Development of Antigen Detection Assay for Diagnosis of Tuberculosis Using Sputum Samples

LENKA M. PEREIRA ARIAS-BOUDA,1,2,* LAN N. NGUYEN,3 LY M. HO,4 SJOUKJE KUIJPER,1 HENK M. JANSEN,2 AND AREND H. J. KOLK1

Department of Biomedical Research, Royal Tropical Institute, 1 and Division of Pulmonary Diseases, Academic Medical Center, 2 Amsterdam, The Netherlands, and The Pham Ngoc Thach Tuberculosis and Lung Diseases Center, Ho Chi Minh City, 3 and National Institute of Hygiene and Epidemiology, Hanoi, 4 Vietnam

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The rising incidence of tuberculosis worldwide means an increasing burden on diagnostic facilities, so tests simpler than Ziehl-Neelsen staining are needed. Such tests should be objective, reproducible, and have at least as good a detection limit as 10^4 bacteria/ml. A capture enzyme-linked immunosorbent assay (ELISA) was developed for detection of lipoolarabinomannan (LAM) in human sputum samples. As a capture antibody, we used a murine monoclonal antibody against LAM, with rabbit antiserum against Mycobacterium tuberculosis as a source of detector antibodies. The sensitivity of the capture ELISA was evaluated by using purified LAM and M. tuberculosis whole cells. We were able to detect 1 ng of purified LAM/ml and 10^4 M. tuberculosis whole cells/ml. LAM could also be detected in culture filtrate of a 3-week-old culture of M. tuberculosis. The culture filtrate contained approximately 100 ng of LAM/ml. The detection limit in sputum pretreated with N-acetyl-L-cysteine and proteinase K was 10^4 M. tuberculosis whole cells per ml. Thirty-one (91%) of 34 sputum samples from 18 Vietnamese patients with tuberculosis (32 smear positive and 2 smear negative) were positive in the LAM detection assay. In contrast, none of the 25 sputum samples from 21 nontuberculous patients was positive. This specific and sensitive assay for the detection of LAM in sputum is potentially useful for the diagnosis of tuberculosis.

It is estimated that the incidence of tuberculosis worldwide and the number of cases attributable to coexisting human immunodeficiency virus (HIV) infection will increase substantially during the next decade (16). Most of this burden occurs among the low-income countries of the world, particularly those in South East Asia and sub-Saharan Africa. The usual means of diagnosing tuberculosis in resource-poor countries where culture facilities are not available is by the detection of acid-fast bacteria (AFB) in sputum by direct microscopy. Sputum smear-positive patients are the most potent sources of transmission in the community. Therefore, the presence of AFB in sputum is an important marker of infectiousness. When done properly, approximately 60 to 70% of all adults with pulmonary tuberculosis can be identified with the current direct microscopy test using Ziehl-Neelsen staining (ZN). In practice, however, this proportion is around 40 to 60% at best (18). This reduced sensitivity is related to problems associated with the stringent requirements of the test (7). For example, if the need for multiple samples and multiple patient visits is ignored, then fewer smear-positive cases will be identified and treated. The International Union against Tuberculosis and Lung Disease recommends on average 20 slides per technician per working day. Due to overloading of the diagnostic facilities and lack of staff, most laboratory workers, especially in developing countries, process an excessive number of slides or have to combine smear examination with other diagnostic procedures, resulting in a lower quality of the diagnostic service. Patients coinfected with HIV are more likely to have negative sputum AFB smears (15).

The challenge is to develop a simple and inexpensive test—with at least as good a detection limit as that of direct microscopic copy (10^4 bacteria/ml)—that can reduce the workload of laboratory personnel.

Most assays developed so far are based on the detection of specific circulating antibodies. The serodiagnosis of tuberculosis has been the subject of investigation for a long time, but we still lack a test with widespread clinical utility. The available tests have both a sensitivity and specificity of around 80% (3). In HIV seropositive patients coinfected with tuberculosis, the sensitivity of antibody tests is much lower, between 10 and 40% (2, 12, 19). More efforts should be directed toward developing assays based on the detection of antigens in body fluids. Such tests could be useful for the diagnosis and follow-up of patients during treatment.

Mycobacterial antigens have been detected by enzyme-linked immunosorbent assay (ELISA) in sputum (22) and cerebrospinal fluid (13) and by latex agglutination assay in cerebrospinal fluid (10). Lipoolarabinomannan (LAM), a major component of the mycobacterial cell wall, has been detected in the serum (14) and sputum (4) of patients with tuberculosis. None of these tests to detect mycobacterial antigens has achieved widespread use for the diagnosis of active tuberculosis.

In this study, we have developed a specific and sensitive assay for the detection of LAM, which can be used for the diagnosis of tuberculosis. The test is based on a capture ELISA using as a capture antibody a monoclonal antibody against LAM with a rabbit antiserum against Mycobacterium tuberculosis bacteria as a source of detector antibodies.

MATERIALS AND METHODS

Patients. We used sputum samples from nontuberculous patients that had been spiked with M. tuberculosis suspension to develop the capture assay. Two Sudanese smear-positive pulmonary tuberculosis patients provided large volumes of sputum to determine the optimal test conditions. The test was then evaluated with the sputum samples as described below.

(i) Patients with pulmonary tuberculosis from Vietnam. A total of 34 sputum samples were obtained from the Pham Ngoc Thach TB and Lung Disease...
TABLE 1. Bacterial strains used for determining the specificity of the antibodies used in the LAM detection assay

<table>
<thead>
<tr>
<th>Non-Mycobacterium</th>
<th>Mycobacterium</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 2</td>
<td>M. tuberculosis 1</td>
</tr>
<tr>
<td>H. influenzae 1</td>
<td>M. bovis 2</td>
</tr>
<tr>
<td>S. pneumoniae 1</td>
<td>M. avium 1</td>
</tr>
<tr>
<td>S. aureus 1</td>
<td>M. africanum 1</td>
</tr>
<tr>
<td>C. albicans 1</td>
<td>M. microti 1</td>
</tr>
<tr>
<td>N. asteroides 1</td>
<td>M. vaccae 1</td>
</tr>
<tr>
<td></td>
<td>M. kansasii 1</td>
</tr>
<tr>
<td></td>
<td>M. marinum 3</td>
</tr>
<tr>
<td></td>
<td>M. nonchromogenic 1</td>
</tr>
<tr>
<td></td>
<td>M. fortuitum 1</td>
</tr>
<tr>
<td></td>
<td>M. abscessus 1</td>
</tr>
<tr>
<td></td>
<td>M. intracellulare 1</td>
</tr>
<tr>
<td></td>
<td>M. gordonae 3</td>
</tr>
<tr>
<td></td>
<td>M. terrae 2</td>
</tr>
<tr>
<td></td>
<td>M. xenopi 1</td>
</tr>
<tr>
<td></td>
<td>M. scrofulaceum 1</td>
</tr>
<tr>
<td></td>
<td>M. davalli 1</td>
</tr>
<tr>
<td></td>
<td>M. leprae 13</td>
</tr>
<tr>
<td></td>
<td>IEBM A5</td>
</tr>
</tbody>
</table>

* Species were determined by biochemical means by the supplier of the strain.

**KIT**, Royal Tropical Institute, Amsterdam, The Netherlands.

† AMC, Academic Medical Center, Amsterdam, The Netherlands; IEBM, Institute for Experimental Biology and Medicine, Borstel, Germany; ITG, Institute for Tropical Medicine, Antwerp, Belgium; RIVM, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands; UvA, University of Amsterdam, Amsterdam, The Netherlands.

Abbreviations: AMC, Academic Medical Center; ATCC, American Type Culture Collection; BSA, bovine serum albumin; CAMA, Center for Mycobacteriology and AIDS Translation; DMSO, dimethyl sulfoxide; F30-5, monoclonal antibody against LAM; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LAM, lipomannan; NALC, N-acetyl-L-cysteine; PBS, phosphate-buffered saline; PBD, pullulan-based detergent; TMB, tetramethylbenzidine; TBS, Tris-buffered saline; WBC, white blood cells; ZN, Ziehl-Neelsen stain; ZIAD, Ziehl-Neelsen immunofluorescence assay.

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and the A450 was measured. By measuring at two wavelengths (630 and 450 nm), we were able to increase the detection range of LAM. High concentrations of LAM could be detected by measuring the A450, and low concentrations of LAM could be detected by measuring the A630. The relationship between the two is A630/A450 = 3. To correct for day-to-day and plate-to-plate variation, M. tuberculosis culture filtrate (20), passed through a 0.2-μm-pore-size filter and frozen in small aliquots at −20°C, was used as a positive control. All values were corrected by multiplying the A450 of the unknown samples by the correction factor (A450 of culture filtrate at day 0/A450 of culture filtrate at day of testing). To control for the background reaction (conjugate control), four wells were filled with PBS. The final results were expressed as the mean A450 of the duplicates, after subtraction of the background A450. Values above the cutoff value, 0.150, were considered to be positive. The cutoff value was slightly higher than the mean A450 plus two times the standard deviation (SD), 0.156, of the sputum samples from the 16 Dutch subjects with diseases other than tuberculosis as mentioned above.

Statistical analysis. Kruskal-Wallis one-way analysis of variance was used to establish differences in ELISA signals between groups of sputum samples with different ZN scores. A P value of ≤0.05 was considered significant.

The coefficient of variation was used when studying the reproducibility of the assay and the effect of individual variation in sputum on LAM detection, to establish whether differences in ELISA signals were acceptable or not.

**RESULTS**

Specificity of the capture antibody and detection antibody. There was no cross-reactivity between the antibody against LAM (F30-5) and sonicates of the common pulmonary pathogens H. influenzae, S. pneumoniae, S. aureus, and B. catarrhalis or with N. asteroides, E. coli, and C. albicans. As expected, F30-5 cross-reacted with sonicates of all 18 mycobacterial species. The rabbit anti-M. tuberculosis serum (Ra-8.106) contained antibodies which reacted slightly with sonicates of E. coli, H. influenzae, and N. asteroides. However, E. coli, H. influenzae, and N. asteroides did not give a positive result in the final capture ELISA (data not shown).

Detection of solutions containing purified LAM, LAM in M. tuberculosis culture filtrate, and LAM in M. tuberculosis suspensions. A calibration curve was made by using purified LAM (kindly provided by Patrick Brennan) diluted in PBS containing 0.005% Tween 20 (Fig. 1). It was possible to detect as little as 1 ng of purified LAM per ml (measurements of A450 or A630 gave the same detection limit).

LAM could be detected by the capture ELISA in the culture filtrate of a 3-week-old culture of M. tuberculosis. The culture filtrate contained approximately 100 μg of LAM/ml (Fig. 1). As a positive control for day-to-day and plate-to-plate variations in the capture ELISA, we used the M. tuberculosis culture filtrate with 0.005% (vol/vol) Tween 20 in PBS as a dilution buffer.

A serial dilution of M. tuberculosis bacteria in PBS containing 0.005% (vol/vol) Tween 20 (see Materials and Methods) was tested in the capture ELISA (Fig. 1). The detection limit was 106 M. tuberculosis bacteria/ml (measurements of A450 or A630 gave the same detection limit). Figure 1 shows that the amount of LAM found in 105 mycobacteria/ml equals 10 ng of purified LAM/ml. Assuming that all LAM was extracted from the mycobacteria, we can then estimate that one M. tuberculosis bacterium contains approximately 100 fg of LAM (10 ng = 107 fg; 107 fg/105 mycobacteria = 100 fg of LAM/mycobacterium).

Detection of LAM in spiked sputum. Sputum samples from 10 Dutch patients not suspected of having tuberculosis were pooled and spiked with M. tuberculosis whole cells. We found that treatment of purified LAM and M. tuberculosis culture filtrate with 0.25 M sodium hydroxide in combination with 15 mM NALC resulted in a 50% reduction of the signal. Sputum therefore was not treated with NaOH-NALC. Instead, we used NALC (1 mg/ml) in combination with proteinase K (0.1 mg/ml). The detection limit in sputum treated this way was 104 mycobacteria/ml (Fig. 2).

Reproducibility of the assay. We tested the reproducibility of the assay, including pretreatment of the sputum with NALC and proteinase K on 3 consecutive days, by using samples of sputum from four patients (Table 2). Two patients had a disease other than tuberculosis, and two Sudanese patients with tuberculosis had ZN-positive sputa. The SD of the daily differences was small (Table 2). For the sputum samples from both tuberculous patients, the coefficient of variation was 5%.

Effect of individual variation in sputum on LAM detection. To explore whether variation in sputum contents or consistency could influence the detection of LAM, six sputum samples from six Dutch patients with a diagnosis other than tuberculosis were tested both unspiked and spiked with 5 × 106 M. tuberculosis bacteria/ml sputum (Table 3). The sputum samples differed in optical appearance and viscosity. All sputum samples were treated by the NALC-proteinase K method de-
The sputum was pooled and contained equal portions of sputum from 10 Dutch patients with chronic obstructive pulmonary disease, with no suspicion of tuberculosis. From The Netherlands with pulmonary tuberculosis.

In total, 59 sputum samples from 39 subjects were tested in the LAM detection assay: 34 sputum samples from tuberculous patients and 25 from nontuberculous patients.

Results are given as the mean $A_{450}$ minus the plate background. •••••, ELISA value of unspiked sputum; +, $A_{450}$ of $>3$. Error bars show $\pm 2\ SD$s. The detection limit was $10^6\ M.\ tuberculosis$ bacteria/ml of sputum.

Detection of LAM in sputum of patients with pulmonary tuberculosis. In total, 39 sputum samples from 39 subjects were tested in the LAM detection assay: 34 sputum samples from tuberculous patients and 25 from nontuberculous patients.

The patient 1 sample was sputum from a patient from The Netherlands with idiopathic pulmonary fibrosis, with no suspicion of tuberculosis. The patient 2 sample was sputum of a patient from The Netherlands with chronic obstructive pulmonary disease, with no suspicion of tuberculosis.

The patient 3 and 4 samples were ZN-positive sputa from two patients from Vietnam, one of whom had a history of tuberculosis.

The patient 1 sample was sputum from a patient who was admitted to the hospital because of hematemesis; B, sputum sample from a patient with pneumonia, from which no organisms (including AFB) were cultured; C, sputum sample from a patient with bronchitis, from which $H.\ influenzae$ was cultured; D, sputum sample from a patient with chronic obstructive pulmonary disease, from which no organisms were cultured; E, sputum sample from a patient with recurrent respiratory tract infections, from which no organisms were cultured; F, sputum sample from a patient with acute bronchitis, from which $S.\ pneumoniae$ was cultured.

None of the patients had any clinical or radiological sign of $M.\ tuberculosis$ infection. A, sputum sample from a patient who was admitted to the hospital because of hematemesis; B, sputum sample from a patient with pneumonia, from which no organisms (including AFB) were cultured; C, sputum sample from a patient with bronchitis, from which $H.\ influenzae$ was cultured; D, sputum sample from a patient with chronic obstructive pulmonary disease, from which no organisms were cultured; E, sputum sample from a patient with recurrent respiratory tract infections, from which no organisms were cultured; F, sputum sample from a patient with acute bronchitis, from which $S.\ pneumoniae$ was cultured.

Results are given as the mean $A_{450}$ of the duplicates in each test minus the plate background.

Coefficient of variation $= 6\%$.

Table 4 shows the correlation between the results of the LAM detection assay and the disease status of the patient (tuberculous versus nontuberculous). A patient was called positive if at least one sputum sample was positive in the LAM detection assay. Seventeen of 18 patients with tuberculosis (including the partially treated, smear-negative tuberculosis patient) were positive, whereas all of the patients with other diseases were negative, resulting in a sensitivity of 94% and a specificity of 100%.

### DISCUSSION

The assay for detection of LAM in sputum is promising and is a potential candidate to replace direct microscopy.

Preliminary results have been published on new diagnostic tests for tuberculosis based on detection of mycobacterial antigens in cerebrospinal fluid (10, 13), serum (11, 14), and sputum (4, 22). The authors reported sensitivity rates of 45 to 88% and specificity rates of 91 to 100%. However, since then, little...
has been published on this subject, suggesting that these tests did not live up to their original promise.

Any test which is to replace direct microscopy must offer advantages in terms of speed and ease of use and preferably have a higher sensitivity. In high-prevalence areas of tuberculosis, the greatest need is for new diagnostic tools which result in a reduction of laboratory workload. Antigen detection assays are promising in this regard, since they enable the analyst to test many samples at once.

In antigen detection assays, sample processing is an important, but often laborious and time-consuming, step. The number of nonspecific reactions can be high because of cross-reacting substances present in untreated human specimens. Mycobacterial antigens have been found as components of circulating immune complexes (1), so it may be necessary to dissociate the immune complexes to achieve a higher sensitivity in the immunoassay (5). Sada et al. (14), who detected LAM in serum, used an ethanol precipitation method (6) to reduce nonspecific reactions. Their detection system, a simple coagulation technique, is suitable for routine use. However, the described method for sample processing is still too laborious for daily use in laboratories in areas where tuberculosis is endemic, where more than 100 specimens per day have to be examined.

Yáñez et al. (22) and Cho et al. (4), who detected mycobacterial antigens and LAM in sputum, respectively, used NaOH for pretreatment of sputum. We found that treatment of LAM with sodium hydroxide, even for a short time at room temperature, resulted in a dramatic decrease in the LAM detection assay. LAM is alkali labile (8), so treatment with NaOH could destroy the epitope which is recognized by the capture antibody. For the LAM detection assay, we have developed an effective pretreatment method for sputum (NALC-protease K method), avoiding the use of NaOH. By this method, the results of the LAM detection assay were reproducible when a single sputum sample was pretreated and tested on 3 consecutive days. The NALC-proteinase K method can be further simplified, since we have found that it is possible to reduce the incubation time at 50°C (unpublished results).

We could detect as little as 1 ng of purified LAM per ml and \(10^4\) \(M.\) tuberculosis whole cells per ml by using the capture ELISA. In spiked sputum, the detection limit was also \(10^4\) \(M.\) tuberculosis whole cells. We have shown that there was a direct correlation between the LAM detection assay results and the numbers of whole mycobacteria in sputum spiked with different amounts of \(M.\) tuberculosis (Fig. 2). On the other hand, we found that there was no significant correlation between the LAM detection assay results and ZN score of the sputum samples from patients with tuberculosis. An explanation is that, for direct microscopy, the sample was selected from the purulent portion of the sputum, while for the LAM detection assay.

TABLE 4. Correlation between the results of the LAM detection assay and disease status

<table>
<thead>
<tr>
<th>Result by LAM detection assay</th>
<th>Proven TB</th>
<th>Non-TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>21</td>
</tr>
</tbody>
</table>

* A patient was positive if in the LAM detection assay the \(A_{450}\) of at least one sputum sample was higher than the cutoff value (0.150).

* Pulmonary tuberculosis (TB) patients from Vietnam with a positive sputum culture.

* Five patients from Vietnam and 16 patients from The Netherlands with a disease other than tuberculosis.
assay, a homogenate of the remaining portion of the sample was taken. Thus, the LAM detection assay may be more representative and give a more accurate indication of the bacterial load in the sputum. It could become a tool in the follow-up of patients during therapy.

Since LAM is present in all mycobacteria, the assay could be used for the diagnosis of any mycobacterial disease, including disease caused by the *Mycobacterium avium-Mycobacterium intracellulare* complex. The methods used then to identify the infecting mycobacteria will depend upon local circumstances (e.g., culture or multiplex PCR) (9). However, the LAM detection assay has been designed for use particularly in developing countries, where tuberculosis is by far the most common mycobacterial disease.

Our results with the LAM detection assay showed that 91% of the sputum specimens in the group of tuberculous patients were positive, and all of the specimens in the group of nontuberculous patients were negative. Based on the definition that a patient was positive in the LAM detection assay if at least one sputum specimen had an *A_50* value higher than the cutoff value, 94% of the tuberculous patients were found to be positive, whereas all of the nontuberculous patients were negative. Our results are promising, but the assay needs further evaluation in the field to determine its sensitivity and specificity, since we have tested only a small study population so far.

ACKNOWLEDGMENTS

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REFERENCES