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Use of Molecular Methods To Identify the *Mycobacterium tuberculosis* Complex (MTBC) and Other Mycobacterial Species and To Detect Rifampin Resistance in MTBC Isolates following Growth Detection with the BACTEC MGIT 960 System

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A prospective study was organized by using a total of 1,585 consecutive clinical specimens to determine whether biomass obtained from positive growth in the MGIT 960 system could be used directly in AccuProbe DNA hybridization tests, the PCR-based Inno-LiPA Rif.TB (LiPA) assay, and a PCR-based DNA sequencing of the *rpoB* gene for the rapid identification of the *Mycobacterium tuberculosis* complex (MTBC) and other mycobacterial species and for the determination of rifampin (RIF) resistance in MTBC strains. The results were compared to routine culture, identification, and susceptibility testing techniques performed on the same samples. The study results revealed that the DNA AccuProbe assay (on the day of growth positivity) readily identified 95.7%, the LiPA assay readily identified 98.6%, and *rpoB* sequencing readily identified 97.1% of the 70 MTBC isolates from mycobacterial growth indicator tubes (MGIT). In addition, application of the LiPA for the identification and RIF susceptibility testing of the MTBC in growth-positive MGIT resulted in a turnaround time of less than 2 weeks after specimen receipt. Although DNA sequencing of *rpoB* required a slightly longer (16 days) turnaround time, this method was capable of identifying several species of nontuberculous mycobacteria in addition to identifying MTBC and determining RIF susceptibility or resistance. The molecular methods were also found to rapidly identify RIF-susceptible and -resistant MTBC in two of the three mixed mycobacterial cultures weeks earlier than conventional methods. In conclusion, the biomass obtained in MGIT at the time of growth positivity in the 960 system is sufficient for use in all three molecular tests, and this approach can reduce the turnaround time for reporting results.

The routine application of the liquid medium-based BACTEC 460 TB system (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) and the DNA hybridization-based AccuProbe *Mycobacterium tuberculosis* complex (MTBC) assay (TB AccuProbe; Gen-Probe, Inc., San Diego, Calif.) has significantly reduced the time required to isolate and identify MTBC (9, 28). Recently, several nonradiometric, fully automated or manual broth-based systems were introduced that are suitable alternatives to the radiometric, semiautomated BACTEC 460 TB system (5, 10, 33). However, in the radiometric BACTEC 460 TB system, the growth index provided by the instrument helps to accurately monitor the growth kinetics and to define a cutoff value (growth index ≥ 100) that is sufficient to reliably perform the DNA AccuProbe test (23). Similar well-defined and routinely applicable values are not yet available for the newer liquid media, such as mycobacterial growth indicator tubes (MGIT; BBL Becton Dickinson Microbiology Systems, Cockeysville, Md.). Thus, it is unknown how standardized the level of growth found in positive samples is, an important consideration if the suspension is to be used in a DNA AccuProbe assay.

Rifampin (RIF) is a key compound among the drugs in the treatment of tuberculosis, such that resistance to RIF often results in treatment failure and fatal clinical outcome in tuberculosis patients (8, 24). The PCR-based reverse hybridization line probe assay (Inno-LiPA Rif.TB [LiPA] assay; Innogenetics N.V., Ghent, Belgium [at present not commercially available in the United States]) has been reported to be an easy-to-use test for the rapid detection of RIF resistance, especially for clinical laboratories that cannot perform DNA sequencing (7, 35). The LiPA kit consists of 10 oligonucleotide probes (19 to 23 bases in length). One is specific for the MTBC, whereas five partially overlapping wild-type probes (S1 to S5) encompass the region from positions 509 to 534 region of the *rpoB* gene. Four other probes (R2, R4a, R4b, and R5) are specific for amplicons carrying the most common *rpoB* mutations: Asp-516-Val, His-526-Tyr, His-526-Asp, and Ser-531-Leu (7; Innogenetics Inno-LiPA Rif.TB test package insert). Therefore, this single test is suitable for both the identification of the MTBC and for the rapid detection of RIF resistance. In contrast to the non-amplification-based TB AccuProbe, the LiPA assay applies a PCR-based nucleic acid amplification step. This amplification step might provide sufficient sensitivity for the

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assay if the level of growth (in indicator or instrument positive samples) is too low to obtain a positive hybridization reaction with the DNA AccuProbe test.

The present prospective study was carried out to determine the sensitivity and accuracy of the DNA AccuProbe test, the LiPA assay, and a verified automated DNA sequencing method for the rapid and direct identification of the MTBC and other mycobacterial species in patient samples and to detect RIF resistance in MTBC, all after growth detection in the MGIT 960 system.

MATERIALS AND METHODS

Clinical specimens. A total of 1,585 consecutive clinical specimens that were received for routine mycobacterial cultivation in the Clinical Mycobacteriology Laboratory at the Wadsworth Center, New York State Department of Health, were processed between November 2000 and April 2001. All clinical specimens were digested and decontaminated by a modified Petroff's NaOH method (18; M. F. Iademarco, M. F., M. Salfinger, P. Zuber, M. Fitzgerald, J. Hotaling, and N. J. Binkin, Abstr. 30th World Conf. Lung Health Int. Union Tuberc. Lung Dis., abstr. S182, 1999). A 3.5% concentration of NaOH was used (starting concentration). After decontamination the concentrated sediment was suspended in 3.0 ml of sterile phosphate-buffered saline (pH 6.8) and smears were prepared by using the Ziehl-Neelsen acid-fast staining method (18).

MGIT inoculation and incubation, and routine testing. Before inoculation, the BACTEC MGIT 960 tubes were supplemented as described by the manufacturer (30; Becton Dickinson MGIT [7 ml] package insert). A 0.5-ml portion of the processed specimen was inoculated into the MGIT, and the tubes were introduced into the BACTEC MGIT 960 instrument as recommended by the manufacturer and then incubated either until they were found to be positive by the instrument or for 8 weeks.

Routine media, including a BACTEC 12B vial, a Lowenstein-Jensen slant, and a Middlebrook 7H10/7H11 selective biplate, were also inoculated, incubated at 37°C, and held for 8 weeks before we reported the sample as negative. When positive, the strains were identified by the DNA AccuProbe and conventional methods with susceptibility testing performed in the BACTEC 460TB system as reported previously (15, 18, 29). If MTBC was identified, final identification was determined by using PCR-based deletion analysis (25). In the case of drug resistance, the BACTEC susceptibility results were confirmed by the proportion method on Middlebrook 7H10 agar as described elsewhere (18). In addition, RIF resistance was confirmed by *rpoB* sequencing as described below.

Identification by AccuProbe from MGIT. On the day of detection in the MGIT 960 instrument, a 150-μl aliquot of the MGIT broth was cytocentrifuged (Cytospin 3; Shandon, Inc., Pittsburgh, Pa.) at 2,000 rpm for 7 min and examined with a Ziehl-Neelsen stain to confirm the presence of acid-fast bacteria (AFB). A similar volume was subcultured onto a chocolate agar plate to check for contaminants. The biomass from an MGIT found to contain AFB was tested directly in the DNA AccuProbe identification test (Gen-Probe, San Diego, Calif.). For the TB AccuProbe tests, 1.5 ml of broth from instrument positive vials were centrifuged at $28,400 \times g$ for 8 min. After centrifugation, the supernatant was removed, and the pellet was resuspended in 0.1 ml of the lysis reagent (reagent 1) and then tested by using the TB AccuProbe protocol according to the manufacturer's instructions.

LiPA test. Prior to amplification by PCR, 200-μl aliquots from instrument positive MGIT vials were incubated at 80°C for 1 h to heat kill the mycobacterial cells. The heat-killed samples were used for production of a biotinylated 256-bp fragment of the *rpoB* gene by using the LiPA kit according to the instructions of the manufacturer (Innogenetics Inno-LiPA Rif.TB test package insert). The biotin-labeled PCR product was denatured and hybridized to a strip with 10 specific oligonucleotide probes (MTBC-specific probe and the S1 to S5, R2, R4a, R4b, and R5 probes). Hybridized PCR product was detected, and the LiPA results were evaluated as described elsewhere (7; Innogenetics Inno-LiPA Rif.TB test package insert).

Since the occurrence of RIF-resistant isolates was expected to be low in the present study, the LiPA was validated for RIF susceptibility testing of the MTBC with a large number of clinical isolates. A total of 101 MTBC strains (37 RIF-susceptible strains, 41 multiple-drug-resistant strains [i.e., resistant to both RIF and isoniazid], and 23 RIF-monoresistant strains) from the collection of the Bureau of Laboratories of the Florida Department of Health were tested with the LiPA to evaluate the sensitivity and specificity of the system for RIF susceptibility testing. Detection of resistance was confirmed in the Wadsworth Center by the agar proportion method. Twenty-one nontuberculous mycobacterium (NTM) isolates (four *M. gordonae*, three *M. kansasii*, two *M. avium* complex [MAC], two *M. fortuitum*, two *M. abscessus*, one *M. peregrinum*, one *M. nonchromogenicum*, one *M. chelonae*, one *M. scrofulaceum*, one *M. asiaticum*, one *M. marinum*, one *M. simiae*, and one *M. smegmatis* isolate) were also included to assure adequate specificity of the test.

In case of a discrepancy between the LiPA and conventional susceptibility testing results, DNA sequence analysis of the 81-bp region of *rpoB* was also completed as described below.

DNA sequencing of *rpoB*. With primers rpo95 (5′-CCACCCAGGACGTGGAGGCGATCACACCG-3′) and rpo397 (5′-GTCAACCCGTTCGGGTTCATCGAAACG-3′) flanking the 81-bp region of *rpoB*, a 329-bp product was also generated from all isolates in order to verify the results of the DNA AccuProbe and the LiPA tests (13). The same primers were used for DNA sequencing of both strands by using the automated Applied Biosystems 377 DNA sequencer (Applied Biosystems, Foster City, Calif.). The sequencing results were compared to the wild-type *rpoB* sequence from *M. tuberculosis* for confirmation of identification and detection of mutations associated with RIF resistance or were analyzed by using the basic local alignment search tool (BLAST) against the GenBank nucleic acid sequence database for identification of NTMs (20). The DNA sequencing was carried out by the Molecular Genetics Core Facility at the Wadsworth Center.

Recently, it was demonstrated that in some RIF-resistant strains with the wild-type sequence in the 81-bp region, a Val-176-Phe mutation was found in the N-terminal region (11). In order to detect the presence of this mutation in these discrepant isolates, amplification and sequencing were performed with the primers Tb176-f (5′-CTTCTCCGGGTCGATGTCGTTG-3′) and Tb176-r (5′-CGCGCTTGTCGACGTCAAACTC-3′) as reported previously (4, 11). A 365-bp product was generated and sequenced with the same primers.

Controls and statistical analysis. Control susceptibility and molecular tests were performed by using *M. tuberculosis* H37Ra ATCC 25177 (15). Master mix with distilled water in the PCR tests and *M. avium* ATCC 25291 and *M. celatum* ATCC 51131 in the TB AccuProbe tests were used as negative controls (29).

The chi-square test was used to evaluate differences between the sensitivities of the different molecular methods.

RESULTS

Cultures positive for AFB were obtained from a total of 104 (6.6%) specimens, of which 68 (65.4%) were smear positive and 36 (34.6%) were smear negative. The mycobacterial species identified by routine methods were MTBC ($n = 70$), *M. gordonae* ($n = 14$), *M. avium* ($n = 12$), *M. kansasii* ($n = 5$), *M. fortuitum* ($n = 1$), *M. intracellulare* ($n = 1$), and *M. xenopi* ($n = 1$). The mean times from inoculation to the detection of growth for MTBC, MAC (*M. avium* and *M. intracellulare*), and other NTMs in the MGIT and BACTEC 12B media are presented in Table 1. Since both the MGIT 960 and the BACTEC 460 TB system detected growth of mycobacteria faster than on solid media, only the mean times to detection in the liquid media are shown (10, 34). The mean times from inoculation to detect RIF susceptibility or resistance in the BACTEC 460 TB system for all MTBC isolates were also determined and the results are summarized in Table 1.

Using biomass from the MGIT, the TB AccuProbe test detected 67 of the 70 (95.7%) MTBC isolates, the LiPA identified 69 of the 70 (98.6%) isolates, and *rpoB* sequencing identified 68 of the 70 (97.1%) isolates. The observed differences in sensitivity were not statistically significant. The main difference between the three tests was their ability to detect MTBC in the presence of a culture mixed with MAC. For one of the mixed cultures, all three assays were sensitive enough to detect the presence of the MTBC, while in a second mixed culture only the LiPA detected the MTBC. In the third mixed culture, all three molecular methods were unable to detect the MTBC in the MGIT broth in the presence of MAC. Finally, the TB

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TABLE 1. Mean times in days (range) from inoculation to detection of mycobacteria in the MGIT and Bactec 460 TB systems and to detect RIF susceptibility or resistance in the Bactec 460 TB system

Organism	Smear category (*n*)	Mean times in days (range) to detection by:		
		MGIT isolation	Bactec 460 TB isolation	Bactec RIF susceptibility testing
MTBC	Smear positive (57)	10.5 (4–35)	10.1 (3–37)	13.5 (8–29)
	Smear negative (13)	15.0 (6–27)	17.4 (11–22)	14.7 (8–30)
	All (70)	11.3 (4–35)	11.4 (3–37)	22.4 (15–30)
MAC	Smear positive (6)	5.8 (4–10)	5.8 (4–8)	NA[a]
	Smear negative (7)	18.6 (11–38)	13.7 (9–20)	NA
	All (13)	12.7 (4–38)	10.1 (4–20)	NA
NTM	Smear positive (5)	12.8 (2–39)	10.8 (3–21)	NA
	Smear negative (16)	23.4 (5–55)	24.1 (3–52)	NA
	All (21)	20.9 (2–55)	20.9 (3–52)	NA

[a] NA, not applicable.

AccuProbe also failed to detect a pure culture of MTBC from a growth-positive MGIT.

It is also noteworthy that, besides its ability to identify MTBC and provide information on RIF susceptibility, *rpoB* sequencing was also able to identify all of the NTMs to the species level accurately and more rapidly than routine identification methods. The turnaround times for TB AccuProbe, LiPA assay, and *rpoB* sequencing are shown in Table 2.

In the validation of the LiPA assay, all of the 37 RIF-susceptible MTBC isolates reacted only with the MTBC-specific and S1 to S5 probes, indicating that no RIF resistance-associated mutations were present. Of the 64 RIF-resistant MTBC isolates, LiPA was able to detect a genetic alteration in 61 (95.3%) and to identify the particular mutation in 46 (71.9%). The mutations present on the LiPA assay that were detected included 34 Ser-531-Leu mutations (53.1%), 6 His-526-Tyr mutations (9.4%), 3 His-526-Asp mutations (4.7%), and 3 Asp-516-Val mutations (4.7%). In two RIF-resistant MTBC isolates with no detectable mutation in the LiPA assay, DNA sequencing of *rpoB* did not detect any alteration in the 81-bp region. However, in one of these two isolates, DNA sequencing of the N-terminal region of *rpoB* detected the rare Val-176-Phe mutation. The third MTBC isolate with discrepant LiPA results showed a mixed wild-type and RIF-resistant LiPA pattern, which might indicate heteroresistance (the mixture of susceptible and resistant strains) (22, 26). The DNA sequencing for the 81-bp and N-terminal regions of *rpoB* did not reveal any mutation for this isolate. Finally, the specificity of the LiPA assay was 100% since there was no hybridization to any of the bound probes with any of the 21 NTM isolates.

In this prospective study, only two RIF-resistant strains were isolated (2.8% of the 70 MTBC isolates), and the LiPA and the *rpoB* sequencing identified both of them correctly. The molecular tests revealed that the two isolates were carrying the most common Ser-531-Leu mutation.

DISCUSSION

The guidelines of the Centers for Disease Control and Prevention (CDC) recommends that the turnaround times for the growth detection, identification, and susceptibility testing of the MTBC should be 2 to 3 weeks and 2 to 4 weeks, respectively, after receipt of the specimen (6, 31, 32). Previous studies have shown that the fully automated MGIT 960 is comparable in sensitivity and rapidity to the BACTEC 460 TB system (10, 34). Therefore, it is a viable alternative to replace the semiautomated radiometric system. However, the rapidity of the MGIT or any other new broth-based system is of limited benefit if it cannot be correlated with accurate and rapid species identification. Rapid identification is best supported by the application of appropriate molecular biologic methods, but only a limited number of evaluations have been reported on the identification of the MTBC from MGIT broth by molecular or other rapid methods (1–3, 12, 14, 17, 27; S. Rusch-

TABLE 2. Characteristics of mycobacterial identification methods

Parameter	TB AccuProbe	LiPA assay	*rpoB* sequencing
Identification	MTBC	MTBC	All mycobacterium spp.
Detection of RIF resistance	No	Only the four most common mutation types (others are detected indirectly only)	All types of mutations
Turnaround time	Within 24 h	Within 24 h	4 to 5 days (using the institutional core facilities)
Labor	Easy to perform	Labor-intensive	Labor-intensive
Instrumentation	Heat blocks, sonicator, luminometer, centrifuge	Thermocycler, shaking water bath, gel casting system	Thermocycler, gel casting system, automated sequencer
Standardization for liquid medium	Nonstandardized sample preparation	Standardized	Home brewed
Quality-controlled kit format	Yes	Yes	No

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Gerdes, A. Ebrahimzadeh, and B. A. Hanna, Abstr. 100th Annu. Meet. Am. Soc. Microbiol. 2000, abstr. C-6, p. 127, 2000).

While amplification-based assays showed an adequate sensitivity to identify the MTBC from the MGIT, reports on the more widely and routinely used TB AccuProbe showed a variable sensitivity when used in conjunction with the MGIT (77.1% as determined by Ichiyama et al., 96.4% as determined by Alcaide et al., and 98.2% as determined by Rusch-Gerdes et al.) (2, 14; Rusch-Gerdes et al., Abstr. 100th Annu. Meet. Am. Soc. Microbiol. 2000). This may be due to interlaboratory differences in sample preparation for the TB AccuProbe (sample volume, centrifugation force, and time) and/or the need for additional days of incubation after detecting growth positivity to achieve a detectable biomass.

Unfortunately, an adequately standardized protocol for the combined use of liquid media and nucleic acid probes is still lacking. Toward this goal, the *Manual of Clinical Microbiology* recommends that, generally, a pellet of a 1.0- to 1.3-ml aliquot of the broth culture should be used for probing after concentration by centrifugation at 9,000 $\times$ *g* to 10,000 $\times$ *g* for at least 5 to 7 min (23). In line with this recommendation, in our study the sample volume was 1.5 ml (as also in the study by Alcaide et al.), whereas Rusch-Gerdes et al. used 2.0 ml and Ichiyama et al. used 1.0 ml (2, 14; Rusch-Gerdes et al., Abstr. 100th Annu. Meet. Am. Soc. Microbiol. 2000). With regard to the relative centrifugation force (RCF), in our study the RCF was 28,400 $\times$ *g*, whereas Alcaide et al. reported 13,000 $\times$ *g*, Rusch-Gerdes et al. reported 26,671 $\times$ *g*, and Ichiyama et al. reported only 3,000 $\times$ *g* (2, 14; Rusch-Gerdes et al., Abstr. 100th Annu. Meet. Am. Soc. Microbiol. 2000). Our and these other findings indicate that the low (77.1%) TB AccuProbe sensitivity with the MGIT reported by Ichiyama et al. was probably due to the low centrifugation force (3,000 $\times$ *g*) rather than due to the relatively lower sample volume in that study (1.0 ml) (14). From the study of Benjamin et al. that was evaluating the MB/BacT walk-away broth-based system, it appears that an adequate sensitivity of the TB AccuProbe at a lower RCF (4,000 $\times$ *g*) requires a significantly higher aliquot (3.0 ml) from the liquid culture (5).

The results of the present study demonstrate that there is no need for further incubation of an instrument-positive MGIT broth since the TB AccuProbe can readily detect the presence of the MTBC on the day of growth positivity. The present study also revealed that the routine application of the LiPA assay on growth-positive MGIT 960 tubes is an excellent approach to shorten the turnaround time for the rapid identification and RIF susceptibility testing of the MTBC in one step. When the MGIT 960 and the LiPA assay are used in combination, the mean time to detect and to identify the MTBC and its susceptibility to RIF could be shortened to less than 2 weeks, which would be well in line with the CDC's recommendations for acceptable turnaround time (Tables 1 and 2) (6, 31, 32). In addition, the LiPA was also capable of detecting the presence of the MTBC in two cultures mixed with MAC, an important result since one of these cultures contained RIF-resistant MTBC. In these mixed-culture cases, the LiPA assay was able to provide the correct identification and RIF susceptibility results weeks earlier than when conventional test results became available.

The advantage of *rpoB* sequencing is that it can also identify NTM strains in addition to the MTBC strains (Table 2). In addition, *rpoB* sequencing can also identify all mutation types in RIF-resistant MTBC as opposed to the LiPA assay that can identify only the four most common mutations (Table 2). Therefore, this method might be more reliable in geographic areas where less common or silent mutations are more frequent (4, 16). Alternatively, while the LiPA is packaged in a kit format, DNA sequencing may not be as readily available for clinical laboratories (Table 2). Recently, a PCR-restriction enzyme pattern analysis for mycobacteria identification has been developed by using a PCR product amplified from the mycobacterial *rpoB* gene (19, 21). This rapid and easily applicable method could be used to identify mycobacterial isolates, followed by DNA sequencing for RIF susceptibility testing on the PCR product only if MTBC is identified by PCR-restriction enzyme pattern analysis.

In conclusion, the TB AccuProbe, the LiPA assay, or *rpoB* sequencing are reliable methods that can be used in conjunction with the MGIT 960 system for clinical and reference laboratories to detect and identify the MTBC with a turnaround time of less than 2 weeks (Tables 1 and 2). The tests (especially the LiPA assay) can also be helpful in the identification of the MTBC in a mixed culture, which might take weeks by conventional methods. The LiPA assay and *rpoB* sequencing also offer the capability of detecting RIF resistance in 2 weeks for all isolates or in 15 to 20 days in smear-negative cases, together with the identification of the pathogen (Tables 1 and 2). The fact that none of the molecular methods were able to detect the MTBC in all mixed cultures indicates that, at present, the inoculation of both liquid and solid media and the confirmation of the molecular results by conventional tests are still warranted.

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Q.S. and A.S. contributed equally to this study.

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