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Detection of Pulmonary and Extrapulmonary Tuberculosis Patients with the 38-Kilodalton Antigen from *Mycobacterium tuberculosis* in a Rapid Membrane-Based Assay

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A rapid membrane-based serologic assay using the 38-kDa antigen from *Mycobacterium tuberculosis* for the diagnosis of tuberculosis (TB) was evaluated with 201 patients with pulmonary TB, 67 patients with extrapulmonary TB, 79 *Mycobacterium bovis* BCG-vaccinated healthy controls, and 77 non-TB respiratory patients. The overall sensitivities, specificities, and positive and negative predictive values were, respectively, 92, 92, 84, and 96% for sputum-positive TB patients; 70, 92, 87, and 79% for sputum-negative TB patients; and 76, 92, 80, and 90% for extrapulmonary-TB patients. Only 2% (1 of 44) of the healthy control BCG-vaccinated subjects gave weak positive signals in the assay, indicating that this rapid serological assay is a valuable aid in clinical diagnosis for both pulmonary and extrapulmonary TB.

The recent resurgence in tuberculosis (TB) worldwide has renewed interest in new methods for accurate and rapid diagnosis. Particular attention has been given to molecular techniques for diagnosis such as PCR, which is highly sensitive and has the capability of distinguishing between different species of mycobacterium (12, 28, 33). However, this technique suffers from false positives due to contamination with DNA fragments from previous PCRs (26), debris from nonviable bacilli, or inhibitors that interfere with the PCR (24, 28). In addition, it is expensive and relies on sophisticated equipment and a clean, preferably aseptic, environment. These conditions are often lacking in developing countries.

Currently, developing countries rely on acid-fast staining of sputa or cultures of *Mycobacterium tuberculosis* in conjunction with assessment of clinical symptoms and radiographic evidence to diagnose TB. Detection by stain and culture lacks sensitivity, particularly in cases of sputum-negative disease, while chest lesions identified by radiograph cannot identify the causal agent (23, 41). Extrapulmonary TB presents even more problems, as sputum samples are often not available and obtaining specimens from the suspected site of infection often involves highly invasive and expensive procedures.

Recently a number of secreted antigens from *M. tuberculosis* have been purified and characterized (8, 22, 36, 37). Initially these antigens held great promise for detecting only active *M. tuberculosis* infection; however, hopes have since faded, as most of these antigens were found to share epitopes with a broad spectrum of bacterial and mammalian proteins as well as other mycobacterium species (42). However, extensive research of the 38-kDa antigen has shown that this antigen is specific for TB complex species and can detect up to 85% of patients with sputum-positive TB when used in a competitive enzyme-linked immunosorbent assay (ELISA) system (7, 14, 17, 36, 41, 44). The gene for this antigen has been cloned and expressed in *Escherichia coli* (1, 30) and is reported to encode an extracellular lipoprotein involved in phosphate metabolism (9).

We have described a rapid nitrocellulose membrane-based assay which used the 38-kDa antigen from *M. tuberculosis* to detect antibody in pulmonary TB patients (10). In this study we have expanded on these data and made comparisons with a PCR test currently available in China. Particular attention has also been given to assessing the sensitivity of the 38-kDa-antibody assay with extrapulmonary TB patients. The relationship between length of disease or length of treatment and antibody level has also been assessed.

MATERIALS AND METHODS

Patients and serum specimens. A total of 424 subjects have been involved in this study. The hospitals involved were the Department of Respiratory Diseases of the First Teaching Hospital of Harbin Medical University, the First Harbin City Hospital, and the Beijing Chest Hospital. All data on the length of disease and treatment and the PCR assay were provided by the Beijing Chest Hospital.

Patients with pulmonary TB (n = 201; average age, 50.7 years; range, 18 to 86 years) were tested by sputum smear for acid-fast bacilli or by culture for *M. tuberculosis*. All the patients were screened by radiograph. The diagnosis of active pulmonary TB was based on the results of sputum smear and culture, radiographic evidence, clinical symptoms, and experimental treatment. All patients with pulmonary TB selected for this study had no prior clinical history of TB. Patients with extrapulmonary TB (n = 67; average age, 35 years; range, 10 to 76 years) were diagnosed by clinical symptoms, radiographic evidence, ultrasound, and computerized tomographic scanning or a combination of these techniques, depending on the location of the infection in the patient. For patients with TB meningitis, the cerebrospinal fluid was examined. All TB patients were receiving treatment by combination anti-TB chemotherapy with isoniazid, streptomycin, ethambutol, *para*-aminosalicylic acid, and rifampin when the blood samples were collected.

All PCR specimens were obtained from sputum samples except that for patients with meningitis cerebrospinal fluid was collected, and for patients with abdominal TB specimens were taken from abdominal fluids.

Control groups. Patients (n = 77; average age, 49.9 years; range, 3 to 84 years) with non-TB respiratory diseases, including pneumonia (n = 19), lung infection (n = 15), lung cancer (n = 11), acute or chronic bronchitis (n = 12), cor pulmonale (n = 5), non-TB pleurisy (n = 5), asthma (n = 3), pulmonary abscess (n = 3), silicosis (n = 2), influenza (n = 1), and hydrothorax (n = 1), were screened by radiographic techniques and diagnosed according to the standard clinical practice for each disease. Two healthy control groups were chosen for this study. The adult healthy control group comprised 44 subjects (average age, 33.8 years; range 20 to 57 years), and the child healthy control group comprised 35 subjects (average age, 7.4 years; range 6 to 13 years). The child healthy control group was selected because we were interested in the response of this assay with a recently *Mycobacterium bovis* BCG-vaccinated population. All adults included in this study were vaccinated in their early teenage years (8 to 13 years). Children and young adults (1 to 16 years) were vaccinated in their early childhood years

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(1 to 5 years). All subjects were checked for vaccination scars prior to their sera being taken for this study.

All the sera of the TB patients and the non-TB respiratory disease patients were tested for antibodies to human immunodeficiency virus (HIV) with the Wellcozyme HIV recombinant enzyme immunoassay. Potential positive sera were retested several times to confirm the results.

Specimen preparation for the PCR assay. Specimens were collected and centrifuged at 12,000 × *g* for 15 min. One hundred microliters of 0.1 M NaOH–2 M NaCl–0.5% sodium dodecyl sulfate (SDS) solution was added to 1.5 ml of sample and incubated at 94°C for 20 min. DNA was extracted with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) and then chloroform-isoamyl alcohol (24:1). The DNA was precipitated by incubation for 2 h at −20°C with the addition of 2 volumes of absolute alcohol. After centrifugation at 10,000 × *g* for 5 min the pellet was washed with 70% ethanol, air dried, and resuspended in 20 μl of distilled water ready for PCR analysis.

PCR assay. A 245-bp fragment of the insertion sequence IS*6110* was used to detect *M. tuberculosis*. The two oligonucleotide primers were 5′-CGTGAGGGC ATCGAGGTGGC, which corresponds to bp 631 to 650, and 5′-GCGTAGGCG TCGGTGACAAA, which corresponds to bp 856 to 875 of IS*6110* (24, 33, 34).

PCR procedure. Fourteen microliters of the PCR solution was added to 10 μl of extracted DNA sample, mixed, and incubated for 8 min at 94°C. After a brief spin, 1 μl of *Taq* DNA polymerase (Promega) was added to the PCR solution and the tube was recentrifuged for 30 s. Twenty five microliters of mineral oil was added to each tube before the specimens and controls were placed in a Perkin-Elmer Thermocycler TC 9600 and amplified according to the following procedure: 93°C for 1 min, 65°C for 1 min, and 72°C for 2 min, for 35 cycles. The last extension cycle was at 72°C for 6 min. Amplified DNA was detected by electrophoresis of 10 μl of sample through a 1.7% gel containing 0.5 μg of ethidium bromide per ml in Tris-borate-EDTA for 1 to 2 h at 4 to 6 V/cm.

Reagents. The conjugate was goat anti-human immunoglobulin G (heavy plus light chain)-linked to 40-nm-diameter gold particles (British Biocell, London, United Kingdom). The 38-kDa recombinant antigen was obtained from Omega Diagnostics, Alloa, Scotland, United Kingdom.

Principles of test. The details of the test have been reported elsewhere (10). Briefly, the test consists of a cardboard folding device containing a nitrocellulose strip on which the 38-kDa antigen from *M. tuberculosis* has been applied. When a serum sample flows past the nitrocellulose strip, antibodies specific for the 38-kDa antigen bind to the antigen line. In a reverse flow, the bound antibodies are detected by a goat anti-human immunoglobulin G antibody conjugated to colloidal gold particles and show a pink to red positive line. The test is completed in a maximum of 15 min and does not require any other equipment.

All serum samples were tested by operators without knowledge of the clinical diagnosis of each patient. Each test was read by two operators working from color charts which displayed the color intensities correlated with scores from + to ++++. The positive results have been classified into four categories: + (weak positive), a faint pinkish red line; ++ (positive), an easily visible pinkish red signal; +++ (strong positive), a pronounced reddish signal; and ++++ (very strong positive), a deep purple-red signal. If an operator could not definitely categorize a signal into one of the four categories listed above the test was repeated and both operators conferred as to which category the signal should be placed in.

Statistical analysis. The χ^2 test was used to measure the significance of differences in frequency (31).

RESULTS

Test sensitivity and specificity. The percentages of patients and control subjects with positive antibody responses to the 38-kDa antigen are shown in Fig. 1. The patients with pulmonary TB were divided according to the results of sputum smears and cultures. The assay detected 32 (91%) of the 35 smear-positive, culture-positive patients, 22 (92%) of the 24 smear-positive, culture-negative patients, and 16 (94%) of the 17 smear-negative, culture-positive patients. Statistically, the differences among these three groups are not significant ($P > 0.05$); therefore, these results were combined into the sputum-positive group.

Twelve percent (9 of 77) of the patients with non-TB respiratory diseases had detectable levels of antibody. Two of 19 patients with pneumonia and 4 of 10 patients with chronic bronchitis had weak positive signals (+). Two of the 11 patients with lung cancer tested positive, with signal strengths of + and +++. Both these patients were males, and they were 53 and 61 years old, respectively. One of the patients with silicosis had a weak positive result (+).

In the adult healthy control group 2% (1 of 44) of subjects had positive antibody responses. A slightly higher number of positive antibody responses (3 of 35 [9%]) were observed in the child healthy control group, but this was not significantly different from the results with the adult control group (1 of 44 [2%] [$P > 0.05$]). All four antibody-positive subjects in the healthy control groups (one in the adult and three in the child control group) gave weak positive signals (+). The detection rates of the 38-kDa antibody for the healthy control groups were not significantly different ($P > 0.05$), compared with those of the non-TB respiratory disease group; therefore, these three groups were combined into a single control group (156 patients) for further analysis.

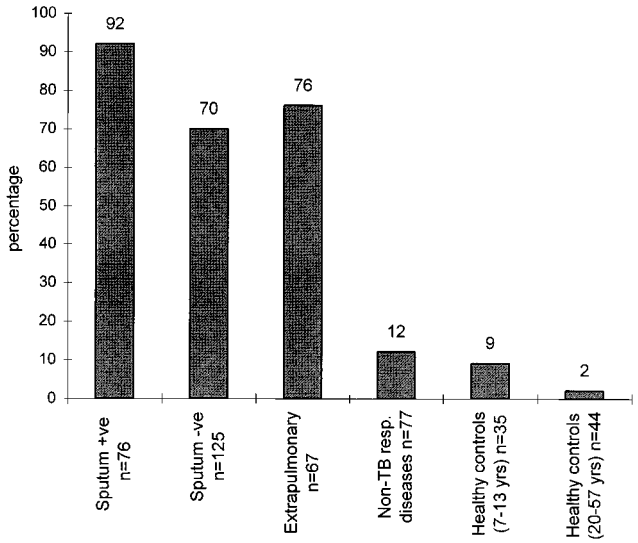

FIG. 1. Percentages of pulmonary and extrapulmonary TB patients with positive antibody responses to the 38-kDa antigen compared with those of non-TB respiratory (Non-TB resp.) disease patients and healthy controls. +ve, positive; -ve, negative.

The percentage of pulmonary TB (sputum-positive and sputum-negative) patients with positive antibody responses is significantly higher than that for the combined control group ($P \leq 0.005$). The sensitivities of the assay in the sputum-positive and sputum-negative groups are 92 and 70%, respectively, for pulmonary TB patients and 76% for extrapulmonary TB patients (percentages based on the combined control group). The overall specificity of the assay is 92%. The predicted positive values are 84% for sputum-positive group, 87% for sputum-negative group, and 80% for extrapulmonary TB patients, and the predicted negative values are 96, 79, and 90%, respectively.

All the TB and the non-TB respiratory sera involved in this study, except one serum that continually tested positive, were HIV antibody-negative. The HIV-positive patient was a 65-year-old male and was diagnosed as having pulmonary TB. He was both sputum smear and culture negative, had clinical symptoms of cough, fever, and weight loss, and showed a lesion in the lung on radiograph. The patient was positive (+++) for the 38-kDa antigen. Since this was the only TB patient with HIV antibodies, he was excluded from the study.

Relationships between length of disease or treatment and antibody level. Evidence of the increase in levels of antibody to the 38-kDa antigen with an increasing length of disease and treatment is shown in Fig. 2. The length of disease group for the sputum-positive patients (culture and/or smear positive) (Fig. 2A) showed that in the first 3 months after the onset of the disease, 63% of patients had positive signals (+ or ++)

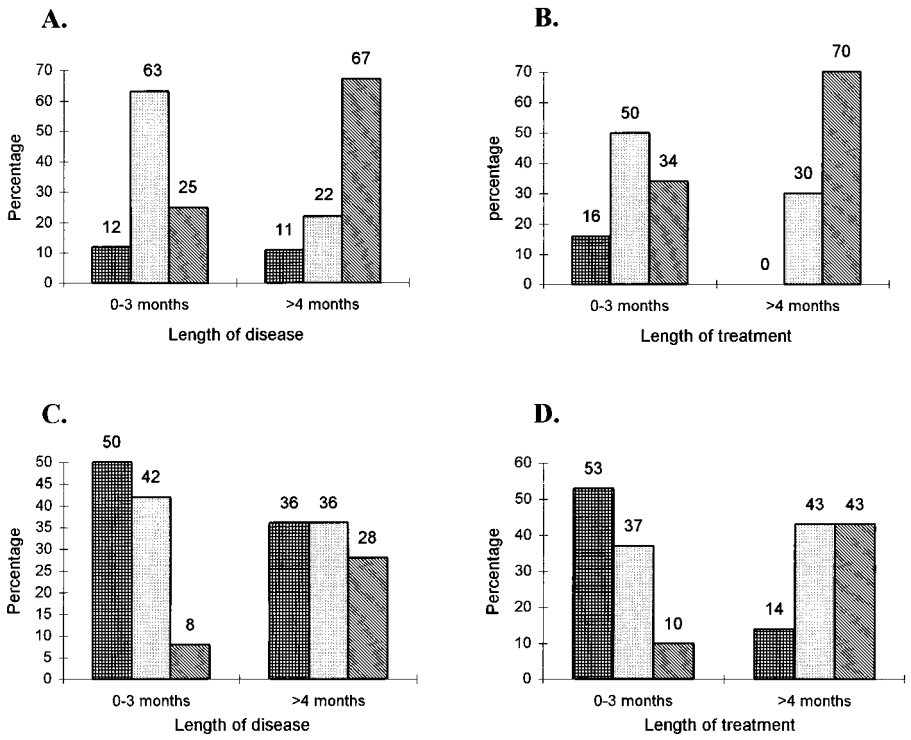

FIG. 2. Percentages of sputum-positive (A and B) and -negative (C and D) TB patients with positive antibody responses to the 38-kDa antigen and correlated to length of disease and treatment. The same groups of sputum-positive ($n = 42$) and sputum-negative ($n = 26$) patients from the Beijing Chest Hospital were used for data on length of disease and length of treatment as this hospital provided the most complete set of patient data. Light-gray bars, no signal; dark-gray bars, positive signal; black bars, strong signal.

and 25% had strong positive signals (+++ or ++++). After 4 months however, the percentage of patients with positive signals had dropped to 22% ($P < 0.01$), while the percentage of patients with strong positive signals had risen to 67% ($P < 0.01$).

Chemotherapy was usually commenced within a few weeks of admission of the patient to hospital. The length of the chemotherapy each patient had undergone prior to being tested was considered (Fig. 2B), and 50% of patients in the first 3 months of treatment had positive signals (+ or ++) compared with 30% after 4 months of treatment ($P > 0.05$). In contrast, 34% of patients undergoing chemotherapy for less than 3 months had strong positive signals (+++ or ++++) compared with 70% of patients after 4 months ($P < 0.05$). This suggests that there is a significant increase in the amount of specific antibody to the 38-kDa antigen during the course of disease and chemotherapy.

Sputum-negative patients (Fig. 2C and D) show trends similar to those of sputum-positive patients when both the length of disease and treatment are considered, although statistically the differences are not significant ($P > 0.05$).

The percentage of sputum-positive patients (88%) detected by the assay was higher than that of sputum-negative patients (50%) in the first 3 months after the onset of disease ($P < 0.05$) and after four months (89 and 64%, respectively) ($P > 0.05$). Similarly, in the first 3 months of treatment more sputum-positive patients than sputum-negative patients tested positive by the antibody assay (84 and 47%, respectively) ($P < 0.005$), while after 4 months all the sputum-positive patients (100%) tested positive by the antibody assay and only 86% of the sputum-negative patients were detected ($P > 0.05$). Overall, this suggests that sputum-positive patients have more antibody to the 38-kDa antigen than do sputum-negative patients.

Analysis of extrapulmonary TB patients. Patients with extrapulmonary TB were divided according to the location of the affected organ (Table 1). The highest degree of sensitivity was obtained with abdominal TB (92%) and TB meningitis (88%) patients. The sensitivity reached 80% for lymph and genitourinary TB. The sensitivity for bone and joint TB was 70%, while the lowest sensitivity was with pleural and pericardial TB (64%). The percentage of extrapulmonary TB patients with positive antibody responses was significantly higher than that for the combined control group ($P < 0.005$).

Comparison of 38-kDa-antibody assay and PCR. The sensitivity of the 38-kDa-antibody assay was slightly higher (84%) than that of PCR (81%) for the sputum-positive group (Fig. 3) ($P > 0.05$). For sputum smear- and culture-negative patients the 38-kDa-antibody assay detected 50% of patients (10 of 20), while PCR detected 40% (8 of 20) ($P < 0.05$). Eighty percent (12 of 15) of the extrapulmonary TB patients had antibodies to the 38-kDa antigen, while PCR detected 5 of 15 (33%) ($P < 0.01$).

TABLE 1. Percentage of extrapulmonary TB patients detected by the 38-kDa antibody assay[a]

Description of disease	No. of patients	% Disease detected
Lymph	5	80
Bone and joint	23	70
Genitourinary tract	5	80
Abdominal	12	92
Meningitis	8	88
Pleural and pericardial	14	64

[a] Total number of patients, 67. Total percent detected, 76.

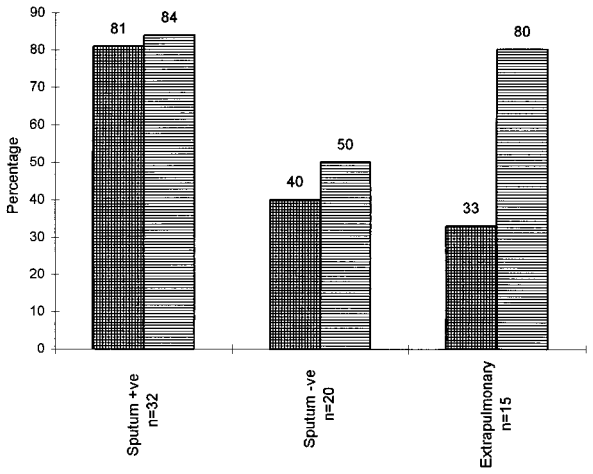

FIG. 3. Percentages of sputum-positive (+ve), sputum-negative (-ve), and extrapulmonary TB patients detected by PCR and 38-kDa-antibody assay. Light-gray bars, PCR results; dark-gray bars, 38-kDa-antibody assay results.

DISCUSSION

Recent studies have indicated that the secreted proteins from actively growing *M. tuberculosis* bacilli play a major role in host immunity (2, 6, 11, 13, 18). This hypothesis has led to renewed and intensive research on the isolation and characterization of many secreted proteins of *M. tuberculosis* (3, 4, 13, 17, 32, 35). The most sensitive and specific of these proteins for detecting antibodies against *M. tuberculosis* is the 38-kDa antigen, which is specific for TB complex species (1, 14, 15, 22, 38, 44). The development of competition ELISAs based on monoclonal antibodies to the 38-kDa antigen has further increased sensitivity (7, 37, 42).

Cole et al. (10) have reported on the sensitivity and specificity of the assay system used in this study with pulmonary TB patients. We have expanded the control and sputum-positive and -negative groups to provide a more accurate picture of the capability of this assay system and added an extrapulmonary TB group of 67 patients. The results (Fig. 1) show sensitivities similar to the sputum-positive (92% compared with 89%) and sputum-negative (70% compared with 74%) results reported previously (10). The overall specificity also remains high (92% compared with 93%).

The ability of extrapulmonary TB to mimic the clinical symptoms of other diseases makes diagnosis extremely difficult (19). Failure to correctly diagnose extrapulmonary TB can have serious consequences, especially in cases of TB meningitis and pericardial TB (43). Conversely, chemotherapy without proper diagnosis can mask other diseases which partly respond to broad-spectrum antibacterial drugs and expose the patient unnecessarily to toxic side effects of the drugs used in the treatment (42).

Table 1 shows the number of extrapulmonary TB patients categorized by the site of infection and the percentage of infections that were detected. For all of the sites of TB infection that were diagnosed by this assay, sensitivities fall in the range of 64 to 92%. This level of sensitivity is similar to that reported by Wilkins and Ivanyi (42), using a competition ELISA based on the 38-kDa TB72 monoclonal antibody. They reported an overall sensitivity of 73% and a specificity of 98% for extrapulmonary TB, while Verbon et al. (37) reported 56% sensitivity. Our overall sensitivity of 76% (Fig. 1) compares favorably with these results.

The degree of positive antibody responses (9%) in the child healthy control group compared with that in the adult healthy control group (2% [1 of 44]) (Fig. 1) suggests that more antibody against the 38-kDa antigen was present in this group than in the adult control group. One possibility is that residue antibody to the 38-kDa antigen from recent BCG vaccinations was present, as this antigen is present in *M. bovis* at about 1/10 of the concentration of that in *M. tuberculosis* (44). A more likely explanation, however, is that this increase in the level of specific antibody represents an increase in the incidence of primary TB infection in Chinese children. The primary TB infection, known as Ghon complex, is seen mainly in early childhood in areas of endemicity, with a maximum incidence occurring between 1 and 3 years (29). The majority of subjects with this primary infection have mild flu-like clinical symptoms and recover in a few weeks without the infection being noticed. The infectious rate of TB in China is over 80%, and 90% of those infected have had the primary disease before the age of 20 years (16, 40). Therefore, antibodies against the 38-kDa antigen are more likely to be present in the child control group.

The clinical diagnosis of pulmonary TB is often problematic. A number of respiratory diseases such as pneumonia, bronchitis, and cancer can mimic both the clinical symptoms and the shadow often seen on a radiograph with pulmonary TB patients (27). Furthermore, coinfection of TB with other respiratory diseases is not uncommon and clinical diagnosis of these cases is particularly difficult and often may involve waiting for the patient to respond to treatment (27). As all patients in the non-TB respiratory disease group in our study were sputum negative, only radiographs and clinical symptoms were used for diagnosis. It is possible, therefore, that concurrent TB infections could also be present in some of the patients diagnosed with antibody to the 38-kDa antigen (9 of 77). We are continuing to monitor these patients to see if they become sputum positive.

Silicotic patients are also very prone to develop active pulmonary TB, because in such patients the function of the alveolar macrophages is impaired. The two diseases often coexist in the same lung site and make the clinical diagnosis very difficult (25).

Ivanyi et al. (21) and later Verbon et al. (37) reported that the patients with the highest antibody levels to the 38-kDa antigen were undergoing chemotherapy prior to serum testing. Figure 2 also confirms this trend and shows a significant increase in antibody levels during the course of disease. High antibody titers have also been associated with more extensive TB (8). This was also evident when the percentages of sputum-positive patients (Fig. 2A and B) and sputum-negative patients (Fig. 2C and D) were compared.

Recent reports have shown that PCR with sputum has a sensitivity comparable to that culture and can produce a result for pulmonary TB in about 6.5 h (5, 12, 39). Figure 3 compares the results of the 38-kDa-antibody assay with those of a PCR assay that is currently available in China. The results of the 38-kDa-antibody assay are comparable to or better than those of PCR for pulmonary TB patients and are more sensitive for extrapulmonary TB (Fig. 3). The increased sensitivity of the 38-kDa-antibody assay for extrapulmonary TB patients can be explained by the frequent paucity of bacilli in the sputum or bronchial fluid. The progression of the disease into the organs does not necessarily mean that the patient will continue to produce a positive sputum sample (20). In the case of extrapulmonary TB, if possible, samples for PCR should be taken from the site of infection. This often involves highly expensive and invasive procedures which are not always available in the developing world.

In summary, the membrane-based assay presented in this work is specific for detecting antibodies in the serum of patients with active TB within 15 min. The assay's lack of re-

sponse with healthy control BCG-vaccinated subjects indicates the clinical diagnostic value of this assay in regions of endemicity. This rapid assay, in conjunction with other diagnostic techniques, may serve as a valuable aid in clinical diagnosis for both pulmonary and extrapulmonary TB.

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