The activation of macrophages induced by IFN- γ produced by T cells appears to be the central event in the elimination of Mtb⁴⁶.

Recruitment and Activation of Natural Killer Cells During TB Infection. 34. **In Vitro Production and Assessment of Activity of Skin Reactive Factors Released by Lymphoid Cells and Associated with Cellular Immunity.** 35. **The Preparation and Purification of Transfer Factor.** 36. **Conversion of Nonsensitive Lymph Node Cell Populations to Sensitive Cells with an RNA-Extract.** 37. **Virus Plaque Assay for Antigen-Sensitive Cells in Delayed Hypersensitivity.** 38. **Preparation and Care of Established Human Lymphoid Cell Lines.** Appendix Tables.

The immune response against mycobacterium tuberculosis (MTB) is cell mediated. T-cells become sensitized when they encounter MTB antigens and subsequently activated effector T-cells produce a number of cytokines including interferon- gamma (INF-gamma) to fight the infecting organisms. Demonstration of either production of INF-gamma or presence of effector T-cells sensitized to MTB specific antigens in vitro can be diagnostic for TB infection. Aim of this study was to determine the efficacy of commercially available T-SPOT.TB kit which is used for the in vitro diagnosis of TB infection and to determine if this test has any cross reactivity in leprosy patients.