Evaluation of a Commercially Available Serologic Assay for Antibodies against Tuberculosis-associated Glycolipid Antigen

Yoshitsugu Linuma1-4*, Kazuyoshi Senda2, Shunji Takakura2, Satoshi Ichiyama2, Masao Tano3, Tomoji Abe3, Tomoko Yamamoto4, Kazumitsu Nakashima4, Hisashi Baba5, Yoshinori Hasegawa5 and Kaoru Shimokata5

1Department of Clinical Laboratory, Nagoya University Hospital, Nagoya, Japan
2Department of Clinical Laboratory, Kyoto University Hospital, Kyoto, Japan
3Department of Respiratory Disease, Higashi-Nagoya National Hospital, Nagoya, Japan
4Department of Respiratory Disease, Chubu National Hospital, Obu, Japan
5Department of Respiratory Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan

A commercially available enzyme immunoassay developed to detect antibodies to a tuberculosis-associated glycolipid antigen was evaluated for serologic diagnosis of tuberculosis. This was a multicenter study comparing the assay with other methods in 78 patients with active pulmonary tuberculosis and in 54 controls with non-tuberculous lung diseases. Sensitivities were highest for sputum culture (91.0%), followed by immunoassay (79.5%), nucleic acid amplification (77.3%), and finally acid-fast staining of sputum smear (60.3%). Immunoassay and amplification, both rapid methods, had similarly high sensitivity in smear-positive subjects (89.4 and 88.9%, respectively); in smear-negative subjects these two techniques showed low sensitivity (64.5 and 60.0%, respectively). Concordance between the two methods was relatively low (72.0%). With regard to specificity, seven out of ten patients with old tuberculosis had positive result by immunoassay (30% specificity). In the control group, 10 out of 54 patients had positive immunoassay result (72.2% specificity), with notably limited specificity in the elderly. The tuberculous glycolipid assay is a rapid method sufficiently sensitive for detection of tuberculosis infection, even in smear-negative patients. Clin Chem Lab Med 2002; 40(8):832–836

Key words: Tuberculous glycolipid; Tuberculosis; Serology; Nucleic acid amplification.

Abbreviations: AFB, acid-fast bacilli; COPD, chronic obstructive pulmonary disease; EIA, enzyme immunoassay; ImCRAC, immuno-cross-reactive antigenic compound; LAM, lipoarabinomannan; NAA, nucleic acid amplification; NTM, non-tuberculous mycobacteria; TBGL, tuberculous glycolipid; TDM, trehalose 6',6'-dimycolate.

Introduction

Rapid and accurate diagnosis of tuberculosis is essential for preventing spread of the disease. The most simple and rapid method is sputum smear examination for acid-fast bacilli (AFB) by microscopy, but about one-half of patients with pulmonary tuberculosis are smear-negative. Culture of sputum is more sensitive, but can require up to 4 weeks in liquid culture and 8 weeks in solid culture. Molecular methods for the diagnosis of tuberculosis based on nucleic acid amplification (NAA) are rapid, highly specific, and more sensitive than microscopic sputum smear examination but are less sensitive than culture in smear-negative cases (1). NAA is also expensive, technically complex, and requires meticulous quality control. Serological detection of antibodies to Mycobacterium tuberculosis (M. tuberculosis) could represent a simple, rapid and inexpensive diagnostic method, and various antigens have been investigated in this context (2, 3). In recent years some of these antigens have been used in commercial kits. Antigen 60 (4, 5), 38 kDa antigen (6, 7), mycobacterial Kp90 immuno-cross-reactive antigenic compound (ImCRAC) (8, 9), or lipoarabinomannan (LAM) antigen (10–14) have been used as the main antibody-detection antigen in many kits. However, sensitivities and specificities have been highly variable, ranging from 20 to 93% and from 62 to 100%, respectively. Variability has been reported even using the same kit, which may be attributable to differences between the studied patient populations. Nonetheless, consistent performance is crucial for a clinical diagnostic kit.

Tuberculous glycolipid (TBGL) antigens including trehalose 6',6'-dimycolate (TDM) and more hydrophilic glycolipids purified from the H37Rv strain of M. tuberculosis have been used recently in a rapid enzyme immunoassay (EIA) kit for serodiagnosis of tuberculosis (Determiner TBGL antibodies, Kyowa Medex, Tokyo, Japan). This kit has been reported to have relatively high sensitivity (81.1%) and excellent specificity (95.7%) (15, 16). We conducted a multicenter study to evaluate the clinical utility and the stability of this kit in diagnosing active tuberculosis.

Materials and Methods

Patients and serum specimens

A total of 78 patients with pulmonary tuberculosis (mean age, 60.5 years; range, 18 to 94) were studied at the Department of
Respiratory Diseases of Chubu National Hospital, the Department of Respiratory Diseases of Higashi-Nagoya National Hospital, the Department of Molecular Medicine and Clinical Science of Nagoya University Hospital, and at the Department of Clinical Laboratory of Kyoto University Hospital. No patient was co-infected with human immunodeficiency virus (HIV). Diagnosis of tuberculosis was based on the results of sputum AFB smear and culture, radiographic findings, clinical symptoms and response to treatment.

Serum specimens from patients with active tuberculosis were obtained upon admission before chemotherapy and stored at $-20^\circ$C until anti-TBGL antibody assay. Sputum specimens for examination by smear and culture were obtained on 3 consecutive days immediately following admission. Sputum smearing was processed for auramine-rhodamine staining, and culture for mycobacteria was carried out with a SeptiChek AFB system (Becton Dickinson, Cockeysville, MD, USA). NAA was performed with the Amplicor PCR assay (Roche Diagnostics, Tokyo, Japan) on any of these three sputum specimens according to the manufacturer's instructions.

Control groups

Because a useful anti-TBGL antibody assay must be able to differentiate patients with active tuberculosis from patients with old tuberculosis or non-tuberculous respiratory disease, we chose 10 patients with old pulmonary tuberculosis (mean age, 69.3 years; range, 34 to 84) and 54 patients with non-tuberculous respiratory disease (mean age, 64.6 years; range, 18 to 89) to investigate specificity of the kit. The patients with old tuberculosis had not received anti-tuberculous chemotherapy for at least 3 years prior to entering the study, and showed stable sclerotic lesions in chest radiographs and no detectable tubercle bacilli in sputum specimens. The non-tuberculous respiratory disease group included 19 patients with neoplasms (17 with lung cancer and 2 with mediastinal tumors), 16 with non-tuberculous lung infections (11 with bacterial pneumonia, 3 with pyothorax and 2 with lung abscess), 18 with chronic obstructive lung disease, 5 with bronchial asthma, 3 with pneumothorax, and 3 with other lung diseases (each with interstitial pneumonia, silicosis, and congestion). All control subjects were HIV-seronegative.

NAA procedure

The NAA assay was performed according to the manufacturer's instructions (17). Briefly, samples were treated with N-acetyl-L-cysteine (NALC)-NaOH, lysed, and neutralized. Polymerase chain reaction (PCR) was performed with 50 µl of the mixture using a GeneAmp PCR System 9600R thermal cycler (Roche Diagnostics, Tokyo, Japan). After amplification, the mycobacterial DNA was detected by an enzyme immunoassay. Absorbances were measured with a Nova Favapath Microplate Reader (Bio-Rad, Richmond, CA, USA) at a wavelength of 450 nm. Specimens with an absorbance value of at least 0.35 were considered positive. Positive and negative amplification controls were included in each run.

TBGL assay

TBGL assay was performed according to the manufacturer's instructions as described previously (15). Briefly, 96-well polystyrene microtiter plates (Polysorp immunoplates; Nunc, Roskilde, Denmark) were coated with TBGL containing TDM and minor glycolipids from the cell walls of the M. tuberculosis strain H37Rv by placing 25 µl of the antigen solution (10 µg/ml in n-hexane) in each well and allowing the plate to dry at room temperature. All samples, sera and calibration samples were diluted 1:41 with dilution buffer, and 100 µl of the diluted samples was added to each well. Plates were then incubated for 60 min at room temperature. After the plates were washed five times with buffer, horseradish peroxidase-conjugated rabbit Fab’ raised against human immunoglobulin (Ig)G was added to each well. Plates were incubated for 60 min at room temperature and were washed three times with washing buffer. Then, 100 µl of 3,3',5,5'-tetramethylbenzidine (TMBZ) solution was added to each well and the plates were incubated for 15 min at room temperature. At that time, 100 µl of 1 M H$_2$SO$_4$ was added to stop the enzymatic reaction. Absorbance at 450 nm was measured with an MTP-120 plate reader (Corona Electric, Tokyo, Japan). Samples with at least 2.0 U of antibody per milliliter were considered positive.

Statistical analysis

Differences in performance between the various laboratory tests for $M. tuberculosis$ infection were analyzed by the chi-squared test.

Results

Positivity rate of AFB smear, sputum culture, NAA and anti-TBGL antibody titers in patients with active tuberculosis

Table 1 shows positivity rates in active tuberculosis for the AFB smear, sputum culture, NAA with Amplicor PCR and anti-TBGL antibody titers. Sensitivity was highest for culture (91.0%), followed by TBGL assay and NAA (79.5 and 77.3%), and finally the AFB smear (60.3%). When two rapid and sensitive methods, TBGL assay and NAA, were compared in active tuberculosis (Table 2), the sensitivity of TBGL assay was nearly the same as that of NAA, both in smear-positive and -negative individuals. Both methods showed high sensitivity in smear-positive individuals but were significantly less sensitive in smear-negative subjects. NAA, but not TBGL assay, showed significantly higher sensitivity in culture-positive than culture-negative subjects. Forty-eight individuals had positive results for both tests, and six had negative results for both. A discrepancy was noted between these tests in 21 individuals. Accordingly, TBGL assay and NAA results were 72.0% concordant.

Table 1  Positivity rate of AFB smear, culture, NAA, and TBGL assay in active tuberculosis.

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of subjects</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Positive</td>
</tr>
<tr>
<td>AFB smear</td>
<td>78</td>
<td>47</td>
</tr>
<tr>
<td>Culture</td>
<td>78</td>
<td>71</td>
</tr>
<tr>
<td>NAA</td>
<td>75</td>
<td>58</td>
</tr>
<tr>
<td>TBGL assay</td>
<td>78</td>
<td>62</td>
</tr>
</tbody>
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NAA, nucleic acid amplification by Amplicor PCR. In the TBGL assay, the cut-off value was set at 2.0 U/ml.
In the group with old tuberculosis, the positivity rate was 70%, which corresponds to 30% specificity (Table 3). Statistical analysis revealed a significant difference (89.5 vs. 62.9%; p=0.037, by chi-squared test) between patients with a neoplasm and those with another respiratory disease. As for age, this test showed higher sensitivity in younger patients (age 59 years or less) than older patients (age 60 years or more), but the difference fell short of significance (88.2 vs. 64.9%; p=0.075, by chi-squared test).

### Discussion

Rapid detection of *M. tuberculosis* in specimens from individuals suspected to be infected has been a mainstay of diagnosis of tuberculosis. While speed, simplicity and low cost of these methods make them suitable screening tests, sensitivity and specificity have varied. Pottumarthy *et al.* (18) reported performance of seven tests for serologic diagnosis of tuberculosis. The Tuberculosis IgA EIA (Kreatech Diagnostics, Amsterdam, The Netherlands), an EIA kit that detects IgA antibody to mycobacterial Kp90 ImCRAC, had the highest sensitivity (57%) in 36 active tuberculosis patients (12/36 AFB smear-positive) but showed a very poor specificity of 62% in negative controls. These authors concluded that the best performing kit was ICT Tuberculosis (ICT Diagnostics, Balgowlah, New South Wales, Australia), an immunochromatographic assay using five highly purified antigens, including 38 kDa antigen. Respective sensitivities were 41% and 55% in active tuberculosis, while specificities were 96% and 89%. Chiang *et al.* (19) compared three specific mycobacter-
ial antigens in the diagnosis of tuberculosis corresponding to three EIA kits. These included Anda TB (Anda Biologicals, Strasbourg, France), which measures IgG antibody to antigen 60; Pathozyme-TB Complex (Omega Diagnostics), which measures IgG antibody to body to 38 kDa antigen; and Tuberculosis IgA EIA (Kreatech Diagnostics). By a receiver operating characteristic (ROC) analysis, sensitivities of these kits were 80.77, 64.12, and 62.58% in 312 active tuberculosis patients (47.4% AFB smear-positive), while specificities were 88.40, 80.74, and 66.30%, respectively.

Serological detection of antibodies against LAM, including a commercially available dot-blot assay kit (Mycodot; Genelabs, Geneva, Switzerland) has been evaluated extensively (10–14). A wide range of sensitivity of anti-LAM IgG was seen among HIV-negative patients with active tuberculosis, ranging from 21.5 to 93%; sensitivity was generally lower in HIV-positive patients. In contrast, specificity was excellent, ranging from 84 to 100%. In a Japanese study of the MycoDot test, sensitivity and specificity were 79 and 96%, respectively (14).

Improvement of serodiagnosis for tuberculosis requires advances enhancing reliability of the results. Heterogeneity of antibody responses to protein antigens of *M. tuberculosis* was reported (20). In this report, heterogeneity between subjects of antigen recognition in general, rather than recognition of particular antigens, appears to characterize the antibody response. When a cell wall component, LAM, has been investigated as a candidate antigen for serodiagnosis, a dot-blot assay kit for antibodies against LAM (MycoDot) also showed variable sensitivity and specificity (10–14). As mentioned above, this kit showed a good sensitivity and excellent specificity in one Japanese population but another study is necessary to confirm reliability (14). This kit, dot-blot immunoassay using visual detection, requires only 20 min to carry out reactions. Results are read with the naked eye, using no special apparatus. However, it does not provide quantitative information about antibody titers, which may lead to misreading of weakly positive results.

The TBGL assay kit uses other cell wall components, glycolipids composed of TDM and a more hydrophilic glycolipid purified from H37Rv (15). Quantitative antibody titers can be obtained with this EIA kit. One study showed a relatively high sensitivity (81.1%), nearly equal to that in the MycoDot study (16). In the clinical setting, the TBGL kit required evaluation as a rapid diagnostic test for tuberculosis, especially in comparison with other rapid methods (AFB smear and NAA). This was our focus in the present study. Sensitivity in our study was 79.5% considering all patients with active tuberculosis, which was almost equal to that obtained previously. As indicated in Table 2, sensitivity was 89.4% in smear-positive and 60.6% in smear-negative patients. Variability of antibody responses against glycolipids and the glycolipid component of the tuberculosis bacilli isolated from patients has been reported. Increased sensitivity in smear-positive patients may suggest that the titer of antibodies against glycolipid may depend on the number of tuberculosis bacilli in the lesion, with low numbers giving a false-negative result. To improve the sensitivity of TBGL assay kit, various combinations of antigens such as LAM and protein antigens together with the glycolipids should be tested as detection antigens (3, 8, 21).

The anti-TBGL antibodies assay should be used mainly to differentiate patients with active tuberculosis from others with non-tuberculous respiratory disease, and we examined specificity of this. Specificity in our study (72.2%) was somewhat lower than in a previous study with a similar age distribution (85.0%) (16). In elderly subjects, the positivity rate was somewhat higher. The reason for apparent low specificity is not clear, but it actually might involve the high prevalence of tuberculosis infection among the elderly in Japan. Maekura et al. (16) also reported excellent specificity of the TBGL assay in young, healthy subjects (95.7%); we found similarly high specificity in similar subjects (92.3%; unpublished data). As an additional reason, the composition of TBGL is similar in *M. tuberculosis* and non-tuberculous mycobacteria (NTM) and cross-reactivity was reported in a previous study (16). The occult infection with NTM may affect the results of the TBGL assay.

Among 10 patients with old tuberculosis, we found 70% to be positive by the TBGL assay. All of these patients showed fibrocalcified old tuberculous scars in chest roentgenograms. Maekura et al. (16) reported that anti-TBGL antibody titers were significantly higher in advanced lesions, and that, while high titers decreased significantly after 6 months of antituberculous chemotherapy, they did not fully return to the normal range. In other reports, about 3 years were required for antibody tests using tuberculosis antigens to become negative. Similarly, Chiang et al. (19) reported high positivity rates, 53% and 49%, in their fibrocalcific tuberculosis group. The old tuberculosis group may have had extensive disease, with fibrocalcific lesions remaining in the lung, causing positive reactions with anti-TBGL antibody.

Usefulness of TBGL assay compared to NAA is an important issue for rapid diagnosis of active tuberculosis. In smear-positive individuals, both methods can detect mycobacterial disease with excellent sensitivity. However, reliability of such rapid diagnostic kits is particularly important to differentiate smear-negative tuberculosis from other pulmonary diseases, given the long time required for culture. In our study, sensitivity of the two tests was almost equal in smear-positive and -negative individuals but showed relatively low concordance. In a study comparing NAA (Amplicor) with a serologic test (Detect-TB, Biochem ImmunoSystems, Montreal, Canada) in patients with minimal pulmonary tuberculosis (22), respective sensitivity was 42% and 33%, which is considerably lower than in our smear-negative patients with active tuberculosis (60.6 and 56.3%, respectively). Patients with particularly minimal lesions might have predominated in that earlier comparison but sensitivity was 73.5% in both smear- and culture-negative patients in that previous study with
TBGL assay (16). NAA is costly and complex, and also requires much ongoing quality control effort; further, results depend upon specimen quality.

In the present study we showed that the TBGL assay is sufficiently sensitive for use in rapid detection of tuberculosis infection even in smear-negative patients. However, further efforts are needed to increase sensitivity, such as testing of multi-antigen cocktails.

References


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Corresponding author: Dr. Yoshitsugu Iinuma, Department of Clinical Laboratory, Nagoya University Hospital, Tsurumai-cho 65, Showa-ku, Nagoya-city, 466-8560, Japan
Phone: +81-52-744-2615, Fax: +81-52-744-2615
E-mail: yiinuma@med.nagoya-u.ac.jp