JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2004, p. 4599–4603 Vol. 42, No. 10
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.10.4599–4603.2004

Performance Assessment of a Nested-PCR Assay (the RAPID BAP-MTB) and the BD ProbeTec ET System for Detection of *Mycobacterium tuberculosis* in Clinical Specimens

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Received 20 April 2004/Returned for modification 14 June 2004/Accepted 30 June 2004

The performance of a nested PCR-based assay (the RAPID BAP-MTB; AsiaGen, Taichung, Taiwan) and the BD ProbeTec ET (DTB) system (Becton Dickinson, Sparks, Md.) for detection of *Mycobacterium tuberculosis* was evaluated with 600 consecutive clinical samples. These samples, including 552 respiratory specimens and 48 nonrespiratory specimens, were collected from 333 patients treated at National Taiwan University Hospital from September to October 2003. The results of both assays were compared to the gold standard of combined culture results and clinical diagnosis. The overall sensitivity and specificity of the RAPID BAP-MTB assay for respiratory specimens were 66.7% and 97.2%, respectively, and for the DTB assay they were 56.7% and 95.3%, respectively. The positive and negative predictive values for the RAPID BAP-MTB were 74.1% and 96.0%, respectively, and for the DTB assay they were 59.6% and 94.7%, respectively. For smear-negative samples, the sensitivity of the RAPID BAP-MTB and DTB assays was 57.1% and 40.5%, respectively. The RAPID BAP-MTB assay produced 14 false-positive results in 14 samples, including one of the six samples yielding *Mycobacterium abscessus*, one of the six samples yielding *Mycobacterium avium intracellulare*, one sample from a patient with a history of pulmonary tuberculosis with complete treatment, and three samples from three patients with a previous diagnosis of tuberculosis who were under treatment at the time of specimen collection. Among the 48 nonrespiratory specimens, the RAPID BAP-MTB assay was positive in one biopsy sample from a patient with lumbar tuberculous spondylitis and one pus sample from a patient with tuberculous cervical lymphadenopathy. Our results showed that the RAPID BAP-MTB assay is better than the DTB assay for both respiratory specimens and nonrespiratory specimens. The overall time for processing this assay is only 5 h. In addition, its diagnostic accuracy in smear-negative samples is as high as in smear-positive samples.

Tuberculosis remains one of the deadliest diseases worldwide. The World Health Organization estimated that in this decade, 300 million more people will become infected with tuberculosis, and 30 million people will die from this disease (24). In 2001, the incidence and mortality of tuberculosis in Taiwan were 64.84 and 5.81 per 100,000, respectively (5). Successful control of tuberculosis depends on effective case finding and rapid detection of *Mycobacterium tuberculosis* complex. The conventional method for laboratory diagnosis of tuberculosis is based on acid-fast staining and culture. Staining and microscopy are a rapid screening method for detection of acid-fast bacilli in clinical specimens but have low sensitivity (3, 14). Moreover, this method does not discriminate *M. tuberculosis* complex from nontuberculous mycobacteria, which are causing increasing numbers of infections in immunocompromised hosts, particularly in patients with AIDS. Culture has acceptable sensitivity and specificity but may take about 10 days on average to detect positive specimens even with a radiometric procedure (15).

Newer diagnostic methods employing nucleic acid amplification and detection may provide very quick and specific tests for identification of *M. tuberculosis* complex (4, 6, 7, 9-12, 16, 17, 19-21, 25). Among them, the BD ProbeTec ET Direct TB System (DTB), which uses an internal amplification control designed to detect the presence of inhibiting substances, has been reported to have an excellent performance (9, 18, 19). Although these newer amplification methods can theoretically detect a single copy of genomic sequence, their sensitivities are considerably less than that of culture (18). Recently, the AsiaGen Corporation in Taiwan developed a new assay for detection of *M. tuberculosis* complex, the RAPID BAP-MTB assay. This technique uses a nested PCR technique, which can improve the sensitivity of detection of *M. tuberculosis* complex in paucibacillary (smear-negative) specimens (13, 25). In addition, the technique can decrease false-positive events because the probability of a wrong locus being mistakenly amplified twice is very low.

The purpose of this study was to compare the value of the DTB assay and the RAPID BAP-MTB assay for the identification of *M. tuberculosis* complex in clinical samples.

MATERIALS AND METHODS

Specimen collection and processing. A total of 600 consecutive clinical specimens were collected, including 552 respiratory specimens (527 sputum and 25 bronchial wash specimens) from 299 patients and 48 nonrespiratory specimens from 34 patients (Table 1). All of these patients were treated at the National

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TABLE 1. Clinical information on the 333 patients and microbiological findings for 600 clinical specimens from these patients

Specimen[a]	No. of patients	No. of specimens
Respiratory	299	552
Active pulmonary TB, not treated	24	60
Pulmonary TB, under treatment	20	38
Old pulmonary TB	5	12
Smear positive	15	21
M. tuberculosis	9	12
M. abscessus	2	2
Culture negative	5	7
Culture positive	37	52
M. tuberculosis	17	30
M. avium intracellulare complex	6	7
M. abscessus	6	6
M. chelonae	3	3
M. fortuitum	2	3
M. kansasii	1	1
Unidentified species	2	2
Nonrespiratory	34	48
Smear positive/culture positive for *M. tuberculosis*	0	0
Smear negative/culture positive for *M. tuberculosis*	1	1[b]
Smear positive/culture negative for *M. tuberculosis*	1	1[c]

[a] TB, tuberculosis.
[b] This was a pus sample from a patient with tuberculosis-related cervical lymphadenopathy.
[c] This was a lumbar spine biopsy sample from a patient with tuberculosis spondylitis.

Taiwan University Hospital from September to October 2003. The 48 nonrespiratory specimens included 20 samples from normal sterile body fluids (10 samples from pleural effusion, four from ascites, three from cerebrospinal fluid, two from synovial fluid, and one from pericardial effusion), 15 pus samples (six from tracheostomy, four from soft tissue abscess, three from surgical spinal wound, one from neck lymphadenopathy, and one from liver abscess), 10 urine samples, and three biopsy specimens (one each from neck lymph node, lumbar spine, and pleura). Specimens that could not be processed on receipt were stored at 2 to 8°C for no longer than 48 h. All specimens were processed and treated as previously described (19). Briefly, each specimen was processed by adding an equal volume of NaOH–citrate–*N*-acetyl-L-cysteine at room temperature for 15 min. After centrifugation, the precipitate was resuspended in 1 ml of phosphate-buffered saline (pH 7.4).

Smear and culture. Smears of the processed specimens for acid-fast bacilli were stained with auramine-rhodamine fluorochrome and examined by standard procedures (15). Fluorochrome stain-positive smears were confirmed by the Kinyoun stain method (15). Cultures were performed by inoculating 0.5 ml of sediment onto Middlebrook 7H11 selective agar with antimicrobials (Remel Inc., Lexena, Kans.) (10) and by the fluorometric BACTEC technique (BACTEC MGIT 960 system; Becton-Dickinson Diagnostic Instrument Systems, Sparks, Md.) as previously described (16).

BD ProbeTec ET (DTB) system. The test was done according to the instructions supplied by the manufacturer (package insert; Becton Dickinson). The procedure consisted of two steps, specimen preparation and combined, fully automated amplification and detection. When the amplification signal was greater than 3,400 units of MOTA (metric other than acceleration), the result was considered positive regardless of the values of the internal amplification control. Values lower than 3,400 were considered negative when the internal amplification control value was greater than 5,000 and indeterminate when the internal amplification control value was less than 5,000 (presence of an inhibitor for this assay). In the latter case, samples were retested, and the results of the repeated tests were used in the analysis (19).

RAPID BAP-MTB assay. Extract DNA was started by mixing double-distilled H_2O with resuspended precipitate in a lysis tube. After 20 s of vortexing and 15 min of centrifugation at 3,800 rpm, the supernatant was removed. The pellet was resuspended in 150 μl of lysis buffer I (KOH, pH 13.1) at room temperature for 10 min and at 100°C in a water bath for another 20 min. Then, 150 μl of lysis buffer II (HCl and acetic acid, pH 1.2) was added. After centrifuging at 10,000 rpm for 2 min, a 3-μl volume of supernatant was transferred to an amplification tube containing 50 μl of amplification reagent (Tris-HCl, $MgCl_2$, dATP, dGTP, dTTP, dCTP, external primer, and AmpliTaq DNA polymerase) for nested PCR.

The primers for nested PCR were derived from the *M. tuberculosis* genome, encoding the insertion sequence IS*6110* with the sequence of the external primer: 5′-GTGAGGGCATCGAGGTGG-3′ and 5′-CGTAGGCGTCGGTCACAAA-3′ and internal primers 5′-GATGCACCGTCGAACG-3′ and 5′-biotin-CCACGGTAGGCGAACCCT-3′.

For each assay, one negative control was prepared. The nested PCR was carried out in a thermal reactor. After a 5-min incubation at 94°C, the first amplification was performed for 27 cycles of 94°C for 30 s, 63.7°C for 15 s, and 72°C for 15 s. After the last cycle, the samples were incubated for 10 min at 72°C. Negative controls contained the PCR mixture without the template DNA. The second amplification was performed with the same extension program except the external primer was replaced by the internal primer.

In a hybridization tube, 10 μl of each amplified DNA sample and 290 μl of hybridization reagent, containing 15 μl of MagProbe (beads with probe; 5′-amine-ACCTAACCGGCTGTGGGTAGCAGA) and 150 μl of hybridization buffer, was added, vortexed, and incubated at 95°C for 5 min and at 60°C for 20 min in a dry bath. Tubes were then transferred to magnetic wells for 5 min. The hybridization buffer was removed by aspiration without disturbing the MagProbe. After adding a 1-ml volume of preheated 60°C wash buffer, the tubes were vortexed and held in a magnetic well for 5 min. The wash buffer was removed by aspiration. The wash steps were then repeated once again.

After adding blocking solution and streptavidin-horseradish peroxidase, the hybridization tube was vortexed, kept from light at room temperature for 20 min, and transferred back to the magnetic well for another 5 min. Then the supernatant was removed by careful aspiration. After washing twice and resuspending with phosphate-buffered saline, the tube was placed in a luminometer to determine the number of relative light units (RLU) produced by the reaction.

The RAPID BAP-MTB results were interpreted as follows. When the control tube showed an RLU value equal to or greater than 25,000, the test was repeated. When the RLU of the corresponding control was less than 25,000, the sample was considered positive for *M. tuberculosis* complex if the sample's RLU was equal to or greater than 100,000 and negative if the sample's RLU was less than 25,000. If the sample's RLU was between these values, the sample was retested to verify the results. The sample was considered positive if the retest RLU value was equal to or greater than 25,000 and negative if it was less than 25,000. For the RAPID BAP-MTB assay, there was no means to monitor for the presence of inhibitor.

Clinical evaluation of patients. All of the medical records, including history, symptoms, signs, radiology, pathology, microbiology results, and follow-up observations, were carefully reviewed in order to obtain the necessary data from the combination of culture results and observation of clinical condition to perform the assessment which served as the gold standard for diagnosis (resolved results). Clinically, two categories of samples were considered true-positives: (i) samples that were culture positive for *M. tuberculosis* complex and (ii) samples that were culture negative for *M. tuberculosis* complex but originated from a patient whose other samples were culture positive or who had a clinical diagnosis of tuberculosis (19, 22). The clinical diagnosis of tuberculosis was established if the biopsy material demonstrated caseating granulomas or the clinical and radiographic presentations were consistent with tuberculosis with marked improvement after antituberculosis therapy. After this analysis, amplification results were reclassified as appropriate.

Statistical analysis. Statistical comparisons were calculated by the chi-square test; $P < 0.05$ was considered significant.

RESULTS

The clinical information on the 333 patients and microbiological findings of 600 clinical specimens from these patients are summarized in Table 1. Altogether, 52 respiratory specimens were culture positive for acid-fast bacilli, 30 (57.7%) isolates were found to be *M. tuberculosis* complex, whereas the remaining 22 (42.3%) strains were classified as nontuberculous mycobacteria. Based on the clinical and microbiological findings of the 299 patients whose respiratory specimens were collected for this study, 24 patients had active pulmonary tu-

TABLE 2. Correlation between two amplification assays for detection of *M. tuberculosis* and culture results among 552 respiratory specimens

Assay	Acid-fast smear result (no. of samples)	No. of samples				Sensitivity (%)	Specificity (%)	Predictive values (%)	
		Culture positive (n = 30)		Culture negative (n = 522)				Positive	Negative
		PCR positive	PCR negative	PCR positive	PCR negative				
RAPID BAP-MTB	Positive (21)	11	1	6	3	91.7	33.3	64.7	75.0
	Negative (531)	18	0	19	494	100.0	96.3	48.6	100.0
	Overall (552)	29	1	25	497	96.7	95.2	53.7	99.8
DTB	Positive (21)	11	1	8	1	91.7	11.1	57.9	50.0
	Negative (531)	11	7	27	486	61.1	94.7	28.9	98.6
	Overall (552)	22	8	35	487	73.3	93.3	38.6	98.4

berculosis, 20 had a history of pulmonary tuberculosis, and 5 had a previous diagnosis of pulmonary tuberculosis and were currently receiving treatment. Of the 48 nonrespiratory specimens, one lumbar spine biopsy sample from a patient with tuberculosis spondylitis was smear positive and culture negative, and one pus sample from a patient with tuberculosis cervical lymphadenopathy was culture positive and smear negative for *M. tuberculosis* complex.

BD ProbeTec ET (DTB) system and RAPID BAP-MTB assay. Tables 2 and 3 show comparisons of the results of amplification with the two assays in respiratory specimens according to the culture results and gold standard diagnosis made on the basis of culture results and clinical findings. Of the 12 respiratory samples that were smear positive and culture positive for *M. tuberculosis* complex, 11 were both RAPID BAP-MTB positive and DTB positive. Eighteen samples were smear negative for acid-fast bacilli but culture positive for *M. tuberculosis* complex; all were RAPID BAP-MTB positive and 11 were DTB positive (P = 0.008). There were 24 samples that were both smear and culture negative, collected from 14 patients with a clinical diagnosis of pulmonary tuberculosis. These samples were both RAPID BAP-MTB and DTB positive in three, only RAPID BAP-MTB positive in three, and only DTB positive in the remaining three. The cumulative difference for all *M. tuberculosis* complex-positive specimens (40 positive by RAPID BAP-MTB and 34 positive by DTB) was not significant (P = 0.348).

As determined by the findings of culture and clinical diagnosis, a total of 14 respiratory samples had a false-positive result on RAPID BAP-MTB and 23 respiratory samples had a false-positive result on DTB. The possible causes of false-positive results in both assays are summarized in Table 4.

Table 5 compares the results of amplification of nonrespiratory specimens with the culture results and results of gold standard diagnosis based on the result of culture and clinical findings. The cumulative difference between these two methods for all *M. tuberculosis* complex-positive specimens was not significant.

Comparison of the diagnostic value of the two assays. In the 60 respiratory samples considered positive for *M. tuberculosis* complex based on clinical and microbiologic findings, the results of both amplification assays were concordantly positive in 30 and negative in 16. The results were RAPID BAP-MTB positive and DTB negative in 10 samples, all of which were smear negative. The results were RAPID BAP-MTB negative and DTB positive in four, all except one of which were smear negative.

DISCUSSION

The major difference between *M. tuberculosis* complex and nontuberculous mycobacterial infections is that the former can spread via person to person contact. For this reason, it is particularly important to diagnose tuberculosis as early as possible. Because conventional methods, including acid-fast staining and culture, are either insensitive or time-consuming, new technological developments which facilitate rapid diagnosis are of great importance. From a clinical standpoint, the key aspect

TABLE 3. Correlation between two amplification assays for detection of *M. tuberculosis* and resolved results (culture and clinical diagnosis) among 552 respiratory specimens

Assay	Acid-fast smear result (no. of samples)	No. of samples				Sensitivity (%)	Specificity (%)	Predictive value (%)	
		Resolved result positive (n = 60)		Resolved result negative (n = 492)				Positive	Negative
		PCR positive	PCR negative	PCR positive	PCR negative				
RAPID BAP-MTB	Positive (21)	16	2	1	2	88.9	66.7	94.1	50.0
	Negative (531)	24	18	13	476	57.1	97.3	64.9	96.4
	Overall (552)	40	20	14	478	66.7	97.2	74.1	96.0
DTB	Positive (21)	17	1	2	1	94.4	33.3	89.5	50.0
	Negative (531)	17	25	21	468	40.5	95.9	44.7	94.9
	Overall (552)	34	26	23	469	56.7	95.3	59.6	94.7

TABLE 4. Characterizations of specimens with false-positive results in either or the two assays

Specimen with false-positive result	No. of specimens	
	RAPID BAP-MTB (n = 14)	DTB (n = 23)
Pulmonary tuberculosis under treatment	3	6
Old pulmonary tuberculosis	1	5
M. abscessus	1	3
M. avium intracellulare complex	1	0
Unidentified species	0	1

of any new rapid assay for detecting *M. tuberculosis* complex is its negative predictive value. In the case of respiratory tract disease, it is critical to identify all cases of active tuberculosis and thereby interrupt the dissemination and transmission of the organism. By contrast, in extrapulmonary tuberculosis, it is critical to ensure that a readily treatable infection is not overlooked. The test should be sensitive, specific, and technically simple as well as able to differentiate between live and dead mycobacteria.

Although the RAPID BAP-MTB assay is entirely manual, whereas the BD ProbeTec ET system is semiautomated, the present study has demonstrated that both of these amplification assays can detect *M. tuberculosis* complex in clinical samples within a few hours, fulfilling some if not all of the key requirements for clinical applicability. The negative predictive values of both assays approached 100%. Both kits include all of the reagents needed for sample amplification and detection, and the assays are easy to perform. The differences of the results from cutoff values, values in controls, and values in samples were broad enough to allow easy discrimination by both assays. Although the lack of a means to monitor for inhibition in the RAPID BAP-MTB assay, the two-lysis-buffer system was stringent enough to inactivate the inhibitors, and only one specimen falsely negative for detection of *M. tuberculosis* complex was found.

The specificity and negative predictive value of the DTB assay in this study were in agreement with previous reports, 95.3 and 94.7%, respectively, for respiratory specimens and 95.7 and 97.8%, respectively, for nonrespiratory specimens (2, 12, 19). However, the sensitivity and positive predictive value of the DTB assay were low, 56.7 and 59.6%, respectively, for respiratory specimens and 50.0 and 33.3%, respectively, for nonrespiratory specimens. No significant differences in the diagnostic accuracy of the assay were observed between respiratory and nonrespiratory specimens. The low sensitivity of the DTB assay in this study probably resulted from the generally low bacterial load in the specimens. The percentage (40%) of smear-positive specimens in all culture-positive respiratory specimens was lower than that (64.7 to 83.5%) in previous reports (2, 12, 19). The low positive predictive value of the DTB assay was due to the high false-positive rate. Because the culture yield rate of nontuberculous mycobacteria in this study (4.0%) was lower than in previous reports (8.2 to 9.7%), it did not explain the high false-positive rate (2, 12, 19). In contrast, of the 23 specimens from patients with a positive DTB result but a negative result based on culture and clinical findings, 47.8% were collected either from patients with a history of pulmonary tuberculosis who were not currently being treated or from patients with a previous diagnosis of pulmonary tuberculosis who were currently receiving treatment. These findings reflect the high prevalence of tuberculosis in the general population in Taiwan. It is possible that the dead mycobacteria in the specimens may have caused the DTB test to yield a false-positive result.

By applying the nested PCR technique, the RAPID BAP-MTB assay is able to detect as little as 10 fg, or the equivalent of 1 to 20 copies of *M. tuberculosis* complex genomic DNA. In addition, false-positive results are decreased because the probability of a wrong locus being mistakenly amplified twice is very low. Thus, the performance of the RAPID BAP-MTB assay in this study was superior to that of the DTB assay in both respiratory and nonrespiratory specimens. For the 18 smear-negative, culture-positive respiratory samples, a small number of mycobacteria, unequally distributed in the test suspension, or a suboptimal target extraction, is perhaps the most likely explanations for the false-negative DTB result in seven (38.9%) samples. However, all 18 samples were RAPID BAP-MTB positive.

The problem of smear-negative pulmonary tuberculosis is worth particular attention, because these patients have been reported to be responsible for about 17% of tuberculosis transmission (1, 8). In addition, although many previous studies which used nucleic acid amplification to test extrapulmonary specimens had encouraging results, the sensitivity of this method is still far from ideal when routinely applied to clinical conditions for which proper evaluation may be crucial to outcome (12, 19, 23). Although only 48 nonrespiratory specimens

TABLE 5. Correlation between two amplification assays for detection of *M. tuberculosis* and culture results or resolved results (culture and clinical diagnosis) among 48 nonrespiratory specimens

Diagnostic criteria and assay	No. of samples				Sensitivity (%)	Specificity (%)	Predictive value (%)	
	Diagnosis positive		Diagnosis negative				Positive	Negative
	PCR positive	PCR negative	PCR positive	PCR negative				
Culture results								
RAPID BAP-MTB	1	0	1	46	100.0	97.9	50.0	100.0
DTB	0	1	3	44	0	93.6	0	97.8
Resolved results								
RAPID BAP-MTB	2	0	0	46	100.0	100.0	100.0	100.0
DTB	1	1	2	44	50.0	95.7	33.3	97.8

were tested in this study, the results of the RAPID BAP-MTB assay were 100% concordant with the findings of the diagnosis based on the results of culture and clinical findings. Further investigation to assess the performance of this assay in nonrespiratory specimens is needed.

In summary, the resurgence of tuberculosis has prompted the need for sensitive, correct, and fast methods for the laboratory detection of *M. tuberculosis* complex. Our results showed that, although the test is entirely manual, the diagnostic value of the nested PCR-based RAPID BAP-MTB assay is superior to that of the semiautomated DTB assay for both respiratory specimens and nonrespiratory specimens. The overall time for processing this assay is only 5 h. In addition, the diagnostic accuracy of the RAPID BAP-MTB assay in smear-negative samples is as high as that in smear-positive samples.

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