JCM
Journals.ASM.org

Novel Real-Time Simultaneous Amplification and Testing Method To Accurately and Rapidly Detect *Mycobacterium tuberculosis* Complex

Zhenling Cui,[a] Yongzhong Wang,[b] Liang Fang,[c] Ruijuan Zheng,[a] Xiaochen Huang,[a] Xiaoqin Liu,[b] Gang Zhang,[b] Dongmei Rui,[b] Jinliang Ju,[a,c] and Zhongyi Hu[a]

Shanghai Key Laboratory of Tuberculosis, Shanghai Pulmonary Hospital, Medical School, Tongji University, Shanghai, China[a]; The 3rd People's Hospital of Changzhou, Changzhou, China[b]; and Shanghai Rendu Biotechnology Co., Ltd., Shanghai, China[c]

The aim of this study was to establish and evaluate a simultaneous amplification and testing method for detection of the *Mycobacterium tuberculosis* complex (SAT-TB assay) in clinical specimens by using isothermal RNA amplification and real-time fluorescence detection. In the SAT-TB assay, a 170-bp *M. tuberculosis* 16S rRNA fragment is reverse transcribed to DNA by use of Moloney murine leukemia virus (M-MLV) reverse transcriptase, using specific primers incorporating the T7 promoter sequence, and undergoes successive cycles of amplification using T7 RNA polymerase. Using a real-time PCR instrument, hybridization of an internal 6-carboxyfluorescein–4-[4-(dimethylamino)phenylazo] benzoic acid *N*-succinimidyl ester (FAM-DABCYL)-labeled fluorescent probe can be used to detect RNA amplification. The SAT-TB assay takes less than 1.5 h to perform, and the sensitivity of the assay for detection of *M. tuberculosis* H37Rv is 100 CFU/ml. The TB probe has no cross-reactivity with nontuberculous mycobacteria or other common respiratory tract pathogens. For 253 pulmonary tuberculosis (PTB) specimens and 134 non-TB specimens, the SAT-TB results correlated with 95.6% (370/387 specimens) of the Bactec MGIT 960 culture assay results. The sensitivity, specificity, and positive and negative predictive values of the SAT-TB test for the diagnosis of PTB were 67.6%, 100%, 100%, and 62.0%, respectively, compared to 61.7%, 100%, 100%, and 58.0% for Bactec MGIT 960 culture. For PTB diagnosis, the sensitivities of the SAT-TB and Bactec MGIT 960 culture methods were 97.6% and 95.9%, respectively, for smear-positive specimens and 39.2% and 30.2%, respectively, for smear-negative specimens. In conclusion, the SAT-TB assay is a novel, simple test with a high specificity which may enhance the detection rate of TB. It is therefore a promising tool for rapid diagnosis of *M. tuberculosis* infection in clinical microbiology laboratories.

The rapid and accurate diagnosis of pulmonary tuberculosis (PTB) plays a critical role in its successful management, as it is essential to treat patients with the appropriate therapy as soon as possible to minimize the risk of transmission. A number of tests based on nucleic acid amplification (NAA) have been developed to identify *Mycobacterium tuberculosis* in clinical specimens rapidly and directly (8, 34, 35, 44). Among the approved commercial NAA methods, the amplified *M. tuberculosis* direct test (MTD test; Gen-Probe) detects *M. tuberculosis* RNA by using a specific isothermal transcription-mediated amplification method (21). The MTD test has been shown to be a sensitive, specific, and rapid method for use with clinical samples (9, 17, 18); however, it requires the use of expensive specialized detection equipment, which prevents the assay from being applied widely in clinical laboratories. The aim of this study was to report and evaluate a simultaneous amplification and testing method for detection of the *M. tuberculosis* complex (SAT-TB assay) for use with clinical sputum specimens, based on real-time fluorescence detection of isothermal RNA amplification using routine real-time PCR equipment.

MATERIALS AND METHODS

Strains and culture media. *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and 20 reference strains were gifts of the National Tuberculosis Reference Laboratory (Beijing, China). The reference strains were as follows: *Mycobacterium bovis* ATCC 19210, *M. africanum* ATCC 25420, *M. kansasii* ATCC 12478, *M. intracellulare* ATCC 13950, *M. chelonae* ATCC 14472, *M. fortuitum* ATCC 6481, *M. gordonae* ATCC 14470, *M. aurum* ATCC 23366, *M. neoaurum* ATCC 25795, *M. marinum* ATCC 927, *M. gilvum* ATCC 43909, *M. aichiense* ATCC 27280, *M. smegmatis* ATCC 19420, *M. parafortuitum* ATCC 19686, *M. nonchromogenicum* ATCC 19530, *M. vaccae* ATCC 15483, *M. avium* ATCC 25291, *M. phlei* ATCC 11758, *M. scrofulaceum* ATCC 19981, and *M. malmoense* ATCC 29571. All mycobacteria were cultured using Middlebrook 7H9 liquid culture supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton Dickinson, NJ). *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, as well as the clinical isolates, were obtained from the National Institute for Food and Drug Control (Beijing, China). *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were cultured on MacConkey agar, *Staphylococcus aureus* was cultured on blood agar, and *Escherichia coli* was cultured on Luria-Bertani agar plates.

Specimen collection and processing. A total of 253 sputum samples were obtained from patients with active PTB, before treatment or within a week of treatment initiation, including newly diagnosed and relapsed patients. In addition, 134 sputum samples were randomly collected from respiratory disease patients for whom TB was excluded. All of the specimens were collected from the Shandong Chest Hospital and the Shanghai Pulmonary Hospital. The active pulmonary tuberculosis case definition was a patient with a positive sputum culture for the *M. tuberculosis* complex or a patient who was diagnosed as having PTB by a clinician, according to clinical diagnostic criteria. Diagnostic criteria should include radiographic abnormalities consistent with active pulmonary tuberculosis, no

Received 20 September 2011 Returned for modification 29 October 2011
Accepted 21 December 2011
Published ahead of print 28 December 2011
Address correspondence to Zhongyi Hu, shtblab@163.com.
Z. Cui and Y. Wang contributed equally to this article.

doi:10.1128/JCM.05853-11

response to a course of broad-spectrum antibiotics (except in a patient for whom there is laboratory confirmation or strong clinical evidence of HIV infection), and the decision by a clinician to treat the patient with a full course of antituberculosis chemotherapy (43). Of the 253 samples from PTB patients, 123 specimens were smear positive for acid-fast bacilli (AFB) and 130 were smear negative for AFB (42). Of the 134 samples from other respiratory disease patients, 2 specimens were smear positive for AFB and 132 were smear negative for AFB. Each specimen of approximately 5 ml was decontaminated using the *N*-acetyl-L-cysteine (NALC)–NaOH method (31). The processed sediment was washed once using a sterile 0.9% NaCl solution and resuspended in 1.5 ml sterile 0.9% NaCl solution; three separate 500-μl aliquots were prepared in 1.5-ml tubes for the SAT-TB, real-time PCR, and Bactec MGIT 960 culture tests.

Principles of SAT-TB assay. Briefly, *M. tuberculosis* 16S rRNA was reverse transcribed by use of Moloney murine leukemia virus (M-MLV) reverse transcriptase to generate a 170-bp DNA fragment, using a pair of specific primers that incorporated the T7 promoter sequence in the sense primer. The DNA was subsequently transcribed to RNA by using T7 RNA polymerase in order to undergo successive cycles of amplification. An internal labeled probe was included, which released a fluorescence signal when hybridized with the target RNA. Kinetic measurements of the real-time amplification fluorescence signal were detected using a real-time PCR instrument. The specific *M. tuberculosis* 16S rRNA sense primer containing the T7 promoter sequence was 5′-AATTTAATACGACTCACTATAGGGAGAGTAGGCCGTCACCCCACCAACAAGCTG-3′, and the antisense primer was 5′-CTGGGAAACTGGGTCTAATAC-3′. The probe sequence was 5′-CCAGCCACGGGAUGCAUGCUGG-3′ and was labeled with 6-carboxyfluorescein (FAM) phosphoramidite at the 5′ end and with 4-[4-(dimethylamino)phenylazo] benzoic acid *N*-succinimidyl ester (DABCYL) at the 3′ end.

SAT-TB method. To process the clinical samples for the SAT-TB assay, the aliquoted processed sediments were centrifuged, the supernatants were discarded, and 50 μl TB dilution solution (10 mM sodium citrate, pH 8.0) was added to each tube and vortexed. Each sample was sonicated for 15 min at room temperature in a water bath sonicator (Shanghai Sheng-Yan Ultrasound Machines Co. Ltd., Shanghai, China) at 300 W and then centrifuged, and the supernatant was used in the SAT-TB assay. The live *M. tuberculosis* attenuated strain H37Ra (ATCC 25177) was used as a positive control, and the negative control was double-distilled water.

For the assay, 2 μl of processed supernatant and 30 μl of a reaction solution containing 40 mM Tris-HCl, pH 8.1, 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine hydrochloride, 5 mM dithiothreitol, 80 μg/ml bovine serum albumin, 200 μM dATP, 200 μM dTTP, 200 μM dGTP, 200 μM dCTP, 1 mM ATP, 1 mM GTP, 1 mM CTP, 1 mM UTP, and 0.5 mM (each) primers and probe were prepared in a 200-μl PCR tube. The mixture was preincubated at 60°C for 10 min, followed by 42°C for 5 min, and then a 10-μl aliquot containing 2,000 units M-MLV reverse transcriptase and 2,000 units T7 RNA polymerase (RD Bioscience, Inc., San Diego, CA) was added and gently mixed. The reaction mix was then placed immediately into a model 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA). RNA isothermal amplification was conducted at 42°C for 1 min, with a total of 40 cycles, and FAM fluorescence data were collected after each cycle of amplification. Specimens with cycle thresholds (C_T) of ≤40 were classified as TB positive.

The above processes were performed in a biological safety cabinet (class II B2) in a biosafety level PII laboratory. RNase-free tubes and tips were required for the SAT-TB assay.

Determination of SAT-TB sensitivity. An *M. tuberculosis* H37Rv bacterial suspension in log-phase growth was adjusted to a McFarland standard of 1 (approximately 10^7 CFU/ml) in sterile saline (40) and then further diluted with progressive 10-fold dilutions to 10^{-10} CFU/ml in tubes with glass beads containing 9 ml sterile saline. A 1-ml aliquot of each dilution was centrifuged in a 1.5-ml tube, and the sediments were processed for the SAT-TB assay as described above. Additionally, a 0.1-ml aliquot of each dilution was inoculated into one L-J culture tube for colony counts of *M. tuberculosis* H37Rv.

At the same time, a pooled sputum sample was prepared from samples from 10 respiratory disease patients with no mycobacterial infection and divided into 10 equal sputum specimens. A 1-ml aliquot of each dilution was spiked into a corresponding sputum specimen and vortexed. The sputum specimens were processed for the SAT-TB assay as described above. All SAT-TB and L-J culture tests were performed in triplicate.

Determination of SAT-TB specificity. The log-phase mycobacterial suspensions and *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* cultures were adjusted to a turbidity equal to 10^6 CFU/ml, and a 1-ml suspension of each bacterial culture was tested using the SAT-TB assay.

Bactec MGIT 960 culture. Bactec MGIT 960 (Becton Dickinson and Company, MD) culture was performed according to the manufacturer's instructions (19). The *M. tuberculosis* complex isolates from positive cultures were identified by conventional biochemical tests according to a standard protocol (31).

Real-time PCR assay. *M. tuberculosis* was detected in processed sediments by using *Mycobacterium tuberculosis* PCR fluorescence diagnostic kits (Da An Gene Co., Ltd., Dalian, China) according to the manufacturer's instructions, using a model 7500 real-time PCR system. The detected target gene of this kit is the *M. tuberculosis* repetitive sequence IS*986*. The sequences of the primers and probe are 5′-TCGCCCGTCTACTTGGTGTT-3′, 5′-TGATGTGGTCGTAGTAGGTC-3, and 5′-ACAACGCCGAATTGCGAAGGGC-3 (labeled with FAM at the 5′ end and with DABCYL at the 3′ end). The sensitivity of this kit is 10 CFU/ml (3), according to the manufacturer, and we also confirmed this level of sensitivity.

Resolution of discrepancies. The real-time PCR assay and sequencing were used to resolve all specimens with discordant Bactec MGIT 960 culture and SAT-TB results. For SAT-TB-positive samples which were negative by both the real-time PCR assay and Bactec MGIT 960 culture, the SAT-TB cDNA amplification products were sequenced using the primer with the sequence 5′-AATTTAATACGACTCACTATAG-3′. For Bactec MGIT 960 culture-positive specimens that were SAT-TB negative and real-time PCR negative, genomic DNA was extracted from the culture, and the 16S rRNA fragment was amplified and sequenced as previously described (4).

Statistical analysis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for the SAT-TB results versus Bactec MGIT 960 culture results as well as for the SAT-TB and Bactec MGIT 960 culture results versus the clinical diagnosis. The agreement between the SAT-TB and Bactec MGIT 960 culture results was analyzed using the kappa value. The detection rates for *M. tuberculosis* by the SAT-TB assay and Bactec MGIT 960 culture were compared using the Mann-Whitney U test.

RESULTS

Methodological sensitivity and specificity of SAT-TB assay. The methodological sensitivity of the SAT-TB assay for *M. tuberculosis* H37Rv in sterile saline was 100 CFU/ml. The sensitivity of the SAT-TB assay for *M. tuberculosis* H37Rv in spiked sputum was 100 CFU per specimen. No other nontuberculous mycobacteria or common respiratory tract pathogens were detected using the SAT-TB method.

Comparison of SAT-TB assay and Bactec MGIT 960 culture. The results for 387 sputum samples tested by the SAT-TB and Bactec MGIT 960 culture methods are shown in Table 1. One active PTB patient specimen was identified as positive by the Bactec MGIT 960 culture system and negative by the SAT-TB assay, and the real-time PCR assay identified it as *M. tuberculosis* by the use of 16S rRNA sequencing. The 16 Bactec MGIT 960 culture-negative and SAT-TB-positive specimens were all positive in the real-time PCR assay. The amplification products of these culture-

TABLE 1 Performances of SAT-TB and Bactec MGIT 960 culture methods with clinical sputum specimens

SAT-TB result	No. of specimens with Bactec MGIT 960 culture result		Mean % (95% CI)			
	Positive	Negative	Sensitivity	Specificity	PPV	NPV
All samples			99.4 (96.5–99.9)	93.1 (89.1–95.7)	90.6 (85.3–94.2)	99.5 (97.4–99.9)
Positive	155	16				
Negative	1	215				
AFB smear-positive samples			100 (96.9–100)	71.4 (35.9–91.8)	98.3 (94.1–99.5)	100 (56.6–100)
Positive	118	2				
Negative	0	5				
AFB smear-negative samples			97.4 (86.5–99.5)	89.74 (85.19–93.0)	72.6 (59.1–82.9)	99.5 (97.4–99.9)
Positive	37	14				
Negative	1	210				

negative specimens were verified as *M. tuberculosis* by sequence analysis of the SAT-TB amplification products. The SAT-TB and Bactec MGIT 960 culture results agreed for 95.6% (370/387 specimens) of the sputum specimens, with a kappa coefficient of 0.91, indicating substantial agreement between the two tests.

Performance of SAT-TB assay for PTB diagnosis. The performances of the SAT-TB and Bactec MGIT 960 culture methods compared to clinical diagnoses are shown in Table 2. For the smear-positive specimens, the TB detection sensitivity of the SAT-TB assay was similar to that of the Bactec MGIT 960 culture method, according to the U test ($U = 1.24$; $P > 0.05$ for sensitivity). For the smear-negative specimens, the TB detection sensitivity of the SAT-TB assay was higher than that of the Bactec MGIT 960 culture system ($U = 2.10$; $P < 0.05$).

DISCUSSION

Culture of *M. tuberculosis* is the gold standard for diagnosis of active PTB from clinical samples. However, cultivation on L-J culture medium is laborious and often takes 3 to 8 weeks. Rapid culture systems, such as the Bactec MGIT 960 and BacT/Alert 3D automated systems, greatly speed up the detection process; however, these methods still take an average of 7 to 10 days for smear-positive specimens (5, 33). NAA techniques, which are mostly based on PCR, have provided additional simple, rapid, and accurate methods for detection of *M. tuberculosis* in clinical specimens (14, 15, 23, 30, 37, 38).

Our novel SAT-TB method provides several distinct advantages over PCR, as the detection target is rRNA. RNA products are much more labile outside the reaction tube than DNA amplification products. Since PCR detection commonly suffers from severe contamination problems, the SAT-TB assay should reduce the risk of laboratory contamination and false-positive results (1). The SAT-TB assay uses the same principles of isothermal RNA amplification as the MTD assay (12, 18, 21, 25, 27, 28, 35, 39). NAA based on isothermal amplification of RNA has been used to detect several pathogens, including *M. tuberculosis*, human immunodeficiency virus type 1, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* (10, 16, 24, 45). In contrast to the MTD assay, the SAT-TB assay can be performed on real-time PCR instruments, which are found routinely in most clinical laboratories. Additionally, since

TABLE 2 Performances of SAT-TB and Bactec MGIT 960 culture methods compared with clinical assessment of patients

Method and group	Result	No. of cases with clinical diagnosis of TB		Mean % (95% CI)			
		Positive	Negative	Sensitivity	Specificity	PPV	NPV
SAT-TB assay							
All samples	Positive	171	0	67.6 (61.6–73.1)	100 (97.2–100)	100 (97.8–100)	62.0 (55.4–68.3)
	Negative	82	134				
AFB smear-positive group	Positive	120	0	97.6 (93.1–99.2)	100 (34.2–100)	100 (96.9–100)	40.0 (11.8–76.9)
	Negative	3	2				
AFB smear-negative group	Positive	51	0	39.2 (31.26–47.8)	100 (97.2–100)	100 (93.0–100)	62.6 (55.9–68.8)
	Negative	79	132				
MGIT 960 culture							
All samples	Positive	156	0	61.7 (55.5–67.43)	100 (97.2–100)	100 (97.6–100)	58.0 (51.6–64.2)
	Negative	97	134				
AFB smear-positive group	Positive	118	0	95.9 (90.8–98.3)	100 (34.2–100)	100 (96.9–100)	28.6 (8.2–64.1)
	Negative	5	2				
AFB smear-negative group	Positive	38	0	30.2 (22.8–38.7)	100 (97.2–100)	100 (90.8–100)	60.0 (53.4–66.3)
	Negative	88	132				

the SAT-TB assay produces a real-time amplification signal, C_T values can be used for relative quantification of *M. tuberculosis*, which could potentially inform the clinical management of PTB therapy.

In this study, the SAT-TB assay did not nonspecifically amplify or detect 18 nontuberculous mycobacteria or several other common pathogenic lung bacteria, suggesting that the SAT-TB assay is highly specific for *M. tuberculosis*. Serial dilution of *M. tuberculosis* showed that the SAT-TB assay has a high sensitivity and can detect as few as 100 CFU/ml. Additionally, all negative-control samples showed negative results in the SAT-TB assay.

The traditional gold standard for the diagnosis of tuberculosis is the culture of clinical specimens; therefore, the most direct assessment of NAA test performance is comparison with culture results (35). This study showed a 95.6% agreement between the results of the SAT-TB and Bactec MGIT 960 culture methods. Compared to Bactec MGIT 960 culture, the sensitivity and specificity of the SAT-TB assay were similar to previously reported values for the MTD assay (2, 11, 20). All specimens with discordant results between the Bactec MGIT 960 culture and SAT-TB methods should be tested by another method. The MTD test is the first alternative choice because it is similar to the SAT-TB method. However, due to the expense of the MTD reagents and equipment, we used a real-time PCR assay as our reference method. Of the 16 specimens with discordant Bactec MGIT 960 culture and SAT-TB results, the real-time PCR results for all samples were the same as those by the SAT-TB assay, and all of the SAT-TB- and real-time PCR amplification-positive products of 16 culture-negative specimens were verified as truly *M. tuberculosis* positive by sequencing, indicating that the SAT-TB assay did not give any false-positive results. The SAT-TB false-negative result for one specimen could potentially have occurred due to the presence of inhibitors of enzymatic amplification, suboptimal target extraction, a low starting concentration, or an uneven distribution of mycobacteria in the sample. The diagnostic accuracy of the SAT-TB assay could be improved by addition of an internal control to detect potential enzymatic inhibitors.

The primary objectives of a new PTB diagnosis method are to enhance the TB detection sensitivity and to have a high specificity. We therefore evaluated the accuracy of the SAT-TB assay relative to clinical diagnosis. The SAT-TB assay showed a 100% specificity and PPV for PTB patients, similar to the reported values for the MTD assay (7, 21). In this study, the SAT-TB sensitivity (67.6%) for all PTB patients was higher than that for Bactec MGIT 960 culture (61.7%); however, the SAT-TB sensitivity was lower than reported values for the MTD assay (80 to 98%) (6, 22, 28, 32, 36, 41). These differences may be due mainly to the different compositions of specimens from a population of PTB patients with various smear results.

The performances of the different methods in this study were assessed by direct comparison to a comprehensive clinical diagnosis, including clinical and laboratory data for each patient. For smear-positive specimens, the SAT-TB assay showed the same excellent performance as the Bactec MGIT 960 culture method, with a high sensitivity and specificity. For smear-negative specimens, the SAT-TB sensitivity for the diagnosis of TB was higher than that of Bactec MGIT 960 culture. Since the Bactec MGIT 960 culture system is not specific for culture of the *M. tuberculosis* complex, culture-positive bacteria need to undergo conventional biochemical or molecular tests for identification of this complex. The SAT-TB assay is significantly simpler than the Bactec MGIT 960 culture method and requires only a few hours to complete, making it a much more rapid method.

As an isothermal nucleic acid amplification technique, the loop-mediated isothermal amplification (LAMP) assay also shows a high sensitivity and specificity for the detection of *M. tuberculosis* (13, 26, 29, 46). However, it can be difficult for the LAMP assay to reflect the changes in viable *M. tuberculosis* bacteria from clinical samples over time. Lee et al. reported a method that combines reverse transcription, loop-mediated isothermal amplification, and enzyme-linked immunosorbent assay (RT-LAMP-ELISA) for the rapid detection of viable *M. tuberculosis* organisms in clinical samples in Taiwan (26), but this technique still reflects indirect changes in RNA composition and requires more manipulation processes than the SAT-TB assay. Additionally, although the LAMP assay reduces the risk of workspace contamination with other amplicons, other DNA contaminations from the workspace can still lead to false-positive results with this assay.

In summary, this study shows that the SAT-TB assay is a sensitive and specific method for the direct detection of the *M. tuberculosis* complex, with a high detection rate for clinical specimens. Furthermore, the SAT-TB assay is a simple and rapid technique compared to traditional culture methods. The SAT-TB assay is a promising tool for the rapid diagnosis of *M. tuberculosis* and other mycobacterial infections in clinical microbiology laboratories.

ACKNOWLEDGMENTS

This work was supported by grant 10411955100 from the Science and Technology Commission of Shanghai Municipality and grant 10QA1405800 from the Shanghai Rising-Star Program.

We are grateful to all members of the Shanghai Key Laboratory of Tuberculosis, Shanghai Pulmonary Hospital, Medical School, Tongji University, for their knowledge, helpfulness, and willingness to share.

REFERENCES

1. **Abe C, et al.** 1993. Detection of Mycobacterium tuberculosis in clinical specimens by polymerase chain reaction and Gen-Probe amplified Mycobacterium tuberculosis direct test. Clin. Microbiol. **31**:3270–3274.
2. **Bergmann JS, Yuoh G, Fish G, Woods GL.** 1999. Clinical evaluation of the enhanced Gen-Probe amplified Mycobacterium tuberculosis direct test for rapid diagnosis of tuberculosis in prison inmates. J. Clin. Microbiol. **37**:1419–1425.
3. **Cheng G, He YS, Zhou XY, Deng WG, Li H.** 2002. Development and clinical trial of fluorescence PCR diagnostic kit to detect Mycobacterium tuberculosis. Di Yi Jun Yi Da Xue Xue Bao **22**:533–535.
4. **Cook VJ, Turenne CY, Wolfe J, Pauls R, Kabani A.** 2003. Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. J. Clin. Microbiol. **41**:1010–1015.
5. **Cruciani M, et al.** 2004. Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria. J. Clin. Microbiol. **42**:2321–2325.
6. **Dalovisio JR, et al.** 1996. Comparison of the amplified Mycobacterium tuberculosis (MTB) direct test, Amplicor MTB PCR, and IS6110-PCR for detection of MTB in respiratory specimens. Clin. Infect. Dis. **23**:1099–1106.
7. **Davis JL, et al.** 2011. Nucleic acid amplification tests for diagnosis of smear-negative TB in a high HIV-prevalence setting: a prospective cohort study. PLoS One **6**:e16321.
8. **Dinnes J, et al.** 2007. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. Health Technol. Assess. **11**:1–196.
9. **Dowdy DW, Maters A, Parrish N, Beyrer C, Dorman SE.** 2003. Cost-effectiveness analysis of the Gen-Probe amplified Mycobacterium tuberculosis direct test as used routinely on smear-positive respiratory specimens. J. Clin. Microbiol. **41**:948–953.
10. **Fang L, Zhang C, Yu M, Wang M, Ju J.** December 2009. A kit for

detection of Neisseria gonorrhoeae based on isothermal transcription-mediated amplification. China open patent number CN101591707.
11. **Gallina M, Troupioti P, Rocco G, Sensalari G, Libanori E.** 2000. Predicting culture results for Mycobacterium tuberculosis complex. Amplified Mycobacterium tuberculosis direct test and acid-fast bacilli microscopy. Chest **118**:28–32.
12. **Gamboa F, et al.** 1998. Comparative evaluation of initial and new versions of the Gen-Probe amplified Mycobacterium tuberculosis direct test for direct detection of Mycobacterium tuberculosis in respiratory and non-respiratory specimens. J. Clin. Microbiol. **36**:684–689.
13. **George G, Mony P, Kenneth J.** 2011. Comparison of the efficacies of loop-mediated isothermal amplification, fluorescence smear microscopy and culture for the diagnosis of tuberculosis. PLoS One **6**:e21007.
14. **Greco S, Girardi E, Navarra A, Saltini C.** 2006. Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. Thorax **61**:783–790.
15. **Greco S, Rulli M, Girardi E, Piersimoni C, Saltini C.** 2009. Diagnostic accuracy of in-house PCR for pulmonary tuberculosis in smear-positive patients: meta-analysis and metaregression. J. Clin. Microbiol. **47**:569–576.
16. **Guatelli JC, et al.** 1990. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc. Natl. Acad. Sci. U. S. A. **87**:7797.
17. **Guerra RL, et al.** 2008. Specimen dilution improves sensitivity of the amplified Mycobacterium tuberculosis direct test for smear microscopy-positive respiratory specimens. J. Clin. Microbiol. **46**:314–316.
18. **Guerra RL, et al.** 2007. Use of the amplified Mycobacterium tuberculosis direct test in a public health laboratory: test performance and impact on clinical care. Chest **132**:946–951.
19. **Hanna BA, et al.** 1999. Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. J. Clin. Microbiol. **37**:748–752.
20. **Hoffner SE, et al.** 1996. Direct detection of Mycobacterium tuberculosis in sputum samples from Guinea Bissau by an rRNA target-amplified test system. Tuber. Lung Dis. **77**:67–70.
21. **Jonas V, et al.** 1993. Detection and identification of Mycobacterium tuberculosis directly from sputum sediments by amplification of rRNA. J. Clin. Microbiol. **31**:2410–2416.
22. **Kambashi B, et al.** 2001. Utility of nucleic acid amplification techniques for the diagnosis of pulmonary tuberculosis in sub-Saharan Africa. Int. J. Tuberc. Lung Dis. **5**:364–369.
23. **Kearns AM, Freeman R, Steward M, Magee JG.** 1998. A rapid polymerase chain reaction technique for detecting M. tuberculosis in a variety of clinical specimens. J. Clin. Pathol. **51**:922–924.
24. **Kwoh DY, et al.** 1989. Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc. Natl. Acad. Sci. U. S. A. **86**:1173–1177.
25. **Lebrun L, Espinasse F, Poveda JD, Vincent-Levy-Frebault V.** 1992. Evaluation of nonradioactive DNA probes for identification. J. Clin. Microbiol. **30**:2476–2478.
26. **Lee MF, Chen YH, Peng CF.** 2009. Evaluation of reverse transcription loop-mediated isothermal amplification in conjunction with ELISA-hybridization assay for molecular detection of Mycobacterium tuberculosis. J. Microbiol. Methods **76**:174–180.
27. **Lumb R, Lanser JA, Lim IS.** 1993. Rapid identification of mycobacteria by the Gen-Probe Accuprobe system. Pathology **25**:313–315.
28. **Musial CE, Tice LS, Stockman L, Roberts GD.** 1988. Identification of mycobacteria from culture by using the Gen-Probe rapid diagnostic system for Mycobacterium avium complex and Mycobacterium tuberculosis complex. J. Clin. Microbiol. **26**:2120–2123.
29. **Neonakis IK, Spandidos DA, Petinaki E.** 2011. Use of loop-mediated isothermal amplification of DNA for the rapid detection of Mycobacterium tuberculosis in clinical specimens. Eur. J. Clin. Microbiol. Infect. Dis. **30**:937–942.
30. **Noordhoek GT, van Embden JD, Kolk AH.** 1996. Reliability of nucleic acid amplification for detection of Mycobacterium tuberculosis: an international collaborative quality control study among 30 laboratories. J. Clin. Microbiol. **34**:2522–2525.
31. **Pfyffer GE, Brown-Elliot BA, Wallace RJ.** 2003. General characteristics, isolation, and staining procedures, p 532–559. *In* Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH (ed), Manual of clinical microbiology, 8th ed. ASM Press, Washington, DC.
32. **Piersimoni C, et al.** 1998. Comparative evaluation of the new Gen-Probe Mycobacterium tuberculosis amplified direct test and the semiautomated Abbott LCx Mycobacterium tuberculosis assay for direct detection of Mycobacterium tuberculosis complex in respiratory and extrapulmonary specimens. J. Clin. Microbiol. **36**:3601–3604.
33. **Piersimoni C, et al.** 2001. Comparison of MB/Bact Alert 3D system with radiometric BACTEC system and Löwenstein-Jensen medium for recovery and identification of mycobacteria from clinical specimens: a multicenter study. J. Clin. Microbiol. **39**:651–657.
34. **Piersimoni C, et al.** 2002. Performance assessment of two commercial amplification assays for direct detection of Mycobacterium tuberculosis complex from respiratory and extrapulmonary specimens. J. Clin. Microbiol. **40**:4138–4142.
35. **Pollock N, Westerling J, Sloutsky A.** 2006. Specimen dilution increases the diagnostic utility of the Gen-Probe Mycobacterium tuberculosis direct test. Am. J. Clin. Pathol. **126**:142–147.
36. **Portaels F, et al.** 1996. Evaluation of the Gen-Probe amplified Mycobacterium tuberculosis direct test for the routine diagnosis of pulmonary tuberculosis. Acta Clin. Belg. **51**:144–149.
37. **Sandin RL.** 1996. Polymerase chain reaction and other amplification techniques in mycobacteriology. Clin. Lab. Med. **16**:617–639.
38. **Suffys P, et al.** 2000. Evaluation of the polymerase chain reaction for the detection of Mycobacterium tuberculosis. Int. J. Tuberc. Lung Dis. **4**:179–183.
39. **Syre H, Myneedu VP, Arora VK, Grewal HM.** 2009. Direct detection of mycobacterial species in pulmonary specimens by two rapid amplification tests, the Gen-Probe amplified Mycobacterium tuberculosis direct test and the genotype mycobacteria direct test. J. Clin. Microbiol. **47**:3635–3639.
40. **Vandepitte J, et al.** 2003. Basic laboratory procedures in clinical bacteriology, 2nd ed. World Health Organization, Geneva, Switzerland.
41. **Vuorinen P, Miettinen A, Vuento R, Hällström O.** 1995. Direct detection of Mycobacterium tuberculosis complex in respiratory specimens by Gen-Probe amplified Mycobacterium tuberculosis direct test and Roche Amplicor Mycobacterium tuberculosis test. J. Clin. Microbiol. **33**:1856–1859.
42. **World Health Organization.** 2003. Manual of basic techniques for a health laboratory, 2nd ed. World Health Organization, Geneva, Switzerland.
43. **World Health Organization.** 2009. Treatment of tuberculosis: guidelines, 4th ed. World Health Organization, Geneva, Switzerland.
44. **Yang YC, et al.** 2011. Evaluation of the COBAS TaqMan MTB test for direct detection of Mycobacterium tuberculosis complex in respiratory specimens. J. Clin. Microbiol. **49**:797–801.
45. **Zhang C, Fang L, Yu M, Wang M, Ju J.** August 2009. A kit for detection of Chlamydia trachomatis based on isothermal transcription-mediated amplification. China open patent number CN101509041.
46. **Zhu RY, et al.** 2009. Use of visual loop-mediated isotheral amplification of rimM sequence for rapid detection of Mycobacterium tuberculosis and Mycobacterium bovis. J. Microbiol. Methods **78**:339–343.