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Evaluation of Etest for Susceptibility Testing of Rapidly Growing Mycobacteria

JON R. BIEHLE,[1]* STEPHEN J. CAVALIERI,[1] MICHAEL A. SAUBOLLE,[2] AND LINDA J. GETSINGER[2]

Department of Pathology, Creighton University Medical Center, Omaha, Nebraska 68131,[1] and Department of Pathology, Clinical Microbiology Section, Good Samaritan Regional Medical Center, Phoenix, Arizona 85006[2]

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MICs of amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, and imipenem were determined by Etest for 100 clinical strains of rapidly growing mycobacteria and compared with MICs determined by a reference agar dilution method. Etest MICs were also determined by an alternative inoculum application (agar overlay) method and compared with MICs determined by the inoculum application method recommended by the manufacturer (swabbing). Agreement between Etest and agar dilution MICs within ±1 log_2 dilution was 85% (511 of 600), and agreement within ±2 log_2 dilutions was 97% (580 of 600). The rate of complete category agreement was 88%, and rates of major and minor errors were 2.2 and 11.7%, respectively. No very major errors were detected for Etest MICs. Interlaboratory agreement between MICs determined at two separate laboratories was 81% (121 of 149) within ±1 log_2 dilution and 92% (137 of 149) within ±2 log_2 dilutions. Agreement between laboratories by interpretive category was 92%. Exact agreement between agar overlay and swab application MICs was 52.3%, and agreement within ±1 log_2 dilution was 82.3%. Diffuse ellipse edges and trailing growth were still a problem with the overlay method, and in some cases results were more difficult to interpret than they were with the corresponding swab-prepared plate. In summary, our data suggest that Etest may be an accurate and reproducible method for determining susceptibility of rapidly growing mycobacteria.

Recent reports describing the differences in susceptibility patterns among subspecies and biovariants of rapidly growing mycobacteria have emphasized the importance of determining antimicrobial susceptibilities in an accurate and timely manner (3, 14, 15). Testing methods currently in use for determining susceptibility of isolates of rapidly growing mycobacteria include broth microdilution (3, 14), agar dilution (5, 8), agar disk elution (12), disk diffusion (16), a tablet diffusion method (7), and Etest (8). Unfortunately, no testing method has been standardized for this group of organisms.

Etest (AB Biodisk, Solna, Sweden) is a new concept for determining MICs of antimicrobial agents. It has been shown to compare well with conventional dilution techniques for numerous bacterial pathogens (1, 4). The relative ease of use of this method is a feature which is attractive to smaller clinical laboratories that do not have the facilities necessary to prepare complex susceptibility testing media.

The purpose of this study was twofold. First, MICs of the antimicrobial agents amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, and imipenem for 100 clinical strains of rapidly growing mycobacteria were determined by Etest and compared with those determined by a reference agar dilution method.

Secondly, Etest MICs determined by an alternative inoculum application (agar overlay) method were compared with Etest MICs determined by the inoculum application method recommended by the manufacturer (swabbing). The agar overlay method was evaluated for its potential to sharpen and define the diffuse ellipse edges or eliminate trailing growth that can occur with the Etest method. Formation of diffuse ellipse edges or trailing growth can make determining the precise MIC difficult, especially with an antimicrobial agent for which the interpretive breakpoints are within 1 twofold dilution, such as ciprofloxacin.

* Corresponding author. Mailing address: Department of Pathology, Creighton University Medical Center, 601 N. 30th St., Omaha, NE 68131. Phone: (402) 449-4952. Fax: (402) 280-5247.

MATERIALS AND METHODS

Mycobacterial strains. One hundred clinical isolates of rapidly growing mycobacteria were used in this study. The isolates were selected from culture collections at the Creighton University Medical Center (CUMC), Omaha, Nebr., and the Good Samaritan Regional Medical Center (GSMC), Phoenix, Ariz. Organisms were identified to the species and biovariant levels by standard methods (9). The species distribution included 42 *Mycobacterium fortuitum* isolates, 14 *Mycobacterium peregrinum* isolates, 6 *M. fortuitum* isolates of the third biovariant, 20 *Mycobacterium chelonae* isolates, and 18 *Mycobacterium abscessus* isolates. Isolates were maintained on Lowenstein-Jensen slants prior to being tested and subcultured onto Trypticase-soy-sheep blood agar plates at 35°C in ambient air for 3 to 5 days. *M. fortuitum* ATCC 6841 was the designated mycobacterial reference strain, and *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used for internal quality control.

Antimicrobial agents. The antimicrobial agents used in this study were amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, and imipenem. Each of these agents has been shown previously to have activity against the various species and biovariants of rapidly growing mycobacteria (3, 5, 13–15). Drugs were obtained from the manufacturers as powders suitable for susceptibility testing. Stock solutions of the agents were prepared on the day of testing according to the recommendations of the manufacturers. Etest strips were obtained from AB Biodisk.

Susceptibility testing. Preparation of agar dilution plates followed National Committee for Clinical Laboratory Standards recommendations for dilution techniques (11), with the exception that Mueller-Hinton medium was supplemented with oleic acid-albumin-dextrose-catalase (OADC). Agar dilution plates were prepared by adding serial twofold dilutions of antimicrobial agents to Mueller-Hinton agar supplemented with OADC (10%, vol/vol) to match the MIC ranges on the respective Etest strips. Inoculum for the agar dilution method was prepared by transferring mature growth from sheep blood agar to sterile distilled water. The turbidity of the bacterial suspension was adjusted with additional sterile distilled water to equal a McFarland 0.5 turbidity standard. The plates were inoculated with 0.01 ml of a 10^6-CFU/ml suspension of bacteria by using a Steers replicator (10^4 CFU per spot). Agar dilution plates were placed in plastic bags and incubated in ambient air at 35°C. The MIC was defined as the lowest concentration that completely inhibited visible bacterial growth.

Etest MICs were determined by two different inoculum preparation and application methods. Inoculum for the recommended Etest method (application by swabbing) was prepared by transferring mature growth from sheep blood agar to sterile distilled water. The turbidity of the bacterial suspension was adjusted with additional sterile distilled water to equal a McFarland 0.5 turbidity standard.

TABLE 1. MIC breakpoints used for categorization of susceptibilities of 100 strains of rapidly growing mycobacteria to six antimicrobial agents

Antimicrobial agent	MIC (μg/ml) for category:		
	Susceptible	Moderately susceptible	Resistant
Amikacin[a]	≤16	32	>32
Cefoxitin[a]	≤8	16–32	>32
Ciprofloxacin[b]	≤1	2	≥4
Clarithromycin[a]	≤1	2	≥4
Doxycycline[a]	≤1	2–8	>8
Imipenem[b]	≤4	8	≥16

[a] Suggested breakpoints were used as previously described (6, 12, 13).
[b] National Committee for Clinical Laboratory Standards breakpoints for aerobic bacteria were used.

This bacterial suspension was applied to the surface of 150-mm-diameter plates of Mueller-Hinton agar by use of a sterile cotton swab, as previously described (16). Each Mueller-Hinton agar plate was supplemented with 2.0 ml of OADC prior to application of the inoculum. The OADC was spread evenly across the plate by tilting the plate from side to side and absorbed into the agar prior to inoculation.

Inoculum used with the agar overlay method was prepared by suspending the organism in sterile distilled water, with the turbidity equal to a McFarland 1.0 standard or approximately 10^9 CFU/ml. A 0.1-ml volume of this suspension was added to 0.9 ml of OADC, producing a suspension containing approximately 10^8 CFU/ml. This 1.0-ml suspension was then added to 9.0 ml of molten Mueller-Hinton agar, mixed by gentle inversion, and poured onto the surface of a 150-mm-diameter Mueller-Hinton agar plate. This suspension was spread evenly over the surface of the plate by gently tilting the plate from side to side and allowed to solidify for 30 min.

Etest strips were then applied to plates prepared by both inoculum methods, and plates were incubated in ambient air at 35°C. The MIC was determined by the intersection of the inhibition ellipse with the concentration of antimicrobial agent on the Etest strip. MICs that were half-scale values (e.g., 6.0 μg/ml) were converted to the next highest traditional twofold dilution scale value (e.g., 8.0 μg/ml).

Breakpoints for determining susceptibility and resistance are shown in Table 1. Breakpoints for ciprofloxacin and imipenem were identical to those for aerobic bacteria other than *Haemophilus influenzae* and *Neisseria gonorrheae*. Breakpoints for amikacin, cefoxitin, clarithromycin, and doxycycline were as suggested by other investigators and were selected from previous reports of susceptibility testing (6, 14, 15).

Intralaboratory accuracy and reproducibility were determined (at CUMC) by comparing the MICs from 20 consecutive determinations with *M. fortuitum* ATCC 6841 with established target values for all antimicrobial agents (3, 6, 14, 15). Interlaboratory reproducibility was determined by performing Etest MIC assays with 25 strains in each of two separate geographical locations (CUMC and GSMC).

RESULTS

Results of Etest MIC determinations were similar to previously published results for these organisms and drugs and are illustrated in Table 2. Amikacin was the most active drug, with 100% of *M. fortuitum* complex (contains *M. fortuitum*, *M. peregrinum*, and the third biovariant) strains susceptible and 76% of *M. chelonae* and *M. abscessus* strains susceptible. Cefoxitin was the least active drug tested, with 13% of *M. fortuitum* complex strains and 0% of *M. chelonae* and *M. abscessus* strains susceptible. For ciprofloxacin, 87% (54 of 62) of the *M. fortui-*

TABLE 2. Distribution of Etest MICs of six antimicrobial agents for 100 strains of rapidly growing mycobacteria

Subgroup (no. of isolates)	Antibiotic	No. of isolates for which MIC (μg/ml) was:													
		≤0.06	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16	32	>32	64	128	>256
M. fortuitum (42)	Amikacin			4	13	17	7			1					
	Ciprofloxacin	12	10	5	5	2	6	1	1						
	Clarithromycin		2	2		2	7	13	4	4	2				6
	Doxycycline	1	3	6	2	2	5	5	5	4	2				7
	Cefoxitin								2	6	22		11		1
	Imipenem			2		4	12	17	6	1					
M. peregrinum (14)	Amikacin		1	2	6	5									
	Ciprofloxacin	5	5	2		2									
	Clarithromycin	4	6	1											3
	Doxycycline		1	1	1	1	2	4	2						2
	Cefoxitin								6	5			3		
	Imipenem				1	3	6	4							
M. fortuitum, third biovariant (6)	Amikacin			1	1	2	1	1							
	Ciprofloxacin			1	3	2									
	Clarithromycin								2		1				3
	Doxycycline	1	1				1	1			1				1
	Cefoxitin										1		5		
	Imipenem				1			3	2						
M. chelonae (20)	Amikacin					1	2	3	4	6	1		2		1
	Ciprofloxacin							1	1		1	17			
	Clarithromycin	1	2	4	8	3	1	1							
	Doxycycline				1				1		1				17
	Cefoxitin												2	2	16
	Imipenem						2	1		1		16			
M. abscessus (18)	Amikacin							2	4	7	1		4		
	Ciprofloxacin											18			
	Clarithromycin		2	2	8	2	1	3							
	Doxycycline									1	1			1	15
	Cefoxitin										3		7	1	7
	Imipenem								1	3	1	13			

TABLE 3. Distribution of differences in Etest versus agar dilution MICs for 100 strains of rapidly growing mycobacteria

Antimicrobial agent	No. of Etest[a] MICs within indicated log_2 concn of agar dilution[b] MICs							% Agreement within ±1 log_2 concn
	>−2	−2	−1	Same	+1	+2	>+2	
Amikacin	1	8	35	28	24	3	1	87
Cefoxitin	0	3	20	40	28	6	3	88
Ciprofloxacin	5	13	19	37	22	3	1	78
Clarithromycin	4	16	31	29	17	2	1	77
Doxycycline	0	6	27	42	21	4	0	90
Imipenem	2	3	27	41	23	2	2	91
Total	12	49	159	217	135	20	8	85

[a] Etest MICs (swab inoculation) were determined by use of Mueller-Hinton agar supplemented with OADC.
[b] Agar dilution MICs were determined by use of Mueller-Hinton agar with 10% OADC supplementation.

tum complex strains were susceptible. Six of the ciprofloxacin-nonsusceptible *M. fortuitum* complex strains were categorized as intermediate (2.0 μg/ml) and two strains were categorized as resistant (≥4.0 μg/ml) by Etest. Two of six intermediate strains and one of two resistant strains were categorized as susceptible by agar dilution and thus accounted for two minor errors and one major error, respectively. All strains of *M. chelonae* and *M. abscessus* were resistant to ciprofloxacin. Clarithromycin and doxycycline MICs were quite variable for strains of *M. fortuitum* complex, ranging from 0.023 to >256 μg/ml for clarithromycin and from 0.032 to >256 μg/ml for doxycycline. Clarithromycin had greater activity against strains of *M. chelonae* and *M. abscessus* (84% susceptible), with a MIC at which 90% of the isolates were inhibited of 2.0 μg/ml. Only 3% of the strains of *M. chelonae* and *M. abscessus* were susceptible to doxycycline. For imipenem, 85% of the strains of *M. fortuitum* complex and 8% of the strains of *M. chelonae* and *M. abscessus* were susceptible.

Table 3 illustrates the agreement between agar dilution MICs and Etest MICs determined by using the manufacturer's recommended method of inoculum application (swab). Exact agreement for individual drugs ranged from 28% for amikacin to 42% for doxycycline, and exact agreement for all drugs was 36% (217 of 600). Agreement within ±1 log_2 dilution for individual drugs ranged from 77% for clarithromycin to 91% for imipenem. Overall, agreement within ±1 log_2 dilution was 85% (511 of 600) and agreement within ±2 log_2 dilutions was 97% (580 of 600). Etest MICs that disagreed with corresponding reference values were evenly distributed, with 57% (220) being lower than the reference MIC and 43% (163) being higher than the reference MIC.

Table 4 illustrates complete agreement and interpretive error rates for MICs determined by Etest and agar dilution. When Etest MICs were compared with agar dilution MICs, the rates of complete category agreement for individual drugs were found to range from 72% for cefoxitin to 95% for doxycycline. No very major errors were detected for any drug. Major error rates (risk corrected) ranged from 0% for doxycycline to 12.5% for cefoxitin (one false-resistant result among eight susceptible strains). Minor error rates ranged from 5% for doxycycline to 27% for cefoxitin. Overall, the rate of complete category agreement was 88% and rates of major and minor errors were 2.2 and 11.7%, respectively.

Table 5 illustrates the differences between Etest MICs determined by use of the two different inoculum application methods. Exact agreement between MICs of individual drugs ranged from 31% for cefoxitin to 68% for doxycycline. Overall, 52% of the overlay MICs were the same as swab application MICs and 82% of overlay MICs were within ±1 log_2 dilution of swab MICs.

Table 6 illustrates the results of interlaboratory reproducibility studies performed at two separate geographical locations (CUMC and GSMC) with 25 study strains. Exact agreement between MICs was 51% (76 of 149). Agreement within ±1 log_2 dilution was 81% (121 of 149), and agreement within ±2 log_2 dilutions was 92% (137 of 149). Overall agreement by interpretive category was 92%. Category agreement rates were highest for amikacin and ciprofloxacin (100%) and lowest for cefoxitin (76%). Intralaboratory accuracy and reproducibility of the expected MIC of each study antimicrobial agent for *M. fortuitum* ATCC 6841 (at CUMC) were excellent. The ex-

TABLE 4. Number of susceptible strains, percent complete agreement, and interpretive errors for 100 Etest MICs

Antimicrobial agent	No. of susceptible strains	% Complete agreement	% Errors		
			Very major	Major[a]	Minor
Amikacin	91	93	0	1.1	6.0
Cefoxitin	8	72	0	12.5	27.0
Ciprofloxacin	54	91	0	1.9	8.0
Clarithromycin	48	88	0	4.2	10.0
Doxycycline	20	95	0	0	5.0
Imipenem	56	88	0	1.8	11.0
Total	274	88	0	2.2	11.7

[a] Risk-corrected error rate (number of major errors/total number of susceptible strains).

TABLE 5. Distribution of differences in Etest MICs for 100 strains of rapidly growing mycobacteria by the two different methods of inoculum application

Antimicrobial agent	No. of Etest overlay MICs[a] within indicated log_2 concn of Etest swab MICs[b]						
	>−2	−2	−1	Same	+1	+2	>+2
Amikacin	0	4	12	62	16	6	0
Cefoxitin	0	7	28	31	24	9	1
Ciprofloxacin	0	8	15	52	17	8	0
Clarithromycin	0	10	10	46	18	16	0
Doxycycline	0	7	11	68	9	5	0
Imipenem	0	10	8	55	12	14	1
Total	0	46	84	314	96	58	2

[a] Etest MICs were determined by use of Mueller-Hinton agar (overlay method) with 10% OADC supplementation.
[b] Etest MICs were determined by use of Mueller-Hinton agar (swab method) supplemented with OADC.

TABLE 6. Interlaboratory reproducibility of Etest MICs for 25 selected strains of rapidly growing mycobacteria

Antimicrobial agent	No. of strains with MIC agreement[a]				Category agreement	
	Exact	±1 log_2 dilution	±2 log_2 dilutions	>±2 log_2 dilutions	No. of agreements/ no. tested	% Agreement
Amikacin	15	7	3	0	25/25	100
Imipenem	14	5	2	4	22/25	88
Ciprofloxacin	10	8	7	0	25/25	100
Cefoxitin	9	13	1	2	19/25	76
Doxycycline	15	5	1	4	22/25	88
Clarithromycin	13	7	2	2	24/24	100
Total (cumulative % agreement)	76 (51)	45 (81)	16 (92)	12	137/149	92

[a] MICs for 25 strains were determined at each of two separate geographic locations (CUMC and GSMC).

pected MIC of each antimicrobial agent was obtained for 100% of all MICs within ±1 log_2 dilution (data not shown).

DISCUSSION

Evaluation of the Etest for susceptibility testing of rapidly growing mycobacteria was the primary focus of this study. We found the Etest method generally easy to perform, and it required materials commonly found in laboratories offering Kirby-Bauer disk diffusion, with the exception of the growth supplement OADC, which is commercially available, used to support or enhance growth for this group of organisms. Recent studies of susceptibility testing of rapidly growing mycobacteria, in which unsupplemented Mueller-Hinton medium was used, have suggested that adequate growth of all species and biovariants was achieved (3, 5, 14, 15). However, Hoffner et al., in a recent article describing Etest results obtained with a comparable group of organisms, noted that some strains, primarily *M. chelonae*, failed to produce visible colonies within 3 days of incubation when grown on PDM ASM II agar at 35°C (8). MICs of the same antimicrobial agents for *M. fortuitum* ATCC 6841 were comparable to what is reported here. Thus, the requirement for this growth supplement appears to vary among species and from strain to strain. In this study, all isolates produced adequate growth for interpretation of MICs within 3 days. However, there was an observable difference in the growth rates of *M. chelonae* and *M. abscessus* compared with *M. fortuitum* complex. Most strains of *M. chelonae* and *M. abscessus* required a full 72 h of incubation at 35°C to produce growth adequate for interpretation, whereas isolates of *M. fortuitum* complex generally required only 36 to 48 h.

An alternative growth medium such as Mueller-Hinton medium with 5% sheep blood or chocolatized Mueller-Hinton agar was not used in this study since acceptable growth of all strains was observed to occur on Mueller-Hinton agar with OADC supplement. Occasional shortages of OADC from commercial suppliers make it imperative that other media be thoroughly evaluated as acceptable alternatives for the Etest method. In a recent study by Koontz et al., Mueller-Hinton blood agar was used as the test medium to determine Etest MICs for 31 strains of rapidly growing mycobacteria (10). The authors reported that all strains produced a confluent lawn of growth at 72 h of incubation at 35°C and that all MICs were easily interpreted. The MICs for *M. fortuitum* ATCC 6841 reported by Koontz and coworkers were similar to those obtained in our study and reported previously (6).

When Etest MICs were compared with reference method MICs, agreement within ±2 log_2 dilutions was 95%. Discrepancies of >2 log_2 dilutions were more frequently seen with ciprofloxacin and clarithromycin, usually in the low concentration range, and did not result in differences in category interpretations.

For interpretive-category data, no very major errors were noted when Etest results were compared with those obtained by the agar dilution method. Overall, a 2.2% major error rate was noted for Etest MICs. The 4.2% (clarithromycin) and 12.5% (cefoxitin) major error rates (risk corrected) were produced by two false-resistant results for clarithromycin (MIC > 4.0 μg/ml) from 48 susceptible strains tested (MIC ≤ 1.0 μg/ml) and one false-resistant result (MIC > 32 μg/ml) from 8 cefoxitin-susceptible strains (MIC ≤ 8 μg/ml). Overall, a 11.7% minor error rate was noted when Etest MICs were compared with agar dilution MICs. The majority of minor errors among individual drugs occurred with cefoxitin (27.0%) and were caused by discrepancies between the modal MIC of 32 μg/ml (moderately susceptible) and the MIC of >32 μg/ml (resistant) determined by Etest.

The secondary goal of this study was to evaluate the potential of the agar overlay method of inoculum application to eliminate the diffuse ellipse edges or trailing growth in the inhibition zone and yet produce a similar MIC. Use of this inoculum application method in the past for agar disk diffusion testing of aerobic bacteria with Mueller-Hinton agar has been shown to result in a more clearly defined inhibition zone edge, allowing for easier measurement (2). Drawbacks to this method were slightly smaller zone sizes due to the use of additional agar and the technically complex method of preparation. In this study, diffuse ellipse edges and trailing growth were still a problem with the overlay method, and in some cases results were more difficult to interpret than they were with the corresponding swab-prepared plate. Additionally, the rate of agreement of MICs between the agar overlay and swab methods of applying inoculum to Mueller-Hinton agar plates was less than the desired rate of ≥90%. Therefore, we recommend using the swab method of inoculation as previously described. Etests prepared by both inoculation methods with cefoxitin, clarithromycin, and imipenem were sometimes difficult to interpret because of diffuse ellipse edges or trailing growth for susceptible strains of *M. fortuitum* complex. In contrast, Etests with ciprofloxacin, amikacin, and doxycycline generally produced sharply defined ellipse edges which lead to easily interpreted MICs for susceptible strains.

Interlaboratory reproducibility of the Etest results was excellent. Both participating laboratories performed the Etest for a representative cross section of the study strains by using the recommendation of the manufacturer for selection of the MIC endpoint. That is, the MIC is read at the point along the Etest strip at which no visible colonies are formed. Since diffuse ellipse edges and trailing growth will occasionally occur with

certain antimicrobial agents, such as cefoxitin and imipenem, laboratories should strictly adhere to the manufacturer's recommendations of determining the MIC by reading the result at the point along the Etest strip at which no colonies are formed.

The designated quality control strain in this study was *M. fortuitum* ATCC 6841. Results obtained with all antimicrobial agents tested were similar to those reported previously (6), with the exception of the clarithromycin MIC. Clarithromycin MICs for ATCC 6841 were erratic and ranged from 1.0 to 8.0 μg/ml in this study. The erratic performance of this strain was noted previously by Brown et al. (3). We agree with their conclusions that *M. fortuitum* ATCC 6841 should not be used as a quality control strain with clarithromycin and that additional reference strains should be evaluated for this purpose. We have subsequently evaluated *M. chelonae* ATCC 14472 for potential use as a quality control strain for clarithromycin. The MIC for this strain was consistent at 0.25 μg/ml (data not shown), and the strain demonstrated good growth on OADC-supplemented Mueller-Hinton agar.

In summary, our data suggest that the Etest method of determining susceptibility of the rapidly growing mycobacteria may be an accurate and reproducible method when compared to agar dilution. Laboratories choosing to utilize this method should consider supplementing the Mueller-Hinton medium with OADC prior to inoculation of the plate, especially for strains of *M. chelonae* or *M. abscessus*. Inoculum application by use of a cotton swab, followed by incubation at 35°C for 72 h in an ambient air incubator, appears to be satisfactory.

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