Rapid, Standardized Method for Determination of Mycobacterium tuberculosis Drug Susceptibility by Use of Mycolic Acid Analysis

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Multidrug-resistant (MDR) Mycobacterium tuberculosis and extensively drug-resistant (XDR) M. tuberculosis are emerging public health threats whose threats are compounded by the fact that current techniques for testing the susceptibility of M. tuberculosis require several days to weeks to complete. We investigated the use of high-performance liquid chromatography (HPLC)-based quantitation of mycolic acids as a means of rapidly determining drug resistance and susceptibility in M. tuberculosis. Standard susceptibility testing and determination of the MICs of drug-susceptible (n = 26) and drug-resistant M. tuberculosis strains, including MDR M. tuberculosis strains (n = 34), were performed by using the Bactec radiometric growth system as the reference method. The HPLC-based susceptibilities of the current first-line drugs, isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA), were determined. The vials were incubated for 72 h, and aliquots were removed for HPLC analysis by using the Sherlock mycobacterial identification system. HPLC quantitation of total mycolic acid peaks (TMAPs) was performed with treated and untreated cultures. At 72 h, the levels of agreement of the HPLC method with the reference method were 99.5% for INH, EMB, and PZA and 98.7% for RIF. The inter- and intra-assay reproducibilities varied by drug, with an average precision of 13.4%. In summary, quantitation of TMAPs is a rapid, sensitive, and accurate method for antibiotic susceptibility testing of all first-line drugs currently used against M. tuberculosis and offers the potential of providing susceptibility testing results within hours, rather than days or weeks, for clinical M. tuberculosis isolates.
TABLE 1. M. tuberculosis strains used in this study and their susceptibility profiles

<table>
<thead>
<tr>
<th>M. tuberculosis strain designation(s)</th>
<th>Total no. of strains with the susceptibility profile</th>
<th>Susceptibility*</th>
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<tbody>
<tr>
<td></td>
<td>INH</td>
<td>RIF</td>
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<tr>
<td>H37Rv, 2 to 25, 32</td>
<td>26</td>
<td>S</td>
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<td>27, 30, 34, 35</td>
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<td>R</td>
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<td>58</td>
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</tr>
<tr>
<td>55, 56, 60</td>
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<td>S</td>
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</table>

* Susceptibilities were determined with the Bactec 460 system. S, susceptible; R, resistant.

has enabled previous investigators to demonstrate the feasibility of performing M. tuberculosis susceptibility testing by measuring the total mycolic acid concentration (10).

In the current work, we describe an improved, standardized, and validated mycolic acid-based susceptibility testing method.

MATERIALS AND METHODS

Organisms and antibiotics. All M. tuberculosis strains (n = 59 clinical strains and strain H37Rv) were maintained on Lowenstein-Jensen agar slants (Difco, Detroit, MI) at 37°C. All drugs (INH, RIF, ethambutol [EMB], pyrazinamide [PZA], and streptomycin [STR]) were purchased from Becton Dickinson (Sparks, MD). All assays were performed in duplicate.

Standard susceptibility testing. Susceptibility testing and determination of MICs were done by using the Bactec 460 radiometric growth system (Becton Dickinson) and/or the agar proportion method by the use of standard protocols (15, 19). Table 1 shows the profiles of susceptibility to INH, RIF, EMB, PZA, and STR of each of the strains used in this study. Briefly, for the Bactec 460 system, a standardized inoculum (a 1.0 McFarland standard) was prepared for each strain to be tested was prepared from solid medium by using commercially available diluting fluid (Becton Dickinson) and glass beads. The suspensions were vortexed and allowed to settle for a minimum of 30 min, and a 0.1-ml aliquot of the supernatant was removed and used to inoculate a Bactec 12B “seed vial.” The seed vials were read at 24-h intervals until a GI of 999 was reached. All strains exhibiting GIs between 9% and 11% of the control GI were classified as borderline resistant.

HPLC susceptibility testing. (i) Time course determination and sample processing. The same standardized inoculum and procedure outlined above for the Bactec 460 system were used. An undiluted control vial was included for each strain tested and was used for comparison of the TMAPs with those of the antibiotic-containing vials. The vials were monitored at 24-h intervals for up to 72 h, and the GI obtained with the Bactec system was recorded. Aliquots (2 ml) were removed from the individual vials at 20 min and 24, 48, and 72 h following the introduction of each antibiotic and were mixed 1:1 with 50% potassium hydroxide, followed by autoclaving for 30 min. Once the mixtures were cooled to room temperature, 3.6 ml of 50% HCl was added and the contents of the tubes were mixed by inverting the tubes two or three times. Subsequently, 3 ml of CHCl3 was added to each tube, the tubes were vortexed for 1 min, and the phases were allowed to separate. Equal volumes of each CHCl3 layer from each strain tested were transferred to a clean glass tube and dried under N2. Fluorescent derivatives of the mycolic acids were prepared as described previously (22). Briefly, the samples were dissolved in 200 μl of CHCl3 containing 200 μg of 4-bromomethyl-6,7-dimethoxycoumarin and 200 μg of 18-crown-6 ether (both from Sigma) and transferred to a 2-ml amber vial containing 100 μl of 2% potassium bicarbonate solution which had previously been evaporated. The vials were heated at 60°C for 10 min and allowed to evaporate. Dried extracts were resuspended in 150 μl of HPLC-grade isopropanol ( Fisher Scientific) and transferred to a vial containing two fluorescently derivatized mycolic acid reference standards (~40 and ~97 carbons) whose retention times do not overlap with those of the mycolic acids present in M. tuberculosis.

(ii) HPLC conditions. HPLC analysis was performed on an Agilent (Wilmington, DE) 1100 HPLC system consisting of an autosampler, quaternary pump, column heater (70°C), and fluorescence detector. The instrument was controlled by the use of Sherlock (MDL Inc., Newark, DE) software coupled with Agilent ChemStation software. The sample injection volume was 5 μl. Separation of the mycolic acids was achieved with an Agilent Zorbax SB-C18 column (4.6 mm by 75 mm), a mobile phase of methanol-isopropanol (IPA), and a linear gradient of 25% IPA to 95% IPA over a 10-min period with a flow rate of 1.7 ml/min. Mycolates were detected with a fluorescence detector by using excitation and emission wavelengths of 345 nm and 425 nm, respectively. To maintain maximum sensitivity, the column effluent was cooled to 22°C before it was placed into the detector.

(iii) Chromatographic data analysis. Raw chromatographic data, expressed as the peak height response, were acquired with ChemStation software and processed by using Sherlock software for calculation of the TMAP. The TMAPs were determined for each isolate-drug combination and were compared to that for the corresponding control. The resulting ratio was used for all further data analysis.

Growth rate determination. For growth rate comparisons, a suspension of each strain to be tested was prepared from solid medium by using commercially prepared diluting fluid (Becton Dickinson) and glass beads. The suspensions were vortexed and allowed to settle for a minimum of 30 min, and a 0.1-ml aliquot of the supernatant was removed and used to inoculate a Bactec 12B “seed vial.” The seed vials were read at 24-h intervals until a GI of 999 was reached. Subsequently, 0.1 ml was removed from each seed vial and used to inoculate a new Bactec 12B vial. The GI was then recorded at 24-h intervals for up to 9 days, and the average daily change in the GI was calculated for each strain.

RESULTS

Time course determination. A time course analysis was done to determine the optimal time point at which quantitative differences in the total mycolic acids could be correlated with drug susceptibility or resistance to a given agent. This was done for first-line antitubercular drugs that act in both a mycolic acid-specific and a non-mycolic acid-specific manner. The chromatograms were analyzed for the total mycolic acid profile for each strain-antibiotic combination and compared to the profile for the corresponding untreated control. Figure 1 shows a characteristic chromatogram for a drug-susceptible strain of M. tuberculosis, strain H37Rv, with and without INH, and the specific region which comprises TMAP. As shown, significant differences in TMAPs were found at 72 h of exposure to the control vial, the strain was classified as resistant. Strains exhibiting GIs between 9% and 11% of the control GI were classified as borderline resistant.

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antibiotic. Differences were noted between antibiotics. For instance, the mycolic acid-inhibiting drug, INH, caused a nearly linear decrease in the percent change in TMAP relative to that for the corresponding control over a 72-h incubation period, ranging from a 34% reduction at 20 min to an 80% reduction at 72 h for strain H37Rv (Fig. 2). However, for a non-mycolic acid-specific inhibitor such as RIF for a susceptible strain, little change in the TMAP was observed at the earliest time points tested: 5.2% at 20 min and 18% at 24 h. At the later time points of 48 and 72 h, the decreases in the TMAPs were 25% and 63%, respectively (Fig. 2). Other first-line drugs, such as EMB and PZA, showed similar patterns for drug-susceptible strains, with the greatest reductions in the TMAPs observed at 72 h of incubation (76% and 67%, respectively). This time point was selected for evaluation because it was the time at which the largest reproducible difference in TMAPs was observed for all drugs, regardless of their mechanisms of action.

**FIG. 1.** Characteristic chromatogram and TMAPs of drug-susceptible *M. tuberculosis* strain H37Rv following 72 h of incubation with and without INH. (Top panel) Control, no INH; (bottom panel) INH (0.1 μg/ml). FU, fluorescence units.

**FIG. 2.** Percent change in TMAP following exposure to INH (0.1 μg/ml) versus RIF (2.0 μg/ml) over time in drug-susceptible *M. tuberculosis* strain H37Rv.

TMAP susceptibility testing and breakpoint determination. TMAP susceptibility assays were conducted at 72 h for all *M. tuberculosis* strain-antibiotic combinations (*n* = 60), the TMAP was calculated, and the ratio of the values for the treated strains to those for the untreated controls were determined. At 72 h, drug-susceptible strains demonstrated an average reduction in TMAP of >78% (range, 52% to 90%) compared with the values for the corresponding controls. These differences varied by antibiotic, with the greatest inhibition in susceptible strains being observed with INH (average, −89%; range, −76% to −100%) and RIF (average, −88%; range, −43% to −100%) compared with the values for EMB (average, −78%; range, −52% to −89%) and PZA (average, 59%; range, −39% to −94%). In contrast, drug-resistant strains demonstrated a change in TMAP which was either a reduction of <25% or a slight increase over the values for the controls (range, +28% to −24%).
Several breakpoints ranging from 20% to 30% were examined to determine which one provided the best agreement between the standard Bactec 460 susceptibility test method and HPLC. For each strain-antibiotic combination, the percent change in TMAP for each treated culture compared with that for the control culture was determined at 72 h and compared with the known susceptibility of that particular strain. Susceptibility was defined as a reduction in TMAP relative to that for the corresponding control of greater than or equal to breakpoints set at 20% or 30%. Strains exhibiting TMAP changes of less than or equal to the breakpoint (20% or 30) were defined as resistant. By using a 20% breakpoint, the concordances were 71%, 88%, 94%, and 82% for INH, RIF, EMB, and PZA, respectively. In contrast, the highest percent agreement for all drugs was obtained by using the 30% breakpoint, which resulted in 99.5% agreement for INH, EMB, and PZA and 98.7% agreement for RIF compared to the values obtained by the Bactec 460 susceptibility testing method. Figure 3 illustrates the mean percent change in TMAP for all drug-susceptible and -resistant strains to each of the first-line agents obtained by use of the 30% breakpoint. With a total of 240 susceptibility tests, the overall concordance was 97.5%. Discordant results indicating false susceptibility were observed for the following antibiotics: INH and EMB (one result each) and RIF (two results). False resistance was demonstrated for RIF and PZA (one result each). Intra- and interassay variabilities were assessed by testing the same strain (strain H37Rv) six times within the same day or individual replicates over 6 consecutive days. For each culture the coefficient of variation was calculated on the basis of the ratio of the TMAP for the control culture to the TMAP for the treated cultures. The overall precision was 13.4%. However, the precision did vary by drug (INH, 9.17%; RIF, 11.2%; EMB, 11.4%; PZA, 21.9%).

STR, a second-line antimycobacterial drug, was also evaluated by the TMAP-based method. The overall agreement was 93.3% between the standard Bactec 460 method and TMAP analysis, which was less than that observed for the first-line drugs at the corresponding time point of 72 h. Susceptibility to STR was correctly identified in all susceptible strains (n = 38) by the TMAP method. However, STR resistance was not detected in 15 of 22 resistant strains. For each strain with discrepant results, a reduction in TMAP of >30% (average, 62.4%; range, 42% to 83%) was observed in each of two separate experiments. The reduction in the TMAP was similar in STR-susceptible strain H37Rv (average, 88%). Resistance to STR (2.0 µg/ml) was confirmed by both the Bactec 460 radiometric method and the agar proportion method. However, differences in growth rates were observed between STR-susceptible strain H37Rv and the resistant strains producing discrepant results. Overall, these strains exhibited slower growth than drug-susceptible strain H37Rv, slowing an average of 54 GI units over 9 days (range, 38 to 386 GI units).

DISCUSSION

*M. tuberculosis* remains a major cause of morbidity and mortality globally, with an estimated 2 million deaths yearly due to active infection. This number continues to rise in light of a latently infected reservoir of approximately one-third of the world’s human population. Although there has been reported success in the treatment of TB through the use of directly observed therapy by utilizing a short course, noncompliance, among other factors, has led to the emergence of MDR and, recently, XDR *M. tuberculosis* strains. In addition, diagnostic methods, in the cases of patients with sputum, smear-negative, or pulmonary or extrapulmonary disease, are slow, requiring days to weeks for diagnosis and susceptibility testing. This necessitates empirical therapy until susceptibility testing is completed, resulting in the potential dissemination of resistant MDR and XDR *M. tuberculosis* strains to others in the population.

A variety of susceptibility testing methods have been developed for *M. tuberculosis*. The oldest and current standard is the proportion method. In this method, drug-free and drug-containing media are inoculated with known dilutions of the test organism and the subsequent ratio of the number of colonies on the drug-free medium/number of colonies on the drug-containing medium yields an estimate of the proportion of drug-resistant organisms present in the sample. This method is not rapid, and its performance may require 3 to 6 weeks after recovery of the isolate in culture. Other broth-based susceptibility testing platforms, such as the semiautomated, radiometric Bactec 460 system and the automated, nonradiometric Bactec MGIT (mycobacterial growth indicator tube) 960 system (Becton Dickinson), have mean turnaround times of 4.5 days (range, 3 to 10 days) and 7.3 days (range, 5 to 12 days), respectively (20). Molecular tests have also been used to determine resistance to many of the first-line drugs. These tests are compared to the standard method for determining susceptibility. However, the TMAP-based method is advantageous because it can be performed in a shorter time period.
sequence the PCR-amplified products of target genes to detect the mutations responsible for resistance. Although they are potentially very effective, molecular tests are hindered by the presence of multiple drug resistance mechanisms for some antibiotics and resistant strains for which no molecular determinant of resistance has been identified. In addition, such tests are expensive, are technically demanding, and are likely beyond the abilities if routine clinical laboratories at this time (1, 12).

In this study and others (10, 21), a clear, nearly linear correlation between TMAPs and the numbers of CFU/ml was demonstrated. As a result, it is possible to use TMAP values as a measure of CFU/ml without having to incubate cultures for up to 3 weeks by standard susceptibility testing methods. Earlier studies focused primarily on INH, RIF, or STR and required 5 days of incubation before susceptibility could be assessed. In the current study, a standardized, time-dependent breakpoint was established for all drugs (INH, RIF, EMB, PZA, and STR) considered together. The extreme sensitivity of the analytical method enabled a definitive correlation to be obtained for all antibiotics at a uniform time point of 72 h and even earlier for isolated antibiotics, such as INH. Since the extraction procedure requires several steps and interpretation of the results relies on quantitative comparisons between control and drug-treated cultures, care must be taken in sample processing. However, the use of the Sherlock software, which automates the operation of the instrument, in conjunction with improvements in the method, including less carryover compared with that described in previous reports (21), and the use of less noxious solvents (isopropanol versus methylene chloride), enabled its routine use by technologists with little or no experience with HPLC.

Discernable differences in TMAP reductions were noted on the basis of the mechanisms of action of the individual drugs. For instance, our results demonstrated that a rapid decrease in TMAP occurred in as little as 20 min for *M. tuberculosis* strains treated with INH. This is not surprising, since the INH-specific mechanism of action in *M. tuberculosis* is inhibition of mycolic acid synthesis. Thus, with a mycolic-acid-specific inhibitor such as INH, the detection of significant differences in the numbers of CFU/ml is not necessary to detect susceptibility or resistance. However, for other drugs that do not directly target mycolic acid synthesis (RIF, STR, PZA) or that are bacteriostatic (EMB), a significant difference in the numbers of CFU/ml is required to determine drug susceptibility by the TMAP method. This requires longer incubation times to maximize differences in the numbers of CFU/ml. In this study, 72 h of incubation and a 30% breakpoint were the optimal TMAP testing parameters for all four first-line drugs (INH, RIF, EMB, and PZA) considered together. However, further improvements in the method could target optimization of drug-specific incubation times and breakpoints. For instance, on the basis of the data generated in this study, the detection of INH susceptibility or resistance could be optimized at a 30-min incubation time, and that for RIF could be optimized at a 50-h incubation time. This would provide for the even more rapid detection of MDR *M. tuberculosis* by the TMAP method. MDR *M. tuberculosis* strains are defined as those strains resistant to both INH and RIF. The ability to detect MDR *M. tuberculosis* in <3 days would be a significant improvement over currently available conventional methods, which require days to weeks to complete. During the delays inherent in conventional methods, inappropriate therapy is often administered, with no clinical improvement in the patient and with the possible dissemination of *M. tuberculosis* to others in the population.

The intra- and interassay precisions also varied by drug and ranged from 9.17% for INH to 21.9% for PZA. These drug-specific differences in precision may be related to the specific mechanism of action of each drug and the rapidity with which a given agent kills *M. tuberculosis* in vitro. INH, which specifically targets mycolic acid synthesis and which is rapidly bactericidal, yielded reproducible differences in TMAP values, with a much narrower range in variation compared with those obtained with RIF, EMB, and PZA. PZA, for which the most variation in precision was observed, was a produg which requires conversion to pyrazinoic acid (POA) via the mycobacterial nicotinamidase/pyrazinamidase (24). This conversion is necessary for PZA-mediated bactericidal activity against *M. tuberculosis*. Once it is converted to POA, it is excreted by the bacilli via a weak efflux pump. Due to the inefficiency of this efflux pump, the POA accumulates within the bacilli, resulting in cellular damage and death. The time required for the conversion of PZA to POA may vary between *M. tuberculosis* strains. This variation may also be related to the inherently different growth rates observed between *M. tuberculosis* strains in slightly acidic media (pH 6.0) (24). In this study, strain-to-strain variation in growth was not assessed. However, it is possible that such differences played a role in the increased amount of variation in the intra- and interday precision studies with PZA.

STR, once considered a first-line drug for the treatment of TB, has been transitioned to second-line status (15). We examined the utility of the TMAP susceptibility testing method in correctly identifying STR susceptibility and resistance in multiple strains of *M. tuberculosis*. Previous investigators have demonstrated that it was possible to detect differences in growth after 3 to 4 days of incubation in STR-exposed cultures of *M. tuberculosis* using mycolic acid analysis (14). It is important to note that the majority of the strains used in that study were susceptible to STR. In the present study, STR susceptibility was correctly identified in 42 of 42 (100%) susceptible strains. However, the TMAP method failed to detect STR resistance in 15 of 22 resistant strains. The molecular mechanism of STR resistance correlates closely with the in vitro activity of the drug (14). STR resistance is mediated by a number of factors, including mutations in *rpsL*, which encodes a ribosomal 30S gene (S12), and *rrs*, a 16S rRNA gene. Mutations in *rpsL* are associated with high-level resistance, whereas mutations in *rrs* are associated with intermediate resistance. Some STR-resistant strains have no mutations in either of these two genes and exhibit low-level resistance. These strains are thought to have altered membrane permeability to the drug, which contributes to resistance, since the use of membrane-active agents in combination with STR significantly increases susceptibility. Altered membrane permeability could also have a negative impact on the growth rate. In this study, all the STR-resistant strains producing discrepant results between the two methods exhibited slower growth relative to that of their STR-susceptible counterparts. The number of CFU/ml at
a given time point would necessarily be lower for the more slowly growing strains, resulting in inaccurate TMAP results. Since the molecular mechanism(s) of STR resistance was not determined in this study, it is not possible to confirm whether these strains had a genotype consistent with that found in STR-resistant isolates for which altered membrane permeability is suspected.

Our data suggest that determination of the mycolic acid concentration by the TMAP method is a rapid and sensitive indicator of cell growth inhibition and death in *M. tuberculosis* compared to the speeds and sensitivities of standardized susceptibility protocols. Further studies are planned, including optimization of the testing parameters with first-line drugs, with extension to second-line agents, and feasibility studies with other testing platforms, such as the Bactec 960 MGIT system.

In summary, the TMAP method is a rapid, sensitive, and accurate method for testing the susceptibility of *M. tuberculosis* strains to all currently used first-line drugs. The results are obtained in 72 h, as opposed to the lengthy period required for conventional methods, leading to the more rapid identification of MDR and, possibly, XDR *M. tuberculosis*.

REFERENCES