Serologic Diagnosis of Tuberculosis Using a Simple Commercial Multiantigen Assay*

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Setting: Seven primary health clinics and a pulmonary disease specialty clinic in Rio de Janeiro City, Brazil.

Objective: To evaluate a commercial immunochromatographic test kit (ICT Tuberculosis; AMRAD-ICT; Sidney, Australia) employing five recombinant Mycobacterium tuberculosis proteins for the detection of pulmonary tuberculosis (TB).

Design: Serology test results were compared with duplicate sputum microscopy and culture in 277 patients with symptomatic pulmonary disease (243 with pulmonary TB and 34 with nontuberculous disease). An additional 110 healthy control subjects were also tested.

Results: The serology test was simple and rapid to perform and detected 64.2% of smear-positive and 46.3% of smear-negative TB patients overall. HIV co-infection was present in 15.3% of TB patients, and serology was much less sensitive (overall 27.6%) in this small group, as was microscopy (13.8%). Specificity of the serology test was 100% in healthy control subjects and 85.2% in the small number of control patients with pulmonary disease, including those with prior TB. Combined with microscopy, serology detected 72.8% of TB patients.

Conclusion: Depending on the population studied, multiantigen serologic tests for TB may be as sensitive as microscopy, but detect a different and overlapping subset of patients. The use of multiple antigens in this kit increased test sensitivity without significant loss of specificity. Bacille Calmette-Guérin vaccination and tuberculin sensitivity did not affect serology results. Estimating specificity in clinical use will require testing a much larger cohort of symptomatic patients with nontuberculous disease. The TB diagnostic performance of this group of antigens in HIV co-infected individuals was poor. (CHEST 2003; 123:107–112)

Key words: developing countries; diagnosis; serology; tuberculosis

Abbreviations: AFB = acid-fast bacilli; BCG = bacille Calmette-Guérin; CI = confidence interval; PPD = purified protein derivative; TB = tuberculosis

The treatment of tuberculosis (TB) is a highly cost-effective medical intervention. Selection of patients for treatment, however, remains complicated by the lack of specificity of clinical findings and the poor performance characteristics of diagnostic methods available in the majority of developing-world laboratories. Many attempts have been made to develop a serologic TB test, challenges to which include the need to discriminate active from latent infection, to avoid cross-reactivity to bacille Calmette-Guérin (BCG) or nontuberculous mycobacteria, and to perform consistently in genetically and immunologically diverse populations.

Early studies using partially purified antigens showed that antimycobacterial antibody was readily detected in tuberculosis patients, but test specificity was poor.1,2 Monoclonal antibodies and highly purified native or recombinant antigens have improved specificity, but at a cost of decreased sensitivity. Recently, the degree to which the humoral response to TB is heterotypic has received greater recognition, stimulating the development of serology tests using multiple antigens.3

Though sensitivity, specificity, and cost are the
characteristics most frequently considered in TB diagnostic tests, robustness and speed are also critical to field effectiveness. Delays of even 1 to 2 days result in loss of patients to follow-up, increased cost from return visits, and diminished physician control and test confidence.4,5 A diagnostic tool for TB that could be performed rapidly in the field would be of great benefit to tuberculosis control programs, especially in developing countries, even if test performance were less than ideal. In this report, we evaluate a rapid immunochromatographic test kit to detect antibody directed against any one of five recombinant antigens in TB patients in Rio de Janeiro City, Brazil. Rio de Janeiro, the fourth-largest city in Latin America, is an evolving mega-city with an expanding epidemic of TB. There were 10,210 cases of TB registered in the city in 1997, and a incidence rate based on passive case finding of 120 per 100,000 population citywide.6

### Materials and Methods

#### Patient Enrollment

Sera were collected between July 1996 and December 1996 from three populations of patients not receiving antituberculous therapy: (1) patients with newly diagnosed TB at seven Rio de Janeiro City primary health clinics; (2) patients with pulmonary disease of indeterminate cause presenting for evaluation by induced sputum and/or BAL at Clementino Fraga Filho Hospital, Federal University of Rio de Janeiro; and (3) healthy subjects, including asymptomatic household TB contacts and purified protein derivative (PPD)-positive and PPD-negative health-care workers at the study site. The control group was thus composed of both asymptomatic volunteers and patients with pulmonary disease in whom Mycobacterium tuberculosis was not detected on mycobacterial culture or microscopic examination of Ziehl-Neelsen–stained sputum smear. BCG vaccination history was not recorded, but the age of the patients and the history of universal BCG vaccination of infants in Brazil predict that between 50% and 75% would have received the vaccine.

#### Exclusions

Symptomatic patients for whom no smear or culture data were available or for whom relevant clinical information was lacking were excluded from analysis. A small number of samples that did not yield test results for technical reasons were also excluded.

#### Clinical and Laboratory Evaluation

All case patients were interviewed and underwent physical and laboratory evaluation including a chest radiograph. Two sputum samples from each patient were submitted for microscopic examination for acid-fast bacilli (AFB) and culture on Löwenstein-Jensen media following standard alkaline decontamination. Smear and culture results were considered positive if either of the two duplicate samples were positive. HIV testing was performed whenever possible. Asymptomatic control patients underwent no laboratory evaluation other than tuberculin skin testing.

#### Serology

From case patients, serum for testing was drawn at the time of diagnosis in all but one patient, who was tested 6 months after initial presentation. Testing was done using a commercial kit (ICT Tuberculosis; AMRAD-ICT; Sydney, Australia) consisting of a folding cardboard device containing a nitrocellulose strip onto which the 38-kd antigen and four other recombinant antigens were fixed in discrete lines. The construction of the device and its use has been described elsewhere in detail.7 Briefly, 30 μL of serum was placed at one end of the nitrocellulose strip and allowed to migrate across four bands containing the five fixed antigens. After the serum had reached a marked limit line, migration was stopped with buffer, and captured antibody detected using an immunogold goat anti-human IgG conjugate. Testing required 5 to 15 min, and ease of use was such that multiple tests could be run concurrently. Interpretation was performed as suggested by the manufacturer, and a visible line at any of the four antigen-binding areas was considered positive. Each positive band was scored for location (A through D) and intensity of staining (1+ to 4+). A single investigator read all strips. Serum was frozen, not fresh, and went through at least one freeze-thaw cycle prior to use in this test.

#### Results

Adequate clinical and laboratory information was available to evaluate 393 of the 494 patients from whom serum was collected. In six subjects (1.5%), kits did not yield readable results, either because of operator error or because of viscous serum that failed to migrate on the nitrocellulose strip. The remaining 387 tests formed the basis for our evaluation.

Patients were classified into four groups for analysis, depending on the results of clinical and laboratory findings: (1) patients with smear-positive TB, (2) patients with smear-negative TB with or without positive mycobacterial culture, (3) patients with symptomatic pulmonary disease and no evidence of TB, and (4) healthy individuals who were hospital workers or household contacts of pulmonary TB patients.

Case patients had a mean age of 36.0 years, whereas healthy control subjects tended to be younger (29.8 years) and patients with non-TB pulmonary disease older (55.4 years). The proportion of men (n = 158) and women (n = 85) among the case patients reflected that routinely reported by the Brazilian National Tuberculosis Program, HIV test results were available from 189 of 243 case patients, of which 29 were positive (15.3%), and from 28 of the 34 control subjects with nontuberculous pulmonary disease, of which none were positive. HIV testing was not performed on healthy control subjects.

The results of the ICT Tuberculosis test kit are shown in Table 1. Overall, 55.1% of TB patients were test positive with detectable antibodies to one
or more of the five recombinant *Mycobacterium tuberculosis* antigens in the test. Smear-positive TB patients were more frequently test positive (64.2%, $p = 0.005$), but nearly half of the smear-negative patients (46.3%) were also detected by the kit, including 50% of the 38 smear-negative, culture-negative patients with diagnoses made on clinical and radiographic grounds. The duration of symptoms ranged from 1 to 58 weeks (average 10.4 weeks) in 49 of 243 TB patients for whom firm dates were available. Duration of symptoms in patients with positive serology results was not significantly longer than in those with negative results (11.5 weeks vs 8.4 weeks, $p = 0.28$).

Band A in the kit used for this study contained the 38 kd antigen. The identity of the other four antigens is proprietary. As shown in Table 1, the most commonly detected antibody in case patients was that directed against the 38-kd antigen, but antibody to the other test antigens was present in roughly 10 to 30% of patients. The addition of antigens beyond the 38-kd antigen increased sensitivity of the assay 51% in smear-positive patients and 63% in smear-negative patients, without markedly diminishing specificity. There were an insufficient number of false-positive test results to evaluate the impact of multiple antigens on specificity.

The serology test result was positive in 5 of the 144 sera from control patients, all of whom were from the subset with symptomatic pulmonary disease. Three of these patients had a history of prior TB. Two additional symptomatic control patients with no history of prior TB were also seropositive using the test kit. One was a patient with bilateral interstitial lung disease, and the other was a patient with COPD and lung cancer of unknown type who died within a few months of presentation. No autopsies were performed. The test kit results from all five of these sera classified as false-positive showed either 3+ or stronger scoring on a single band or had multiple positive bands. Of 113 control subjects who had skin tests performed, 23 subjects (20.3%) had positive tuberculin reactions, none of whom had positive serology test results.

The results of smear, culture, and serology tests for tuberculosis case patients, divided by HIV-infection status, are shown on Table 2. All patients submitted two or more sputum specimens for examination; AFB microscopy and culture were each considered positive if any one of the respective examinations were positive. Laboratory results from 54 TB patients for whom HIV results were unavailable were not statistically different from those from HIV-negative TB patients ($p > 0.5$ for all tests), and these results were pooled for analysis into a single HIV-uninfected group. Among the 214 HIV-uninfected TB patients, microscopy was positive in 54.2%, culture in 79.0%, and serology in 58.4%.

### Table 1—TB Test Result in Each Study Group and Antibody Detection by Test Antigen Line

<table>
<thead>
<tr>
<th>Patient Group Tested</th>
<th>Positive Test Result to Any Antigen, No. (%; 95% CI)</th>
<th>% Positive by Individual Antigen Line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Line A</td>
</tr>
<tr>
<td>All TB patients (n = 243)</td>
<td>134 (55.1; 48.8–61.4)</td>
<td>42.5</td>
</tr>
<tr>
<td>Smear-positive TB (n = 120)</td>
<td>77 (64.2%; 55.6–72.8)</td>
<td>25.5</td>
</tr>
<tr>
<td>Smear-negative TB (n = 123)</td>
<td>57 (46.3%; 37.5–55.1)</td>
<td>5 (3.5; 0.3–6.5)</td>
</tr>
<tr>
<td>All non-TB patients (n = 144)</td>
<td>5 (14.8; 2.9–26.7)</td>
<td>5.9</td>
</tr>
<tr>
<td>Non-TB pulmonary disease (n = 34)†</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Healthy control subjects (n = 110)‡</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*For test-positive proportion in smear-positive and smear-negative patients ($p = 0.005$).
†Including prior TB.
‡Including PPD-positive and PPD-negative subjects.

### Table 2—Results of Duplicate Microscopy (AFB) and Culture and of TB Kit in All TB Patients, Divided by HIV-Infection Status

<table>
<thead>
<tr>
<th>HIV Status</th>
<th>TB. No.</th>
<th>Positive AFB†</th>
<th>Positive Culture†</th>
<th>Positive Serology</th>
<th>Positive AFB or Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>29</td>
<td>13.8 (12.6–26.4)</td>
<td>69.0 (52.2–85.8)</td>
<td>27.6 (11.3–43.9)</td>
<td>37.9 (20.2–55.6)</td>
</tr>
<tr>
<td>Uninfected</td>
<td>214</td>
<td>54.2 (47.5–60.9)</td>
<td>79.0 (73.5–84.5)</td>
<td>58.4 (51.8–65.0)</td>
<td>77.1 (71.5–82.7)</td>
</tr>
<tr>
<td>Overall</td>
<td>243</td>
<td>49.4 (43.1–55.7)</td>
<td>77.8 (72.6–83.0)</td>
<td>55.1 (48.8–61.4)</td>
<td>72.4 (66.8–78.0)</td>
</tr>
</tbody>
</table>

*Values shown as percentage of test-positive patients (95% CIs).
†Smear and culture considered positive if either of the two samples were positive.
‡Includes 54 patients without HIV test result. Microbiology and serology results from untested group was not statistically different from those of uninfected (p > 0.5 for all tests).
Among the 29 HIV-infected TB patients, microscopy was positive in 13.8%, culture in 69.0%, and serology in 27.6%, but confidence intervals (CIs) were wide. CD4 counts were not available on enough of these HIV-infected patients to evaluate any association between the degree of immunosuppression and the likelihood of false-negative serology results. Combining acid-fast sputum smear microscopy with serology yielded sensitivity of 77.1% in HIV-uninfected TB patients, which was equivalent to culture (p = 0.80). Combined sensitivity of microscopy plus serology was 37.9% in HIV-coinfected patients and 72.4% overall.

**DISCUSSION**

The need for improved TB diagnostics has long been recognized. Local diagnostic needs may vary markedly, and it is unlikely that a single diagnostic test be ideal for all situations. However, an accurate, simple, rapid, point-of-care assay would be widely useful and could be expected to improve case holding, empower health-care workers at the peripheral level, and decrease diagnostic confusion and delay.

Recognizing these needs, serologic approaches to TB diagnosis have been tried on and off since the seroagglutination work of Arloing in 1898. Early TB serology tests used PPD or other crude antigens and showed relatively poor specificity, as many of the dominant antibody responses are to shared antigens.

The availability of monoclonal antibodies, recombinant antigens, and methods for the accurate application of controlled amounts of immunologic reagents to nitrocellulose or other solid matrix have enhanced the specificity and reproducibility of such tests. The best studied *M. tuberculosis* diagnostic antigen is the 38-kd antigen, and its availability as a recombinant expressed in *Escherichia coli* led to the development of tests that could easily distinguish between healthy PPD-positive controls and tuberculous patients.

Development of immunochromatographic strips has put these tests into a form appropriate for disease endemic countries, though the change in format from enzyme-linked immunosorbent assay has sometimes been accompanied by a loss of favorable operating characteristics. A number of simple serologic assays using highly purified or recombinant proteins are now being marketed in the developing world, many based on detection of antibody to 38 kd, but limited data exist to support their use.

Variations in specific antibody responses to mycobacterial antigens in different human populations may be linked to human leukocyte antigen-DR phenotype, and tests designed to detect responses to a single antigen could show important geographic variability and limited sensitivity. To overcome these problems, multiantigen tests have been developed. Loss of specificity is additive, however, so each of the antigens used must be very highly specific.

The test kit used in this study employed five different protein antigens to detect an IgG response to *M. tuberculosis*. Each individual antigen showing a specificity of at least 98% (differences not statistically significant), resulting in an overall specificity of 96.5% in the study group. However, this result is relatively arbitrary, as antibody was detected in none of the healthy control subjects, and simply increasing their number would increase the reported specificity of the test. Of the 34 patients with nontuberculous pulmonary disease included here, 5 patients (14.7%) had a positive serology test result, but because of the small size of this group, CIs were wide (2.9 to 26.7). Symptomatic, culture-negative patients in endemic areas may often have positive results of nucleic acid amplification assays or serology tests for TB, as was found in studies of a previous version of the ICT Tuberculosis kit. Determining the true specificity of nonculture-based tests in symptomatic patients will require evaluation of patients from nonendemic sites and prolonged and careful follow-up of study patients in disease endemic countries.

The performance characteristics of diagnostic tests for TB vary widely with the population studied. The poor performance of microscopy in HIV-coinfected patients in this study, for example, was due in part to an enrollment bias with the purposeful inclusion of smear-negative HIV-infected patients with pulmonary disease undiagnosed at initial evaluation. Differences in patient populations studied may also account for the higher sensitivity found in previous trials of a serology test using the 38-kd antigen alone in a similar format to that employed here. Two studies of a single antigen ICT Tuberculosis kit carried out in China demonstrated 70 to 90% sensitivity in smear-negative and smear-positive patients, respectively. Greater duration or severity of illness, which have been correlated with likelihood of positive serologic test for TB, may be one explanation. In the current study, duration of illness did not correlate with test results in the group from which such data were available. Comparison with other published studies of the ICT Tuberculosis test is confounded by limitations in the available data. For example, a study in California performed with the same multiantigen kit used in the present trial found only 20% sensitivity in a small number (n = 59) of culture-positive TB cases. The lack of supporting
clinical and microbiologic data, including an absence of smear microscopy results, make that study difficult to interpret. There were a number of limitations to the current study. The first is that the serology testing was done retrospectively, using stored frozen sera that had passed through at least one freeze-thaw cycle. The use of fresh serum may increase sensitivity. A second limitation to the study is the lack of inclusion of patients with known nontuberculous mycobacterial infections, as cross-reactive antibodies to antigens from other mycobacterial species could result in loss of specificity as mentioned by others. Thirdly, patients from a single geographic region were included, limiting the certainty that these data apply to other regions. Lastly, the study enrolled a limited number of HIV-coinfected patients and patients with other comorbidities, decreasing its resemblance to most field-use conditions. The most pertinent group, persons with nontuberculous pulmonary disease, represented a minority of our study subjects, though among individuals seeking care for respiratory symptoms in primary care clinics they generally greatly outnumber TB patients.

CONCLUSION

The principal findings of the current study are that serologic testing for TB with a simple multiantigen format is feasible in field settings with minimal technical requirements for the user. The use of multiple antigens allowed detection of a significant number of additional patients over those detected by the 38-kd band alone without a loss of specificity, but did not result in an overall sensitivity markedly greater than that reported in previous studies. In HIV-uninfected patients, combined use of serology and sputum microscopy was as sensitive as culture, thus representing an opportunity to greatly shorten the time to diagnosis in a substantial subset of patients. As has been found in other serologic studies, test performance in HIV co-infected patients in this study was poor, albeit superior to smear microscopy. Specificity was very high in healthy individuals and was not compromised by tuberculin sensitivity or childhood BCG vaccination. The positive predictive value in true use situations, however, was not calculable due to the artificial composition of the study population. Larger trials with more representative populations are needed before the appropriate role of TB serology can be ascribed.

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