

Rapid Diagnosis of Active Tuberculosis by Detecting Antibodies from Lymphocyte Secretions

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In the present study, we investigated the tuberculosis (TB) diagnostic performance of an assay on the basis of detection of TB-specific antibodies from peripheral blood mononuclear cells (PBMCs), to determine whether antibodies in lymphocyte secretions obtained from PBMCs would better reflect active disease than antibodies in serum. PBMCs from patients with and without TB cultured in various concentrations for different times were assessed. Immunoglobulin G (IgG) specific for antigen (bacille Calmette-Guérin [BCG] vaccine and purified protein derivative [PPD]) was measured in lymphocyte secretions. Patients with active TB had higher BCG- or PPD-specific IgG antibody responses than patients without TB or healthy subjects ($P = .001$). This method can be used as a quick diagnostic aid to facilitate rapid detection of TB cases.

Tuberculosis (TB) remains a major global health problem and is the most frequent cause of death from a single infectious agent [1]. The appearance of multi-drug-resistant strains of *Mycobacterium tuberculosis* and the human immunodeficiency virus (HIV)/AIDS epidemic have contributed to the resurgence of active TB in humans. Therefore, the World Health Organization declared TB to be a global emergency in 1993. Surveys carried out in Bangladesh from 1987 to the present suggest the smear-positive TB case rate in Bangladesh to be 1%–1.8% [2–5].

Early diagnosis of TB is crucial to prevent the spread of the disease in the community. However, the clinical and laboratory diagnosis, follow-up of the infection

activity, and response to therapy are not always easy to evaluate [6, 7]. Although culture of bacteria is the reference standard in diagnosis and follow-up of disease, it can take up to 6–8 weeks to isolate *M. tuberculosis*. It is estimated that a false-negative culture result may be obtained for 10%–20% of TB cases [8, 9]. A rapid serological test for diagnosis, follow-up of disease activity, and response to therapy would be useful to clinicians [10, 11]. The purified protein derivative (PPD) skin test (Mantoux test) is an important tool for diagnosis of latent TB infection and disease in the developed world, but it has low predictive value in bacille Calmette-Guérin (BCG)-vaccinated individuals, as well as in individuals living in areas where TB is endemic. The low predictive value is a result of cross-reactivity with BCG and atypical mycobacteria, as well as false-negative reactions in malnourished children [12–14].

BCG has been used as an antigen in EIAs in *in vitro* studies to determine disease activity, but its use was suspended because of difficulties in interpretation, problems differentiating between active or past disease, and low sensitivity and specificity [15–19]. With the identification of regions of the *M. tuberculosis* genome that are missing in BCG and nontuberculous mycobacterium, new antigens have been identified that provide better opportunities for the development of novel

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diagnostic tools [20–22]. The introduction of these antigens has resulted in a much higher sensitivity and specificity in cell response assays [23]. However, serological tests based on mycobacterial antigens to detect circulating antibodies have been hampered by decreased sensitivity and cross-reactivity with other mycobacteria [24–28], or have relatively limited utility in the diagnosis of TB in countries where TB is endemic [29]. Several molecular biological techniques have been proposed as indicators of disease activity in pulmonary and extrapulmonary TB [30, 31] and are currently the most sensitive and specific diagnostic tests. However, a recent study on interlaboratory comparisons of polymerase chain reaction (PCR)-based TB diagnosis have demonstrated the complications of obtaining reproducible results with such sensitive techniques, that false-positive results can be a major problem [32].

The antibodies from lymphocyte secretion (ALS) assay was earlier used to detect specific antibody response after oral vaccination with a killed cholera vaccine in healthy adults without any requirement for in vitro antigen stimulation [33]. In the present study, we sought to evaluate the diagnostic potential of TB-specific ALS responses in Bangladeshi subjects for the assessment of active pulmonary TB.

MATERIALS AND METHODS

Study subjects and sampling. Adult patients with suspected pulmonary TB who had attended the National Institute of Diseases of the Chest and Hospital (IDCH) in Dhaka, Bangladesh, were prospectively studied. The diagnosis of TB was established by the clinical presentation, chest radiograph examination, and sputum smear positivity. Clinical evaluation included lung opacity, pyrexia, weight loss, high erythrocyte sedimentation rate, and positive sputum smear. Sputum samples were collected twice on consecutive days for mycobacterial culture from each patient after enrollment onto the study. Diagnosis was further confirmed when sputum culture was found to be positive.

All patients received standard therapy that included rifampicin, isoniazid, pyrazinamide, and ethambutol. Most of these patients had been ill for 3–5 months before inclusion in the study. Tuberculin skin test was not performed in these patients, because, in Bangladesh, BCG is provided to ~80% of people; exposure to environmental mycobacteria is considered to be widespread, and incidence of TB infection is high, rendering the skin test less specific [5, 34, 35]. To determine whether a patient had been vaccinated for TB, we obtained a medical history from each patient and inspected patients for the scar that typically forms after previous vaccination with *M. bovis* BCG. Although it is possible that some patients may not have developed a scar after vaccination and thus may be falsely grouped as nonvaccinated, most of these patients were able to

tell us whether they had been vaccinated, with the exception of 2 patients who were unsure of their vaccination status and who did not have scars. They were grouped as nonvaccinated.

Patients attending the hospital who presented with symptoms suggestive of TB with lung opacity and high erythrocyte sedimentation rate, but who were sputum smear negative and culture negative, were enrolled as patients without TB. Healthy laboratory personnel with no known exposure to *M. tuberculosis* were selected as healthy control subjects. The tuberculin skin test was performed on these healthy control subjects only. Blood samples were collected from each patient at enrollment; patients had undergone <4 weeks of antimycobacterial drug treatment. In addition, blood samples also were collected from patients without TB and from healthy subjects. The study was approved by the ethical review committee of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B): International Centre for Health and Population Research (Dhaka, Bangladesh). Signed informed consent was obtained from each study subject according to the guidelines of the ethical review committee.

Sputum culture. Sputum specimens from patients were collected at the IDCH and were cultured for *M. tuberculosis* at the ICDDR,B on Lowenstein-Jensen medium by means of standard culture techniques.

Antibodies from lymphocyte secretions. PBMCs were separated from blood on Ficoll-Paque by differential centrifugation and were suspended in 24-well tissue culture plates (Costar) in RPMI 1640 culture medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco), 2 mmol L-glutamine, and 1% amphotericin B–penicillin–streptomycin mix (Sigma Chemical). Different dilutions of PBMCs (1×10^6 , 2×10^6 , 5×10^6 , and 1×10^7 cells/mL) were incubated at 37°C with 5% CO₂. Culture supernatants were collected at 24, 48, 72, and 96 h after incubation. A cocktail of protease inhibitors (0.2 μmol 4-aminoethyl benzenesulfonyl fluoride, 1 μg/mL aprotinin, 10 μmol leupeptin, and 1 mg/mL sodium azide in PBS) were added to the supernatants and were stored at –70°C until used for the assay.

Antigen-specific IgG ALSs. Antigens tested for the method were BCG (freeze-dried, glutamate BCG vaccine for intradermal use; lot 1861, Japan BCG Laboratories; no preservatives were added, and saline was used as a diluent) and PPD (Statum Serum Institut, Tuberculin Department). Antibody (IgG) titers were measured in supernatants by ELISA. Polystyrene microtiter plates (Nunc-Maxisorp) were first coated with BCG vaccine (1 μg/well) or PPD (1 μg/well) in carbonate buffer (0.1-mol sodium bicarbonate and 5-mmol magnesium chloride [pH 9.8]) and incubated overnight at 4°C. After washing, the plates were blocked with 10% FBS in PBS (pH 7.2) and incubated at 37°C for 60 min. Lymphocyte supernatants were thawed and brought to room temperature. After washing with PBS-Tween, lymphocyte supernatants of appropriate dilutions (diluted in 10% FBS in PBS) were added (100 μL/well) and incubated for 2 h at 37°C.

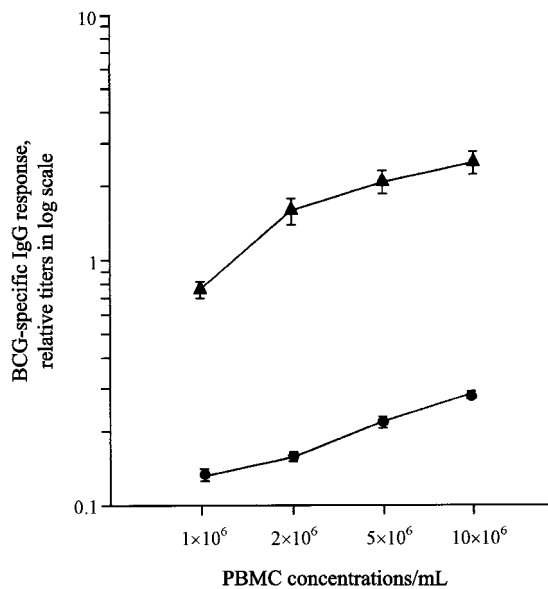


Figure 1. Comparison of bacille Calmette-Guérin (BCG)-specific IgG responses (relative titers) in lymphocyte secretions at various cell concentrations in patients with tuberculosis (▲) and healthy control subjects (■). Each point represents average titers of BCG-specific IgG with standard error of mean (\pm SE). Significantly higher BCG-specific IgG titers were obtained at higher cell concentrations ($2-10 \times 10^6$ cells/mL), compared with that at 1×10^6 cells/mL ($P < .001$). Healthy control subjects had consistently low BCG-specific IgG titers at all cell concentrations.

Plates were washed, and rabbit anti-human IgG horseradish peroxidase conjugate (1:100) in PBS containing 10% FBS was added and incubated for 2 h at room temperature. After washing, freshly prepared substrate (1-mg/mL *O*-phenylenediamine in 0.1-mol sodium citrate [pH 4.5] buffer and H_2O_2) was added, and the plates were developed. The enzyme reaction was stopped with 1.0-mol H_2SO_4 , and optical density was measured after 20 min at 492 nm. Pooled serum samples from *M. tuberculosis* culture-positive patients were used as positive control ($OD > 1.0$). Antigen-specific responses were expressed as relative titers, which were defined as the optical density multiplied by the dilution factor of the specimen [36].

Statistical analysis. Statistical analyses were performed by SigmaStat software (Jandel Scientific). Comparisons between the groups were made by 1-way analysis of variance (ANOVA) or ANOVA on ranks, as appropriate. $P = .05$ was considered to be statistically significant. Receiver-operator characteristic curves were constructed to describe the relation between the sensitivity and specificity at varying cutoff levels of BCG- or PPD-specific IgG titers in ALS.

RESULTS

Demographics of patients. Forty-nine patients with suspected pulmonary TB were recruited from the IDCH. Only

those patients who had 2 consecutive sputum specimens positive for acid-fast bacilli were included in the study. Of 49 patients with smear-positive pulmonary TB, 45 patients (92%) were culture positive for *M. tuberculosis*; 2 had contaminated cultures, and 2 were culture negative. All patients received the standard treatment, and, for therapy-resistant patients, the treatment was modified. Median age of the patients was 30 years (range, 18–57 years). Thirty-six of 49 patients with TB were men and 13 were women. Of these 36 patients, 35 had been vaccinated with BCG (that is, they had a vaccination scar).

Patients with non-TB illness ($n = 35$) included patients with bronchiectasis ($n = 22$), lung cancer ($n = 7$), lung abscess ($n = 4$), and aspergillosis ($n = 2$). The diagnosis was confirmed by histologic or cytologic analysis. Thirty-five healthy individuals (laboratory personnel) were included in the study as healthy control subjects; all but 1 had been BCG vaccinated.

Lymphocyte numbers and supernatant dilution. Culture supernatants from different concentrations of cell suspensions and different incubation time points were used to determine antigen-specific IgG titers. A higher concentration of PBMCs allowed for higher BCG-specific IgG titers to be obtained (figure 1). BCG-specific IgG titers were significantly higher in supernatants of 2×10^6 , 5×10^6 , and 10×10^6 cells, compared with that in 1×10^6 cells ($P < .001$). For $2-10 \times 10^6$ PBMCs/mL, the supernatants need to be diluted 2–4 times. However, with the

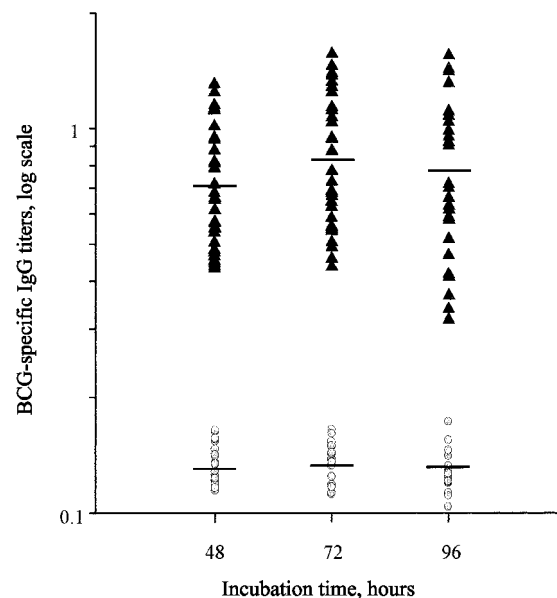


Figure 2. Comparison of bacille Calmette-Guérin (BCG)-specific IgG responses (relative titers) in lymphocyte secretions at various incubation time intervals in patients with tuberculosis (TB) and healthy control subjects. Horizontal bars, geometric mean titers of specific IgG. Patients with TB (▲) had significantly high specific IgG titers at all time points, compared to healthy control subjects (○) ($P < .001$). Healthy control subjects had consistently low BCG-specific IgG titers at all incubation time points.

cell concentration of 1×10^6 PBMCs/mL, undiluted supernatants had to be used. Because the PBMC counts are usually low in moderately to severely ill patients with TB, we opted for 1×10^6 cells/mL suspensions.

BCG- and PPD-specific ALS. A gradual increase in relative titers of BCG-specific antibody was found at 48–72 h, with a slight decline in the titers at 96 h (figure 2). The titers at 24 h were low and were studied in only a few subjects. The optimum time point was found to be 72 h. Patients with pulmonary TB had significantly higher BCG-specific IgG antibody titers than healthy subjects ($P < .001$) and patients without TB ($P < .001$) at all time points (figure 3A). Response to PPD (figure 3B) was similar to that seen with BCG vaccine. There was no significant difference in the BCG-specific antibody titers between patients with BCG vaccination (35 vaccinated; geometric mean of relative titer, 0.67) and patients without BCG vaccination (14 non-vaccinated; geometric mean, 0.75) ($P = .5$).

Cutoff level to define a positive test result. Receiver-operator characteristic curves were constructed from the ALS responses to BCG or PPD, comparing patients with TB with healthy control subjects. The selection of the best cutoff point value was based on the level at which the accuracy was maximum. The best cutoff point was found to be 0.42, with a sensitivity of 92.5% and a specificity of 80% for the BCG-ALS assay (figure 4A). For PPD-specific response, the best cutoff value was 0.32 with a sensitivity of 73% and a specificity of 80% for the ALS assay (figure 4B). The sensitivity and specificity for BCG-ALS assay were higher than those of the PPD-specific ALS assay. The positive predictive value of the assay was 97%.

DISCUSSION

A rapid diagnostic assay that can detect patients with active TB is urgently needed to control and prevent the spread of pulmonary TB. We report a novel technique to rapidly identify such patients by culturing PBMCs and detection of TB-specific ALS. Comparison between bacteriologically confirmed patients with TB and patients without TB (having illness in which TB was part of the differential diagnosis) or healthy control subjects showed a significant difference in the BCG antigen-specific IgG antibody responses in the ALSs. The sensitivity and specificity of the test were ~93% and 80%, respectively, indicating that the combination of the ALS and ELISA assays that used BCG vaccine as an antigen would enable rapid detection of *M. tuberculosis* infection (within 4–5 days) in patients with active TB. Previous vaccination with BCG did not hamper the test for identification of TB; we could successfully differentiate between BCG-vaccinated and *M. tuberculosis*-infected patients. The positive predictive value of the test was 97%. However, it may be noted that the performance characteristic (positive predictive value) of the assay may vary on the basis of the prevalence of TB in the tested population.

Detection of antigen-specific antibody secreting cells have been used for monitoring therapeutic responses in patients with TB [37]. We evaluated the ALS technique, because we hypothesized that active TB would provide continuous antigen stimulation resulting in antibody-producing cells in circulation. In contrast, inactive TB might result in high antibody titers in

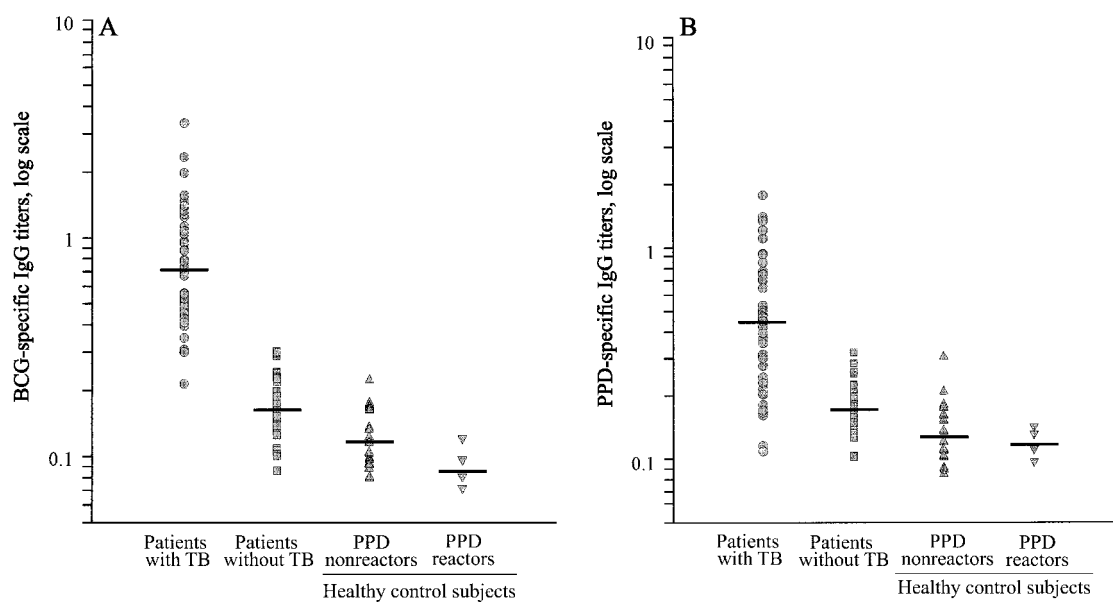


Figure 3. Antibodies from lymphocyte secretion responses to bacille Calmette-Guérin (A) and purified protein derivative (PPD) (B) in patients with tuberculosis (TB) (○) were significantly higher than in patients without TB (■) and healthy control subjects (▲) ($P < .001$). Statistical comparison between groups were performed by analysis of variance (ANOVA) or ANOVA on ranks. Short horizontal lines, geometric mean titers for the groups.

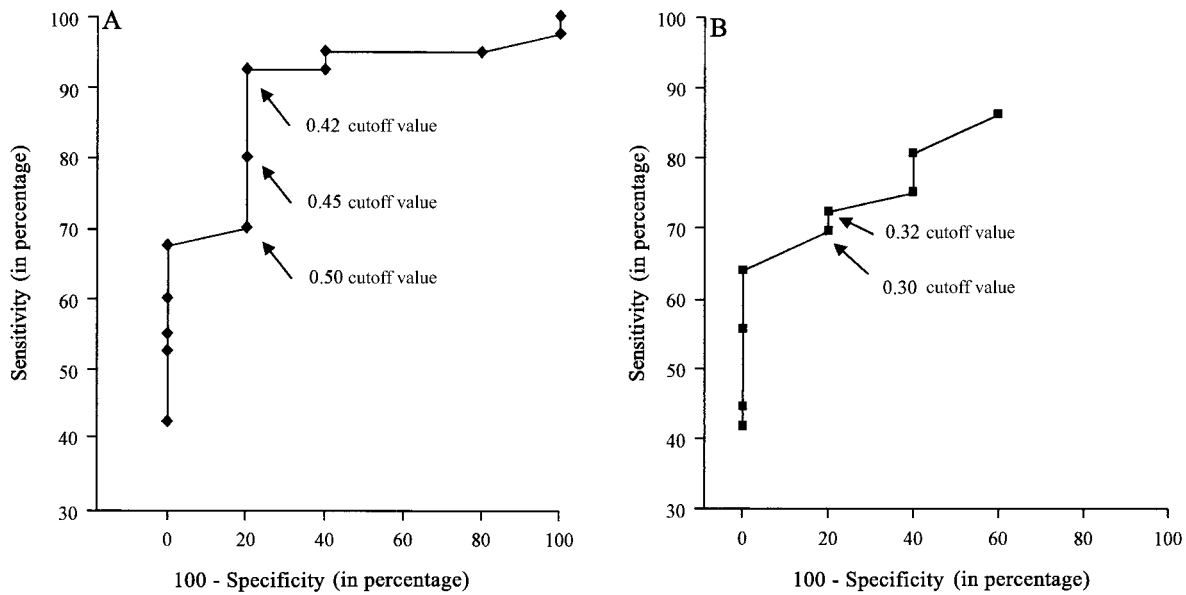


Figure 4. Receiver-operator characteristic curves were constructed from antibodies from lymphocyte secretion responses to bacille Calmette-Guérin (A) and purified protein derivative (B) in 49 patients with tuberculosis and in 35 healthy control subjects. Arrows, some potential cutoff levels.

serum, but would be less likely to stimulate antibody-producing cells in circulation. In addition, it is easier to perform an ALS assay, and the supernatant also can be stored for future use to detect antigen-specific antibodies, novel antigens, cytokines, and other mediators. BCG vaccine and PPD were chosen as antigens for their easy availability and for the assessment of a broad spectrum of TB-specific antibodies, because they cover a vast array of protein and lipid antigens. ALS response to both BCG and PPD were similar; however, the sensitivity and specificity of the BCG-specific ALS response were higher.

Our results strongly demonstrate the need for larger prospective studies in the community to establish the actual predictive value of ALS response to BCG and/or PPD in patients suspected to have TB. Our ongoing follow-up study of family contacts indicates that increased ALS responses to BCG or PPD are associated with an increased risk of developing active TB (authors' unpublished data).

Various purified protein antigens have been tested for diagnostic applications—for example, ESAT-6, a small-molecular-weight peptide expressed by *M. tuberculosis*, *M. bovis*, and *M. africanum* and absent from all strains of *M. bovis* and most of environmental mycobacteria. Recent studies have found ESAT-6 to be a highly promising antigen for immunodiagnosis of active *M. tuberculosis* infection in regions where TB is not endemic [38–41]. However, in regions where TB is endemic, such as The Gambia, India, and Bangladesh, contacts of patients with TB had significantly higher ESAT-6–specific response than patients with TB [29, 42] (R.R., personal observation), thereby limiting the use of the method to countries where TB is not endemic. There is a long-term persistence of ESAT-6–specific

antibodies in patients in remission from pulmonary TB in areas where TB is endemic, making it difficult to discriminate between latent TB or remission from TB [43].

The ALS method is an immunological assay in which antibodies are secreted by B lymphocytes or plasma cells in the supernatant, and the antibody levels are measured by ELISA. We did not use ELISA in patients with TB coinfecting with HIV and/or AIDS or in children; therefore, we cannot predict how the test will perform in these groups. Moreover, in Bangladesh, the prevalence of HIV infection is very low (<1%) in people with high-risk behavior [44, 45], and coinfection of TB with HIV is even lower (<0.2%) (T. Azim, personal communication).

In conclusion, the use of the ALS specimens with the standard ELISA technique holds potential as a future TB-specific diagnostic test. With the extensive availability of ELISA technology in developing countries, this method may be applicable both in developing countries where TB is endemic, as well as in industrialized countries, for screening of patients suspected to have TB. Because this method does not require a specimen taken from the site of disease, it also may be useful in diagnosis of paucibacillary childhood TB. Large-scale evaluations are needed to determine the true usefulness of the ALS assay in the diagnosis of TB in countries where TB is endemic and in various clinical groups.

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