Research Article

# Reproducibility of Immuno-Dot Blot Assay for Diagnosis of Tuberculosis using M. tuberculosis Hsp 65 KDa Protein

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## ABSTRACT

Studies had been attempted to define the mycobacterial antigens by standard biochemical and immunological techniques. One particular antigen, a 65-kilodalton (KDa) protein, is present in a wide range of mycobacterial species and has been most intensively studied. It is designated as 65K antigen or the cell wall protein a (CWP-a) antigen, since it appears to co-purify with cell walls. The 65K antigen is one of the major immunoreactive proteins of the mycobacteria. This antigen contains epitopes that are unique to a given mycobacterial species as well as epitopes that are common to various species of mycobacteria. The purified Hsp 65 KDa and monoclonal antibody against this antigen was used for immuno dot blot assay for detection of IgG against M. tuberculosis. Study with secondary conjugated IgG antibody and IgG Gold conjugate antibody the high prevalence of active TB/latent TB in the test population was observed i.e., 35 of the 104 subjects (33.65%) had active TB whereas for latent TB (21.15%). The positive and negative predictive values of anti-Hsp 65 IgG for active TB in a high-risk population were compared to determine patients with active TB showed sensitivity of anti-Hsp 65 IgG for active TB as 100% with 74% specificity.

**KEY WORDS:** conjugated antibody IgG; Hsp 65 KDa protein; Immuno Dot Blot Assay; M. tuberculosis

## INTRODUCTION:

The rapid and accurate detection, identification of active tuberculosis (TB) is important in Indian condition due to overall increase in incidence [1]. Acid-fast staining in sputum smears is generally used for detection of active TB, sensitivity is less because 104 bacilli per ml of sputum are required for reliable detection and only chest radiographs, with subsequent laboratory confirmation by culture remain the gold standard[2]. According to World Health Organization (WHO)-sponsored TB Diagnostics Workshop (1997), two priorities were set for the development of new diagnostics. First, to replace acid-fast microscopy for smear-positive TB and second, to tests to improve the differential diagnosis of smear-negative TB[3].

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The search for a rapid and reliable diagnostic test for active TB based on examination of sputum, blood, or other clinical specimens has been the focus of a number of studies. Detection of M. tuberculosis using polymerase chain reaction (PCR) is highly sensitive in most countries, where TB is prevalent[4,5]. However, analysis of molecular diagnostic techniques described PCR to be highly accurate on respiratory/pulmonary cases[6], the sensitivity and specificity of PCR is reduced in serum specimens[7]. Recently, Rep-PCR and Amplified ribosomal DNA restriction analysis (ARDRA) profiles of different mycobacterium species provide better segregation amongst the family[8]. The detection of TB by serological means has increased in years due to patients unable to produce adequate sputum, smear-negative, or suspected to have extrapulmonary TB[9,10].

A number of studies have attempted to define the mycobacterial antigens by standard biochemical and immunological techniques, including analysis of the target antigens of monoclonal hybridoma antibodies directed against mycobacteria[10].

One particular antigen, a 65-kilodalton (KDa) protein, is present in a wide range of mycobacterial species and has been most intensively studied as an antigen of M. leprae[11]. This antigen has been designated the 65K antigen or the cell wall protein a (CWP-a) antigen, since it appears to co-purify with cell walls in some isolation procedures[12]. The 65K antigen is one of the major immunoreactive proteins of the mycobacteria. This antigen contains epitopes that are unique to a given mycobacterial species as well as epitopes that are common to various species of mycobacteria[11]. Antibodies directed against this protein can be detected in the sera of patients with tuberculosis or leprosy, and T-cells reactive with this antigen can be isolated from patients with leprosy or tuberculosis as well as from BCG-vaccinated persons. Overall, recently the 65K antigen appears to be a major, medically important B- and T-cell immunogen and antigen in humans[3,13-15].

In the present study, serum specimens from patients with TB were examined for the presence of M. tuberculosis Ag-65 KDa by a sensitive and specific immune dot blot assay developed in our laboratory. We hypothesized that detectable level of anti- Ag 65 KDa IgG will be present in the sera of patients with active TB or have latent infection but not in subjects who are not infected.

## MATERIALS AND METHODS:

***Study groups***: Sera from 35 patients with culture-confirmed active TB were obtained from People's Hospital, Bhopal, MP, India. In addition, 22 patients with latent tuberculosis confirmed by AFB and culturing along with 47 healthy individuals with no signs of clinical impairment and normal chest radiographs were included as controls. All subjects were negative for HIV. Serum samples were obtained and were stored at -20 °C until they were tested. Samples were collected from all study groups for which patient's consent was obtained. To diagnose active TB, sputum microscopy was done on two serial sputum samples by staining with Ziehl-Neelsen stain as per the guidelines of India's Revised National Tuberculosis Control Programme (RNTCP). All the 57 patients had positive results with +1 to +4 for AFB in sputum samples. Final cultures for M. tuberculosis on Lowenstein-Jensen (LJ) medium obtained after 6 weeks were positive. TB was confirmed if AFB and/or culture of sputum specimens were positive for M. tuberculosis.

***Specimens:*** Sputum specimens for ordinary examination by AFB and cultivation were obtained over three consecutive days. The sputum sample was digested and decontaminated with 3% sodium hydroxide and then processed for further investigation (Petroff's method). Ziehl-Neelsen (ZN) acid fast staining was used to confirm the presence of acid-fast bacilli. Venous blood was collected from all the patients and control subjects. Blood was allowed to clot, and after centrifugation (1500×g, 10 min) the serum was separated and stored at -20°C until it was used.

***Antigen and antibody*:** The purified Hsp 65 KDa and monoclonal antibody against this antigen were obtained from AbD Serotec, and Bangalore Genei, India respectively.

### Immuno-dot blot assay for detection of IgG against *M. tuberculosis* Hsp 65 KDa protein

***1. Study with secondary conjugated IgG antibody*:** Diluted M. tuberculosis Hsp 65 antigen (0.1 µg/25 µl in 0.1M NaHCO3, pH 8.6) coated on nitro nitrocellulose discs, blocked for nonspecific binding with skim milk (3% skim milk in 1XPBS), were incubated for 1 hour under continuous agitation at 37°C. After incubation, the discs were amended with 5 µl serum and incubated for 1 hour at 37°C. The discs were washed (10X) free of unbound particles using the 1XPBS/Tween 20 (0.05%) to draw excessive antibody from undesired binding with Hsp 65 through the nitrocellulose. Thereafter, discs were amended with antibody enzyme conjugate (Goat anti-human IgG-HRP at 1:1000 in 1% BSA in 1XPBS) and incubated at 37°C for 1 hour. Repeatedly the discs were washed (10X) free of unbound particles using the 1XPBS/Tween 20 (0.05%). The air-dried discs were then added with substrate solution (TMB/H2O2, Bangalore Genei) to examine development of blue colour. The intensity of blue colour for positive, latent and negative serum was indicative of the binding of the Goat anti-human IgG-HRP to the M. tuberculosis Ag-65 KDa protein (Figure 1). The entire experiment was performed in triplicate.

***2. Study with IgG Gold conjugate antibody:*** Colloidal gold-Goat anti-human IgG conjugate was used to bind IgG present in serum specimens, which were kept frozen until use. For each assay, 5µl of serum was thoroughly mixed with 0.1 ml of colloidal gold Goat anti-human IgG conjugates suspended in phosphate-buffered saline (pH 7.4) at a concentration of 3 to 4 optical density units determined spectrophotometrically at a wavelength of 530 nm. IgG rapidly binds to the colloidal gold-Goat anti-human IgG

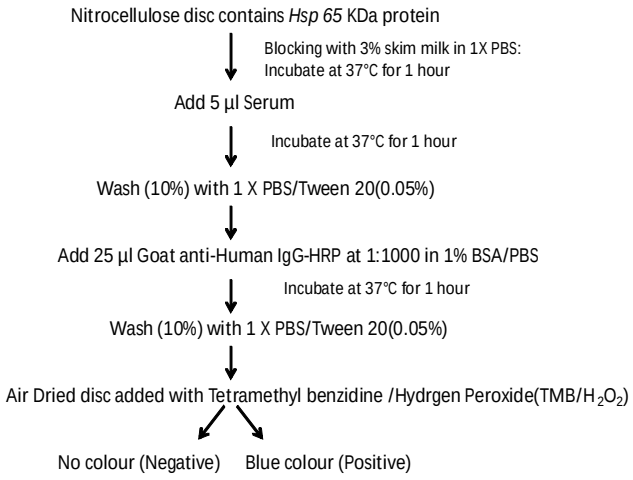


**Figure1**: Immuno-Dot blot assay to detect serum Anti-Hsp 65 KDa IgG using secondary antibody conjugate.

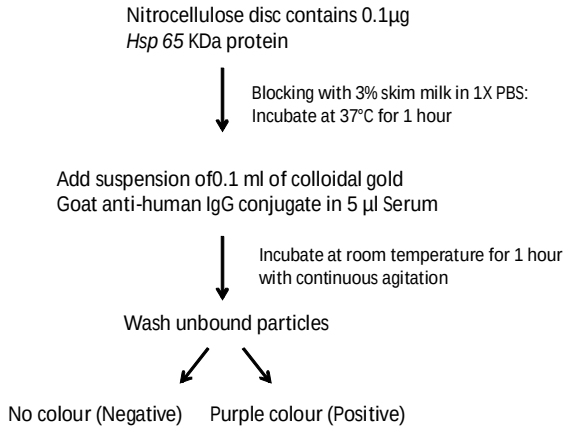


**Figure 2**: Immuno-Dot blot assay to detect serum Anti-Hsp 65 KDa IgG using colloidal gold conjugate.

conjugates owing to high affinity and extensive surface area. The M. tuberculosis Ag-65 KDa containing nitrocellulose discs (0.1 µg/25 µl in 0.1M NaHCO3, pH 8.6), blocked for nonspecific binding with skim milk (3% skim milk in 1XPBS), and was incubated in the serum–colloidal gold mixture for 1 hour under continuous agitation at room temperature (RT). After incubation, the discs were removed and washed free of unbound particles using the capillary action of an absorbent pad to draw buffer through the nitrocellulose. The air-dried discs were then examined for the presence of a deep purple spot indicative of the binding of the anti-Ag-65 KDa IgG-colloidal gold complex to the M. tuberculosis Ag-65 KDa blot. A small number of the serum specimens apparently contained components that caused the colloidal gold to bind nonspecifically over the entire surface of a nitrocellulose disc. This diffuse signal precluded a definitive interpretation and thus these specimens were labeled as "non-determinate (ND)" (Figure 2). The entire experiment was performed in triplicate.

***Statistical analysis:*** The sensitivity and specificity of developed Immuno-dot blot assay based test for diagnosis of TB and Non-TB groups were calculated. The positive and negative predicative values were calculated by using different rates of TB prevalence. Comparison between TB and non-TB groups was done by the chi-square test.

## RESULTS:

***Anti-Hsp 65 IgG is highly sensitive for active and latent TB using secondary Ab and colloidal gold conjugates:*** To test the accuracy of anti-Hsp 65 IgG in the diagnosis of active TB, we compared the sera of patients with culture-confirmed active TB to sera from latent and healthy individuals (Table 1). Thirty-five patients with active TB were positive for anti-Hsp 65 IgG (sensitivity of 100%; 95% confidence interval [CI] of 93 to 100%) whereas out of 22 latent TB individuals eighteen individuals exhibited positive response for Hsp 65 (sensitivity of 81.8%; 95% CI of 77 to 92%). In contrast, none of the 47 control sera from healthy individuals were found to be positive for anti-Hsp 65 IgG (specificity of 100%; 95% CI 97 to 100%). Difference was observed in the color intensity of active and latent TB. Therefore, based on these three classes, the positive predictive value of the anti-Hsp 65 IgG test for active TB is 100% (95% CI 97 to 100%) and the negative predictive value is 25% (95% CI 13 to 59%). The high positive predictive value is likely to be due, in part, to the high prevalence of active TB/latent TB in the test population; i.e., 35 of the 104 subjects (33.65%) had active TB, whereas it was 21.15% for latent TB.

***Accuracy and predictive values of Anti-Hsp 65 IgG for a active TB in a high-risk population:*** To determine the positive and negative predictive values of anti-Hsp 65 IgG for active TB in a high-risk population, we compared patients with active TB with those who did not have active disease in the Pulmonary Department of People's Hospital, Bhopal, MP, India. The overall sensitivity of anti-Hsp 65 IgG for active TB was 100% (35 of 35 patients) and the specificity was 74% (51 of 69 subjects) (Table 2). In this population where the prevalence of active disease was 33.65% (35 of 104 had active disease), the positive predictive value was 52% (i.e., of 53 subjects with a positive anti-Hsp 65 IgG, 35 had active

**Table 1**: Presence of anti-Hsp 65 IgG in sera from patients with active TB, latent TB and healthy individuals for secondary Ab and colloidal gold conjugates.

| Classes | No. +ve | No. -ve | No. samples | Sensitivity % (95% CI)* | Specificity % (95% CI)* |
|---|---|---|---|---|---|
| Active TB | 35 | - | 35 | 100 (93-100%) | - |
| Latent TB | 18 | 4 | 22 | 81.8 (77-92%) | - |
| Control | - | 47 | 47 | - | 100 (97-100%) |
| (healthy) | 53 | 51 | 104 | - | - |
| Total | | | | | |

*95% confidence interval

**Table 2**: Positive and negative predictive values of anti-Hsp 65 IgG for secondary Ab and colloidal gold conjugates.

| | Active TB (+)! | (-)* | Total |
|---|---|---|---|
| Anti-*Hsp*-65 IgG | | | |
| (+) | 35 | 18 | 53 |
| (-) | - | 51 | 51 |
| Total | 35 | 69 | 104 |

!active TB patients
*latent and healthy subjects
Accuracy= [true (+) + (true (-)]/all test = [35+51]/122= 70.49

disease). The accuracy of the test, defined as true positive, true negative divided by all test results, was calculated to account for the indeterminate results. Thus, the accuracy of the application of the anti-Hsp 65 IgG test was (35+51)/122=70.49%. Difference was observed in the color intensities of active and latent TB samples.

## DISCUSSION:

High prevalence and resurgence of TB worldwide describes the prevalence of socio-economic condition. It is estimated that India and China together possess about 50% cases of tuberculosis. The increase in infection requires proper approach for diagnostics capable of discriminating active and latent infections. Serological assay is specific and practical applicability is more for active pulmonary cases with overall sensitivity rate of 55%.

We have compared the IgG response to the mycobacterial cell wall protein Hsp 65 in patients with active TB versus uninfected or latently infected subjects. None of the control sera (healthy individuals) tested positive, resulting in a specificity rate of 100% (Table 1). The incidence of the disease in the study population is subjective to the positive and negative predictive values of a diagnostic test (Table 2). Serologic studies conducted previously used purified extracted glycolipids[16], adsorbed mycobacterial sonicates[17] and an antigen mixture from immunoabsorbent affinity chromatography[18], PPD [19], or more specific mycobacterial antigens such as antigen 5, antigen-16, antigen 60, a 30-kD antigen, P32 antigen (derived from PPD), trehalose-6,6'-dimycolate (cord factor), an 88-kD antigen, Kp90 antigen, and LAM[20-24].

High variability have been observed in sensitivities and specificities obtained from these assays. The mycobacterial antigen 5 or 38-kD antigen was evaluated using ELISA showed variable sensitivity (45 to 90%) and high specificity (90 to 100%). Smear-negative TB patients displayed low sensitivity rate for antigen 5 compared to smear-positive cases which were highest, signifying that a higher burden of organisms is due to seropositivity[25].

Another antigen 60 also present in Nocardia and Corynebacterium species is used in the diagnosis of TB with sensitivity rate of 60-89% in adult pulmonary cases and lower in children/ extrapulmonary cases [26, 27]. The specificity of anti-antigen 60 for healthy individuals was 82 to 100%[28]. Efficiency of testing does not increased with combination of antigen 5, antigen 60, and another mycobacterial antigen Kp- 90. Moreover, similar results were obtaines as of individual tests[29,30].

Rajpal et al. (2011) evaluated Hsp 16, 65 and 71and described elevated level of Hsp 65 in both latent and active cases in comparison to control population [21]. IgG antibody response to mycobacterial heat-shock proteins (Hsp) (the 70-kDa antigen from Mycobacterium tuberculosis and M. bovis BCG; the 65-kDa antigen from M. leprae and M. bovis BCG) and to the fibronectin-binding antigen 85 from M. bovis BCG was analyzed in a dot-blot assay. The differences in binding to the Hsp 65 from M. leprae and to antigen 85 could be helpful in distinguishing different forms of the disease[31]. Dubaniewicz et al. (2013) demonstrated opposite presence of mycobacterial heat shock proteins (Mtb-Hsp) 70 kDa, 65 kDa, 16 kDa in sera and lymph nodes in sarcoidosis (SA). Higher occurrence of serum Mtb-Hsp70 than Mtb-Hsp 65 and 16 could be caused by sequestration of Mtb-hsp 65 and Mtb-Hsp 16 in circulating immune

complexes (Cis). And described higher Mtb-Hsp 16 expression than Mtb-Hsp70 and Mtb-Hsp 65 in CIs formation and initiate an autoimmune response in SA related to mycobacteria's stationary-phase[32].

In this study, we have used an immune blot-assay to detect M. tuberculosis Ag 65 complex in sera from TB and non-TB patients. Immuno-blot assay is technically very simple and affordable in underdeveloped and developing countries. In the absence of sophisticated methods, such as molecular methods based on nucleic acid amplification and T cell-based immunological tests, immuno-Dot blot assay has become widely accepted for diagnosing tuberculosis and would potentially decrease the cost of the antibody reagent compared to the ELISA system.

## CONCLUSION:

The present study concludes that anti-Hsp 65 IgG is a sensitive marker of active TB. Using sputum culture as the gold standard, anti-Hsp 65 IgG was positive for active TB who was smear-positive. Furthermore, in individuals with no or with latent infection, defined by negative and positive tuberculin skin tests, respectively, anti-Hsp 65 IgG was quite specific for active disease. The high positive predictive value of anti-Hsp 65 IgG in a population at risk for tuberculous infection makes it a potentially valuable screening test for active TB.

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