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Comparison of Minocycline Susceptibility Testing Methods for Carbapenem-Resistant *Acinetobacter baumannii*

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Treatment options for infections due to carbapenem-resistant *Acinetobacter baumannii* are extremely limited. Minocycline is a semisynthetic tetracycline derivative with activity against this pathogen. This study compared susceptibility testing methods that are used in clinical microbiology laboratories (Etest, disk diffusion, and Sensititre broth microdilution methods) for testing of minocycline, tigecycline, and doxycycline against 107 carbapenem-resistant *A. baumannii* clinical isolates. Susceptibility rates determined with the standard broth microdilution method using cation-adjusted Mueller-Hinton (MH) broth were 77.6% for minocycline and 29% for doxycycline, and 92.5% of isolates had tigecycline MICs of ≤2 μg/ml. Using MH agar from BD and Oxoid, susceptibility rates determined with the Etest method were 67.3% and 52.3% for minocycline, 21.5% and 18.7% for doxycycline, and 71% and 29.9% for tigecycline, respectively. With the disk diffusion method using MH agar from BD and Oxoid, susceptibility rates were 82.2% and 72.9% for minocycline and 34.6% and 34.6% for doxycycline, respectively, and rates of MICs of ≤2 μg/ml were 46.7% and 23.4% for tigecycline. In comparison with the standard broth microdilution results, very major rates were low (~2.8%) for all three drugs across the methods, but major error rates were higher (~5.6%), especially with the Etest method. For minocycline, minor error rates ranged from 14% to 37.4%. For tigecycline, minor error rates ranged from 6.5% to 69.2%. The majority of minor errors were due to susceptible results being reported as intermediate. For minocycline susceptibility testing of carbapenem-resistant *A. baumannii* strains, very major errors are rare, but major and minor errors overcalling strains as intermediate or resistant occur frequently with susceptibility testing methods that are feasible in clinical laboratories.

Acinetobacter baumannii has become a major health care-associated pathogen over the past 2 decades, due to its intrinsic resistance to several classes of antimicrobial agents, its propensity to acquire resistance to other drug classes, and its ability to resist desiccation in environments typically found in hospitals (1). *A. baumannii* causes a variety of infections, with respiratory tract infections being the most common (1). Significant clinical challenges are posed by carbapenem-resistant *A. baumannii*, which accounted for over 75% of *A. baumannii* clinical isolates tested in a recent global survey (2). Mortality rates for carbapenem-resistant *A. baumannii* infections may be as high as 76% (3). Treatment options for carbapenem-resistant *A. baumannii* infections have not been well defined but generally include polymyxins (colistin and polymyxin B), tigecycline, or sulbactam, alone or in combination with a second agent, such as rifampin or a carbapenem, with the expectation of synergistic activities (4). However, toxicity (polymyxins), suboptimal pharmacokinetics (tigecycline), and the propensity for development of resistance (sulbactam) limit these options (1).

Minocycline is a semisynthetic tetracycline derivative that was introduced into clinical practice in the 1960s (5). With the recent reintroduction of an intravenous formulation of minocycline, there is increasing interest in this agent as an additional treatment option for carbapenem-resistant *A. baumannii* infections. In the aforementioned global survey, over 70% of the clinical isolates were susceptible to minocycline, using the Clinical and Laboratory Standards Institute (CLSI) breakpoints of ≤4 μg/ml for susceptibility, 8 μg/ml for intermediate resistance, and ≥16 μg/ml for resistance. In most hospitals, however, *A. baumannii* is not routinely tested for minocycline susceptibility; at those institutions, testing is conducted upon request by health care providers, using approaches such as the Etest system, disk diffusion, or commercially available MIC testing plates. For tigecycline, several studies have suggested discrepancies in the interpretation of susceptibility results, depending on the testing methods employed (6, 7). Data for minocycline remain limited (8). The aim of the present study was to assess the agreement, correlation, and very major, major, and minor error rates for these three methods, compared with the standard broth microdilution method, for a collection of carbapenem-resistant *A. baumannii* clinical isolates.

MATERIALS AND METHODS

Strains and reagents. A total of 107 carbapenem-resistant *A. baumannii* clinical strains were included in the study. They were collected at hospitals in Pennsylvania, Missouri, New York, Nevada, California, and Florida between 2009 and 2015, and some were reported previously (9–12). Minocycline, tigecycline, and doxycycline were purchased from Sigma-Aldrich (St. Louis, MO). Etest strips were purchased from bioMérieux (Durham, NC). Sensi-Discs for disk diffusion testing were purchased

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TABLE 1 Susceptibility of carbapenem-resistant *Acinetobacter baumannii* strains based on testing methods ($n = 107$)

Testing method[a]	Testing result (% [no. of strains])								
	Minocycline			Doxycycline			Tigecycline		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant	≤2 μg/ml	>2 to <8 μg/ml	≥8 μg/ml
BMD with CAMHB (BD)	77.6 (83)	20.6 (22)	1.9 (2)	29 (31)	4.7 (5)	66.4 (71)	92.5 (99)	7.5 (8)	0
BMD with MHB (BD)	73.8 (79)	22.4 (24)	3.7 (4)	30.8 (33)	3.7 (4)	65.4 (70)	99.1 (106)	0.9 (1)	0
BMD with MHB (Oxoid)	91.6 (98)	7.5 (8)	0.9 (1)	35.5 (38)	0.9 (1)	63.6 (68)	100 (107)	0	0
Sensititre BMD with CAMHB-TES	67.3 (72)	25.2 (27)	7.5 (8)	29.9 (32)	6.5 (7)	63.6 (68)	97.2 (104)	2.8 (3)	0
Etest with MHA (BD)	67.3 (72)	27.1 (29)	5.6 (6)	21.5 (23)	8.4 (9)	70.1 (75)	71 (76)	27.1 (29)	1.9 (2)
Etest with MHA (Oxoid)	52.3 (56)	28 (30)	19.6 (21)	18.7 (20)	10.3 (11)	71 (76)	29.9 (32)	67.3 (72)	2.8 (3)
Disk diffusion with MHA (BD)	82.2 (88)	17.8 (19)	0	34.6 (37)	9.3 (10)	56.1 (60)	46.7 (50)	50.5 (54)	2.8 (3)
Disk diffusion with MHA (Oxoid)	72.9 (78)	24.3 (26)	2.8 (3)	34.6 (37)	5.6 (6)	59.8 (64)	23.4 (25)	72.9 (78)	3.7 (4)

[a] BMD, broth microdilution; MHB, Mueller-Hinton broth; CAMHB, cation-adjusted Mueller-Hinton broth; MHA, Mueller-Hinton agar.

from BD (Franklin Lakes, NJ). Mueller-Hinton (MH) broth (catalog no. 211443), cation-adjusted MH II broth (catalog no. 212322), and MH II agar (catalog no. 211438) were purchased from BD. Oxoid MH broth (catalog no. CM0405) and Oxoid MH agar (catalog no. CM0337) were purchased from Thermo Scientific Remel (Lenexa, KS). Sensititre GNX3F plates and cation-adjusted MH broth with TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (catalog no. T3462) were purchased from TREK Diagnostic Systems (Cleveland, OH).

Susceptibility testing. Minocycline, doxycycline, and tigecycline susceptibility tests were conducted in duplicate by different operators, on separate days, using freshly prepared media and antimicrobial solutions. The testing methods included standard broth microdilution testing using cation-adjusted MH II broth from BD (the reference method in this work) as well as regular MH broth from BD and Oxoid, disk diffusion testing and Etest analysis using MH II agar from BD and MH agar from Oxoid, and Sensititre GNX3F broth microdilution testing using cation-adjusted MH broth with TES, which is the medium recommended by the manufacturer of Sensititre plates. When the results from the duplicates of the same methods were discordant (i.e., >2-fold MIC differences for broth microdilution, Etest, or Sensititre assays and any categorical disagreement for disk diffusion assays), the testing was repeated until the discrepancies were resolved. The concentration ranges tested with the standard broth microdilution method were 0.06 to 128 μg/ml for minocycline and doxycycline and 0.01 to 32 μg/ml for tigecycline. For disk diffusion assays, the Sensi-Discs contained 30 μg of minocycline and doxycycline and 15 μg of tigecycline. For Etest assays, the concentration ranges were 0.016 to 256 μg/ml for all three drugs. The inocula were in accordance with CLSI recommendations (13, 14) or the manufacturer's recommendations, in the case of Sensititre plates. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains.

Interpretive criteria. The current interpretive criteria from the CLSI were used, as follows: minocycline, ≤4 μg/ml (≥16 mm) for susceptible, 8 μg/ml (13 to 15 mm) for intermediate, and ≥16 μg/ml (≤12 mm) for resistant; doxycycline, ≤4 μg/ml (≥13 mm) for susceptible, 8 μg/ml (10 to 12 mm) for intermediate, and ≥16 μg/ml (≤9 mm) for resistant (15). For the purposes of this study, tigecycline susceptibility was categorized based on the U.S. Food and Drug Administration susceptibility breakpoints for *Enterobacteriaceae*, i.e., ≤2 μg/ml (≥19 mm), 4 μg/ml (15 to 18 mm), and ≥8 μg/ml (≤14 mm). Etest MICs of 6 μg/ml for minocycline and doxycycline were interpreted as intermediate, and MICs of 12 μg/ml for minocycline and doxycycline were interpreted as resistant (16, 17). For tigecycline, Etest MICs of 3 μg/ml and 6 μg/ml were considered equivalent to findings of 4 μg/ml and 8 μg/ml, respectively, for categorization.

Evaluation of concordance among methods. The results were analyzed by using the standard broth dilution method with cation-adjusted MH II broth as the reference method. In addition, comparisons were made within the disk diffusion and Etest methods on the basis of the MH agar used (BD or Oxoid). Essential agreement was defined as an Etest or Sensititre MIC equal to or within ±2-fold of the standard broth microdilution MIC (18). Categorical agreement was defined as a result from any of the three methods that belonged to the same interpretive category (i.e., susceptible, intermediate, or resistant [MICs of ≤2 μg/ml, >2 to <8 μg/ml, or ≥8 μg/ml, respectively, in the case of tigecycline]) as that determined with the standard broth microdilution method. A very major error was defined as a result in the susceptible category when the standard broth microdilution method gave a result in the resistant category (MIC of ≥8 μg/ml for tigecycline). A major error was defined as a result in the resistant category when the standard broth microdilution method gave a result in the susceptible category (MIC of ≤2 μg/ml for tigecycline). A minor error occurred when a result was interpreted as susceptible or resistant and the standard broth microdilution result was interpreted as intermediate or when a result was interpreted as intermediate and the standard broth microdilution result was interpreted as susceptible or resistant (or when shifts in the corresponding categories occurred for tigecycline). The same analysis was performed for the within-method comparisons of disk diffusion and Etest results obtained with BD and Oxoid MH agar.

RESULTS

Susceptibility rates with reference broth microdilution method. The rates of susceptibility of carbapenem-resistant *A. baumannii* strains to minocycline and doxycycline, based on broth microdilution assays with cation-adjusted MH broth from BD (the reference method for this study), were 77.6% and 29%, respectively (Table 1). The rate of MICs of ≤2 μg/ml was 92.5% for tigecycline. The MIC ranges were 0.125 to 16 μg/ml for minocycline, 0.125 to 128 μg/ml for doxycycline, and 0.06 to 4 μg/ml for tigecycline. The scatter plots of the actual MICs and inhibitory zones from all of the methods tested in this study are provided in Fig. S1 in the supplemental material.

When broth microdilution assays were performed with regular MH broth from BD, the susceptibility rates were comparable to those determined with the reference method for all three agents (for tigecycline, MIC of ≤2 μg/ml). With regular MH broth from Oxoid, however, the susceptibility rate was substantially higher (91.6%) for minocycline but not for doxycycline or tigecycline (for tigecycline, MIC of ≤2 μg/ml) (Table 1).

Etest method. When Etest assays were conducted with MH agar from BD and Oxoid, higher rates of susceptibility and MICs of ≤2 μg/ml were observed for minocycline and tigecycline, respectively, with the former product, i.e., 67.3% versus 52.3% for minocycline and 71% versus 29.9% for tigecycline, whereas the susceptibility rates were comparable for doxycycline (21.5% ver-

TABLE 2 Very major, major, and minor error rates, compared with standard broth microdilution method with cation-adjusted Mueller-Hinton broth

Testing method[a]	Error rate (% [no. of strains])								
	Minocycline			Doxycycline			Tigecycline		
	Very major	Major	Minor	Very major	Major	Minor	Very major	Major	Minor
Sensititre BMD with CAMHB-TES	0.9 (1)	0.9 (1)	17.8 (19)	0	0	6.5 (7)	0	0	6.5 (7)
Etest with MHA (BD)	0.9 (1)	0.9 (1)	19.6 (21)	0.9 (1)	2.8 (3)	10.3 (11)	0	1.9 (2)	23.4 (25)
Etest with MHA (Oxoid)	0.9 (1)	5.6 (6)	37.4 (40)	0	2.8 (3)	10.3 (11)	0	1.9 (2)	63.6 (68)
Disk diffusion with MHA (BD)	0.9 (1)	0	14 (15)	1.9 (2)	0	13.1 (14)	0	0.9 (1)	48.6 (52)
Disk diffusion with MHA (Oxoid)	0.9 (1)	0	18.7 (20)	2.8 (3)	0	9.3 (10)	0	1.9 (2)	69.2 (74)

[a] BMD, broth microdilution; CAMHB, cation-adjusted Mueller-Hinton broth; MHA, Mueller-Hinton agar.

sus 18.7%). Against the standard broth microdilution method, very major error rates were low (only up to 0.9%), but major error rates were higher (0.9 to 5.6%) and high minor error rates were observed for minocycline (19.6% with BD agar and 37.4% with Oxoid agar) and tigecycline (23.4% with BD agar and 63.6% with Oxoid agar) (Table 2). Most of the minor errors were intermediate results interpreted as susceptible or vice versa (Table 2).

Disk diffusion method. Similar to Etest assays, higher rates of susceptibility and MICs of ≤2 μg/ml were observed with MH agar from BD versus Oxoid for minocycline (82.2% versus 72.9%) and tigecycline (46.7% versus 23.4%), respectively. Very major and major error rates were low across the three tetracyclines (~2.8% and ~1.9%, respectively). Minor error rates were high for both minocycline (14% with BD agar and 18.7% with Oxoid agar) and tigecycline (48.6% with BD agar and 69.2% with Oxoid agar). Most of the minor errors were intermediate findings interpreted as susceptible, susceptible findings interpreted as intermediate, or comparative shifts for tigecycline.

Sensititre method. The manufacturer of Sensititre plates recommends the use of cation-adjusted MH broth with TES, which is specifically marketed for this product. The use of this medium resulted in rates of susceptibility and MICs of ≤2 μg/ml for doxycycline and tigecycline, respectively, that were very comparable to those obtained with the standard broth microdilution method, while the susceptibility rate for minocycline was slightly lower (67.3% versus 77.6%), resulting mostly from minor errors (susceptible findings to intermediate and intermediate findings to resistant). Major/very major error rates were low (~0.9%). To elucidate this phenomenon further, we conducted additional Sensititre plate testing, once per strain, using cation-adjusted MH broth from BD, which was also used for the standard broth microdilution assays. This method yielded results more concordant with those from the reference method, compared with the cation-adjusted MH broth with TES that was recommended and provided by the manufacturer of Sensititre plates (susceptibility rates of 72.9% versus 77.6% for minocycline and 29.9% versus 29% for doxycycline and rates of MICs of ≤2 μg/ml of 94.4% versus 92.5% for tigecycline).

DISCUSSION

Carbapenem-resistant *A. baumannii* has become one of the most difficult-to-treat pathogens for nosocomial infections, due to the lack of adequate treatment options. Minocycline (both oral and intravenous forms) has been approved by the U.S. Food and Drug Administration for the treatment of minocycline-susceptible *Acinetobacter* infections, including infections involving strains resistant to carbapenem and/or polymyxin classes. However, susceptibility testing for minocycline and tigecycline (another tetracycline agent with anti-*Acinetobacter* activity) is not routinely performed in most hospitals; therefore, it needs to be conducted manually when it is requested. For most clinical laboratories, disk diffusion testing and Etest assays are the easiest tests to perform, while testing with Sensititre plates (commercially available MIC testing plates) is also an option for laboratories with access to it. However, data correlating the performance of these minocycline susceptibility testing methods are scarce for carbapenem-resistant *A. baumannii* strains, the subgroup of *A. baumannii* strains for which most requests for testing are likely to be generated. The present study was conducted to compare the performance of these testing methods, using the standard broth microdilution method as the reference method.

The rates of susceptibility of carbapenem-resistant *A. baumannii* strains to minocycline, doxycycline, and tigecycline (rate of MICs of ≤2 μg/ml for the latter) using the reference method were 77.6%, 29%, and 92.5%, respectively, which were in line with larger surveillance data from the United States and thus were reflective of findings encountered in clinical practice (2, 19, 20). Cation adjustment for calcium and magnesium is recommended for broth microdilution testing, since aminoglycoside MICs may be falsely low for *Pseudomonas aeruginosa* without adjustment, compared with the agar dilution method, due to the function of the MexXY-OprM efflux pump (21). When we performed broth microdilution using cation-unadjusted MH broth from BD and Oxoid, we saw excellent categorical agreements across the combinations of three drugs and three broth formulations, with the exception of minocycline and regular MH broth from Oxoid, which had a substantially higher susceptibility rate (91.6%), compared with the reference method (77.6%). Tet-group tetracycline efflux pumps such as Tet(B), which are present in some *A. baumannii* strains, require divalent cations for their function (22). While divalent cation contents were not measured in this study, the findings suggest that broth microdilution testing of *A. baumannii* with minocycline may be sensitive to the composition of the medium.

Etest assays generally provided lower rates of susceptibility for minocycline and MICs of ≤2 μg/ml for tigecycline, compared with the reference method, regardless of the MH agar used. This resulted from high minor error rates (19.6 to 37.4% for minocycline and 23.4 to 63.6% for tigecycline), most of which represented a shift from the susceptible category to the intermediate category (or the corresponding categories for tigecycline). This phenomenon has been well documented for tigecycline (23, 24) and more

recently for minocycline as well (8). Many of these minor errors could be attributed to incremental MIC differences afforded by the granularity of Etest readings, i.e., a shift from 4 μg/ml (reference) to 6 μg/ml (Etest) for minocycline and from 2 μg/ml (reference) to 3 μg/ml (Etest) for tigecycline. Minor error rates were also high with the disk diffusion method (14 to 18.7% for minocycline and 48.6 to 69.2% for tigecycline), as has been reported by others for tigecycline (6, 25). Our data suggest that this trend also applies to minocycline, based on the current CLSI breakpoints. However, major and very major error rates were low regardless of the medium used (~2.8% for minocycline and tigecycline and ~1.9% for doxycycline).

Sensititre testing is a commercially available broth microdilution method using preformulated 96-well plates. Specifically, the GNX3F plates are optimized for testing non-lactose-fermenting Gram-negative bacteria and include minocycline, doxycycline, and tigecycline. The manufacturer recommends the use of cation-adjusted MH broth with TES buffer, which is also commercially available. Using this method, the categorical interpretations were highly concordant with those from the reference method, with ~1.9% major/very major error rates for each agent, which were lower than those for Etest or disk diffusion assays. Minor error rates (~17.8%) were comparable to those for the other methods, except for Etest assays performed with MH agar from Oxoid (~63.6%). Interestingly, comparable or higher concordance was observed even when Sensititre testing was performed with cation-adjusted MH broth from BD.

Overall, we did not identify a single testing method that was the most concordant with the reference method for minocycline, with all three testing methods having moderately high minor error rates, but the disk diffusion method using MH agar from BD gave the least discordant results. For doxycycline, the error rates were much lower across the methods due to the MICs distributing mostly in the resistant range; therefore, any of these methods appear to be acceptable. For tigecycline, Sensititre testing clearly yielded the most concordant results with respect to the reference method, whereas both agar-based methods suffered from very high minor error rates, especially when MH agar from Oxoid was used.

Our study has several limitations. The study was performed at a single research laboratory. To mitigate biases, the measurements were performed in biological duplicates by blinded researchers on separate days, using independently prepared antimicrobial solutions and media, and discrepancies were resolved with additional measurements. Also, some but not all of the clinical strains used in the present study were subjected to molecular typing in previous studies. On the basis of those findings, most strains likely belonged to worldwide clone 2, which is the prevalent lineage among carbapenem-resistant *A. baumannii* strains in the United States and many other countries (26).

In conclusion, major and very major error rates were low across the susceptibility testing methods that are feasible in clinical laboratories (Etest, disk diffusion, and Sensititre testing methods) for testing of carbapenem-resistant *A. baumannii* strains for minocycline, doxycycline, and tigecycline susceptibility. However, minor error rates were high for minocycline and tigecycline with any of the MH agar-based methods, and the majority of the errors were due to overcalling strains as intermediate or resistant.

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