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Testing of *Mycobacterium tuberculosis* Susceptibility to Ethambutol, Isoniazid, Rifampin, and Streptomycin by Using Etest

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Etest (AB BIODISK, Solna, Sweden) is a precise MIC method and the practical method of choice for the susceptibility testing of many fastidious organisms, including rapidly growing mycobacteria. Methods recommended by the National Committee for Clinical Laboratory Standards for the susceptibility testing of *Mycobacterium tuberculosis* include the Bactec (Becton Dickinson, Sparks, Md.) broth and agar proportion methods. A comparison of Etest with the Bactec broth method for testing the susceptibility of *M. tuberculosis* to four first-line antituberculous agents demonstrated equivalent interpretive results for 100% of the isolates tested. Agreements with agar proportion MICs, within ± 2 $\log_2$ dilutions, were 90, 93, 100, and 94% for ethambutol, isoniazid, rifampin, and streptomycin, respectively. Etest MICs were easily read within 5 to 10 days of inoculation. Preparation of the inoculum with a turbidity equivalent to a McFarland 3.0 standard prepared from growth on an agar surface and with a broth with a Bactec growth index of >999 yielded equivalent results. Clinical isolates for which the MICs were reproducible were also identified as possible quality control strains. The Etest method appears to be an alternative method for testing the susceptibility of *M. tuberculosis* isolates to the four most commonly used therapeutic agents.

The incidence of tuberculosis in the United States has increased dramatically since 1986, following an earlier decline (3). Concomitantly, a significant increase in the number of drug-resistant cases of tuberculosis has also been observed (8). This increase in drug-resistant strains has been attributed to a lack of patient compliance with the prolonged multidrug regimens required for adequate therapy, as well as an increase in susceptible AIDS and homeless populations (9, 12). For these reasons, rapid identification of mycobacterial isolates, along with rapid susceptibility testing of all isolates of *Mycobacterium tuberculosis*, has become critical for therapy selection and for the prevention of the spread of resistant organisms (14, 15). Despite recommendations by the Centers for Disease Control and Prevention, only a small proportion of the participants in the College of American Pathologists proficiency survey program actually use rapid methods for the isolation, identification, and susceptibility testing of *M. tuberculosis* (18).

Current methods for the susceptibility testing of *M. tuberculosis*, as described in the tentative standard (M24-T) of the National Committee of Clinical Laboratory Standards (NCCLS), include the agar proportion and Bactec (Becton Dickinson, Sparks, Md.) radiometric methods (13). Unfortunately, both methods suffer from limitations, such as standardization for only the four first-line antituberculous agents (ethambutol, isoniazid, rifampin, and streptomycin) and reliance on a single critical concentration of an antimicrobial agent for susceptibility categorization. Both methods also require technical expertise for the interpretation of results. The agar proportion method has the additional disadvantage of requiring 3 weeks of incubation (6, 7). Although it is more rapid, the Bactec broth method requires expensive equipment and supplies.

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Etest (AB BIODISK, Solna, Sweden), an accurate MIC method, has emerged as the method of choice for the susceptibility testing of fastidious organisms (16), including rapidly growing mycobacteria (2, 5, 11). Preliminary results have shown that Etest is a promising new method for the susceptibility testing of more slowly growing mycobacteria as well (19). To evaluate the potential of Etest for the susceptibility testing of *M. tuberculosis*, we performed a comparative study of Etest and the Bactec and agar proportion methods by using clinical isolates of *M. tuberculosis* tested against first-line antituberculous agents.

MATERIALS AND METHODS

Strains of *M. tuberculosis*. Seventy-one isolates of *M. tuberculosis* recovered from individual patients seen at several hospitals and community clinics in Houston, Tex., were maintained as stock cultures in glycerol at −70°C for use in this study. *M. tuberculosis* H37Rv (ATCC 27294) and mutant strains resistant to each antimycobacterial agent (ATCC 35822 [resistant to isoniazid], ATCC 35838 [resistant to rifampin], ATCC 35820 [resistant to streptomycin], and ATCC 35837 [resistant to ethambutol]) were maintained as stock cultures for use as quality control strains. All clinical isolates were grown on Lowenstein-Jensen (Remel, Lenexa, Kans.) agar slants and identified presumptively as mycobacteria

TABLE 1. Time when Etest ellipse becomes visible for *M. tuberculosis*

Medium	Inoculum density[a]	Avg time in days (range)
Lowenstein-Jensen agar slant	McFarland 1.0	7 (7–9)
	McFarland 3.0	5 (5–7)
	McFarland 4.0	5
Bactec broth	200–300	10
	500–700	9 (8–10)
	999–>999	6

[a] Results for Bactec broth are given as growth indices measured by a Bactec 460 instrument.

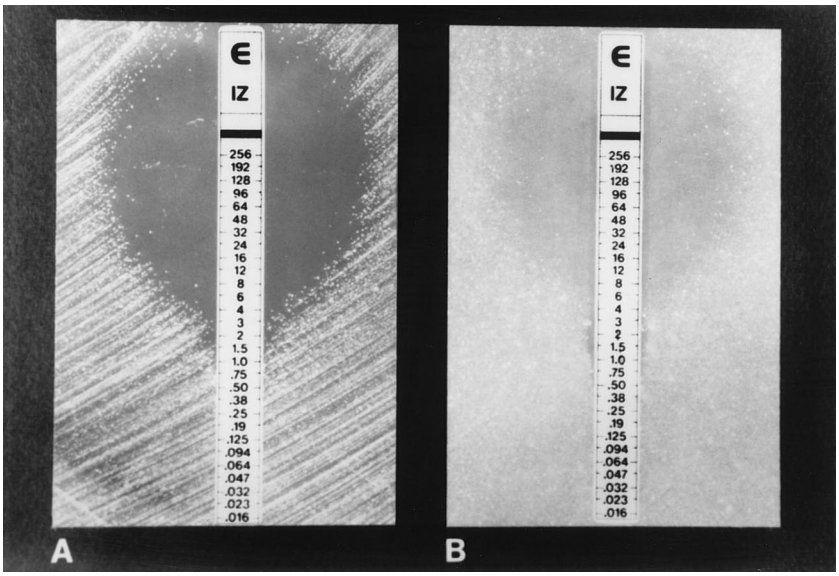

FIG. 1. Etest MICs for a clinical isolate of *M. tuberculosis* inoculated by swabbing a McFarland 3.0 suspension of colonies growing on an agar surface (A) and by flooding growth from a Bactec bottle with growth index of >999 (B).

by colony morphology and Kinyoun stain (7, 10). Identification was confirmed by using a DNA probe (Gen Probe, San Diego, Calif.) according to the manufacturer's instructions. A portion of the 71 isolates were tested by the Bactec, agar proportion, and Etest methods to determine the MICs for the four first-line antimycobacterial agents. Because of the unavailability of Etest strips for isoniazid and ethambutol at the start of this study, a larger number of isolates were tested with rifampin and streptomycin.

Bactec susceptibility testing. Isolates were tested against ethambutol, isoniazid, rifampin, and streptomycin at concentrations of 2.5, 0.1, 2.0, and 2.0 μg/ml, respectively, by using the Bactec 460 (Becton Dickinson) radiometric method as described by the manufacturer (17).

Agar dilution susceptibility testing. Agar dilution testing was performed according to the proportion method as described in NCCLS tentative standard M24-T (13), with the following modifications. Because of the more rapid growth of resistant strains of *M. tuberculosis*, Middlebrook 7H11 agar (Remel) supplemented with OADC enrichment (Remel) was used. For comparison with Etest MICs, quantitative inhibitory endpoints were achieved for the proportion method by using twofold dilutions, ranging from 0.016 to 32 μg/ml, of rifampin (Ciba-Geigy Pharmaceutical Co., Summit, N.J.), isoniazid (Ciba-Geigy), ethambutol (Lederle Laboratories, Pearl River, N.Y.), and streptomycin (Pfizer Inc., New York, N.Y.). Each dilution in 5 ml of 7H11 medium, was added to a quadrant of a plate. The plates were inoculated by spotting 100 μl from a 10^{-2} dilution of a McFarland 1.0 in three spots on each quadrant; then they were incubated at 35°C in 5% CO_2 for 3 to 4 weeks (13). Endpoints were interpreted by comparing the number of colonies growing on the quadrants containing drugs with those on the quadrants with no drug, as described in the NCCLS M24-T document (13).

Etest susceptibility testing. Etest strips, containing gradients of ethambutol, isoniazid, rifampin, and streptomycin (0.016 to 256 μg/ml), were provided by AB BIODISK. Freshly prepared Middlebrook 7H11 agar with OADC supplement (depth, 4 ± 0.5 mm) was used, as well as commercially prepared Middlebrook 7H11 double pour plates (Remel). Plates were inoculated in several different ways as described below and preincubated at 35°C in 5 to 10% CO_2 for 24 h, after which time the Etest strip was placed on the agar surface. The plates were then incubated under the same conditions until an inhibition ellipse was visible (5 to 7 days). The MIC was interpreted as the point at which the ellipse intersected the

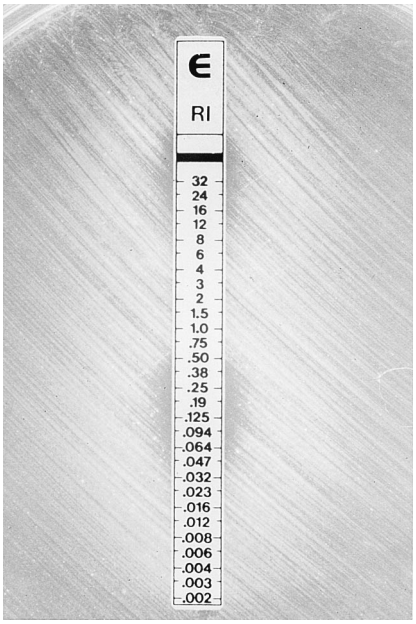

FIG. 2. Partial areas of inhibition for a strain of *M. tuberculosis* resistant to rifampin (MIC > 256 μg/ml).

TABLE 2. Strains and Etest MIC quality control ranges for susceptibility testing of *M. tuberculosis*

Strain	Antimicrobial agent	No. of times tested	MIC range (μg/ml)
H37Rv (ATCC 27294)	Ethambutol	7	0.06–0.25
	Isoniazid	5	0.016–0.06
	Rifampin	16	0.06–0.25
	Streptomycin	11	0.25–1.0
386	Ethambutol	8	0.25–1.0
	Isoniazid	8	0.06–0.25
	Rifampin	5	0.06–0.25
AWC	Ethambutol	5	0.5–4.0
	Isoniazid	12	4.0–16
	Rifampin	5	0.06–0.25
AWB	Ethambutol	5	0.125–0.5
	Isoniazid	5	0.06–0.25
	Rifampin	5	0.06–0.25
	Streptomycin	5	0.25–1.0

TABLE 3. Comparison of Etest and agar proportion MICs for *M. tuberculosis*

Antimicrobial agent	No. of isolates	Etest MIC variation in $\log_2$ dilutions (%)							% within ±2 dilutions	Intermethod interpretive agreement (%)
		>−2	−2	−1	Same	1	2	>2		
Ethambutol	28	10	7	46	30	7	0	0	90	100
Isoniazid	27	4	0	4	78	7	4	4	93	96[a]
Rifampin	68	0	9	13	75	0	3	0	100	100
Streptomycin	50	0	8	18	14	16	8	6	94	96[b]
Overall	173	4	6	20	49	8	4	3	93	98

[a] Very major (false-susceptible) error; however, the Etest results agreed with the Bactec interpretation (susceptible).
[b] Includes one major and one very major error. The Etest major error agreed with the Bactec result.

Etest strip, as described in the Etest technical guide (1). When hazes and isolated mutant colonies were seen in the ellipse, the MIC was read where these were completely inhibited.

(i) Preparation of inoculum from growth on solid media. Colonies were scraped from a freshly growing (3 to 4 weeks) Lowenstein-Jensen agar (Remel) slant into 3 ml of Middlebrook 7H9 broth (Remel) containing four to five 3-mm-diameter glass beads in a conical tube. The tubes were vortexed vigorously for 3 to 5 min to homogenize the suspension. The large particles were allowed to settle, and the supernatant was adjusted to a turbidity equivalent to a McFarland 3.0 for comparison with the agar proportion and Bactec methods. Inoculum variations between McFarland 1.0 and 4.0 were also investigated. The inoculum was swabbed onto the plate by streaking the entire surface in three directions (1).

(ii) Preparation of inoculum from growth in broth. Bactec 12B bottles (Becton Dickinson) containing Middlebrook 7H9 broth were inoculated with a suspension of *M. tuberculosis* and incubated for approximately 5 days at 35°C or until a growth index of >999 was achieved. One milliliter was carefully removed from the bottle and transferred with a needle and a syringe to a sterile disposable tube. The surface of a 7H11 agar plate was then flooded by using a disposable plastic transfer pipette. Excess liquid was removed, and the plate was preincubated as described above prior to the application of the Etest strips.

RESULTS

Comparison of inoculum preparation methods. MICs for seven isolates of *M. tuberculosis*, including the quality control strain H37Rv, were determined by using several inoculum preparations with the Etest method. These isolates were chosen because they demonstrated reproducible results upon repeat testing, and they included strains that were susceptible and strains that were resistant to individual antimycobacterial agents. MICs were equivalent when inocula were prepared from fresh growth from both solid and broth media (Table 1). The time for visualization of an Etest ellipse varied from 5 to 10 days, depending on the inoculum density. Although growth was more rapid with a heavier inoculum, MICs remained equivalent. Once an ellipse was formed, no change in MIC was observed when incubation was increased to 3 weeks. Figure 1A demonstrates a typical inhibitory ellipse for solid media, with a density equivalent to a McFarland 3.0; Fig. 1B demonstrates an ellipse for broth media, with a Bectec growth index of ≥999. Therefore, subsequent studies were performed with an inoculum equivalent to a McFarland 3.0 prepared from colonies growing on solid media.

Reading and interpretation of Etest endpoints. The majority of strains demonstrated clear ellipses which were easy to interpret after 5 days of incubation. Some strains required an additional 1 to 5 days of incubation before the MIC could be interpreted. MICs were interpreted conservatively, and all growth was taken into consideration. Partial areas of inhibition (Fig. 2) were occasionally observed around the Etest strip of resistant (MIC, >256 μg/ml) isolates. The mechanism of this inhibition is unknown but was ignored in the interpretation of the MIC.

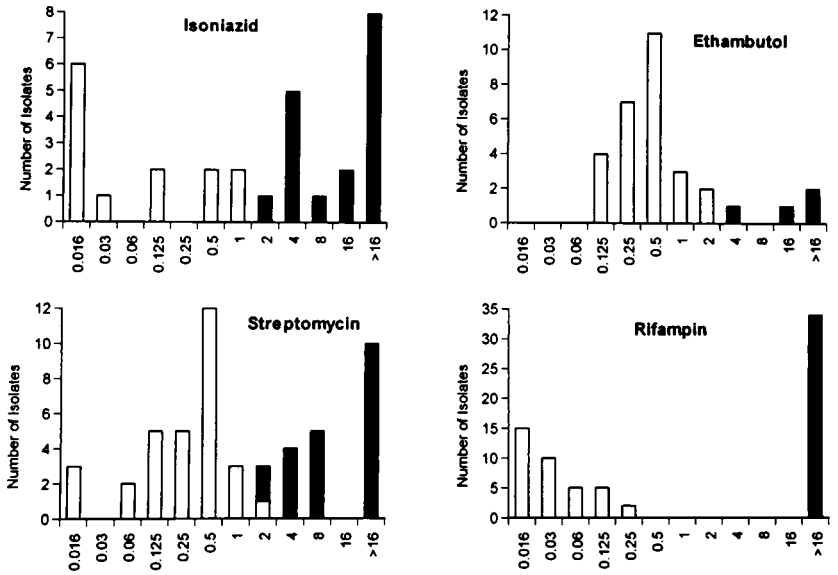

FIG. 3. Comparison of Etest MICs (*x* axis; in micrograms per milliliter) and Bactec interpretive criteria for clinical isolates of *M. tuberculosis* tested against isoniazid, ethambutol, streptomycin, and rifampin. Symbols: open bars, numbers of isolates susceptible by the Bactec method; solid bars, numbers of isolates resistant by the Bactec method.

Evaluation of quality control strains. NCCLS document M24-T recommends the use of several strains of *M. tuberculosis* for quality control of susceptibility testing. These strains include H37Rv (susceptible to all antituberculous agents) and other ATCC strains, each resistant to an individual antimycobacterial agent. Unfortunately, the MICs for the highly resistant ATCC strains are off scale (>1,000 μg/ml), which makes these strains suboptimal for quality control and intermethod comparisons. We evaluated several clinical isolates for which the MICs are on scale for potential use as quality control strains. Although additional multilaboratory testing needs to be performed, preliminary data demonstrated the reproducibility of the MICs for these isolates and drugs upon repeated testing (Table 2).

Comparison of Etest and agar proportion MICs. The correlations between the Etest and agar proportion methods, with agreement within ±2 dilutions, were 90, 93, 100, and 94% for ethambutol, isoniazid, rifampin, and streptomycin, respectively (Table 3). Discrepancies in the interpretive criteria of isolates were found with only three strains. For one strain the MICs of streptomycin were 0.75 μg/ml (indicating susceptibility) by the Etest and 8.0 μg/ml (indicating resistance) by the agar proportion method. For a second strain the MICs of streptomycin were 3.0 μg/ml (indicating resistance) by the Etest and 0.5 μg/ml (indicating susceptibility) by the agar proportion method. (The isolate was resistant by Bactec.) The third strain with which a discrepancy was found was an isolate for which the MICs of isoniazid were 0.125 μg/ml (indicating susceptibility) by the Etest and 4.0 μg/ml (indicating resistance) by the agar proportion method. (The isolate was susceptible by the Bactec method.) A subset of the isolates ($n = 10$) was simultaneously tested with commercially prepared agar plates (Remel), and the Etest MIC results were found to be equivalent (results not shown).

Comparison of Etest MICs and Bactec interpretive results. Excellent agreement (100% for isoniazid and rifampin, 97% for ethambutol, and 98% for streptomycin) was demonstrated between Etest MIC distributions and Bactec interpretive criteria for all clinical isolates of *M. tuberculosis* tested (Fig. 3).

DISCUSSION

A tentative standard for the susceptibility testing of slowly growing mycobacteria by the agar proportion and Bactec methods has been described by the NCCLS (13). These methods have similar disadvantages, the most significant being the use of a single critical concentration of a drug to define susceptibility. Limited clinical and pharmacokinetic data supporting the choice of these concentrations for testing exist (4). The use of a broad range of MICs for testing *M. tuberculosis* was originally proposed by the World Health Organization in 1961 (4). However, at that time it was deemed impractical because of the expense and lack of suitable technology.

Because of the significant increase in the number of tuberculosis cases as well as in drug resistance among *M. tuberculosis* isolates, a rapid and accurate method of testing has become essential. Etest, an accurate and precise MIC method covering 15 twofold dilutions, has previously been shown to hold promise for the testing of slowly growing mycobacteria (19). In our evaluation with numerous clinical isolates of *M. tuberculosis*, this method was found to be reasonably fast, accurate, and easy to perform. Equivalency with the methods currently recommended by the NCCLS as the tentative standard was demonstrated (13). Excellent agreement between Etest MIC distributions and Bactec interpretive results was also observed. Even though it is difficult to compare two very different methods (one based on interpretation of 1% resistance and the other based on a precise MIC), good agreement (±2 dilutions, 90 to 100%) between Etest and agar proportion MICs for the four first-line antituberculous agents tested was found.

Clinical isolates for which the MICs of each antituberculous agent were reproducible and on scale by the Etest method were identified. These isolates should be useful candidates for further evaluation as quality control strains; identification of such strains would facilitate expanded interlaboratory comparison studies.

Etest MICs were easily read in 5 to 10 days when the inoculum was prepared from either agar or broth media at inoculum densities ranging from McFarland 1.0 to McFarland 3.0. Since more-rapid visual growth was achieved with the heavier inoculum, a density of McFarland 3.0 was preferred. Etest MICs were easy to interpret for the vast majority of isolates and antimicrobial agents. All growth, including isolated colonies at the intersection point or within the ellipses, was considered significant, and the MIC was read at the point of complete inhibition.

Etest was found to be equivalent to the current NCCLS methods (13) and had the advantage of being quantitatively precise over a continuous gradient covering 15 twofold dilutions. Etest appears to be cost-effective, rapid (giving results in 5 to 10 days), and independent of costly instrumentation and may be a particularly useful testing alternative for susceptibility determination in less developed countries. Etest can also be used for the evaluation of new investigational drugs for which no established critical concentrations exist and for the surveillance of drug resistance. Preliminary data also indicate that Etest may be useful for MBC determinations and synergy testing. Studies to further evaluate these applications are currently in progress in our laboratory.

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