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Evaluation of Alamar Colorimetric MIC Method for Antimicrobial Susceptibility Testing of Gram-Negative Bacteria

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The Alamar (Alamar Biosciences, Inc., Sacramento, Calif.) colorimetric antimicrobial susceptibility testing method is a new approach to the determination of broth microdilution MICs. The method uses a color indicator to detect growth of microorganisms within the wells of a microdilution tray. The color changes can be read visually or with a fluorometer. The system contains growth and sterility control wells and 20 antimicrobial agents per MIC tray with eight twofold dilutions for each antimicrobial agent. We tested 186 multiresistant, gram-negative bacterial isolates against 33 antimicrobial agents and compared the results to those obtained by agar dilution. Categorical agreement for all agents was 90.9% and ranged from 78.2% for ampicillin-sulbactam to 98.1% for amikacin. Percent agreement for MIC results (within ± 1 $\log_2$ dilution) was 91.0% for all agents and ranged from 69.1% for gentamicin to 97.9% for ciprofloxacin. Most of the disagreements were with the penicillins and cephalosporins for β-lactamase-producing strains. The Alamar MIC system is very easy to read visually and appears to be a satisfactory addition to currently used MIC determination methods.

The method most commonly used to determine MICs in clinical laboratories is the broth microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS) (17). This method is convenient to use, since the reagents can be prepared in house or purchased from commercial manufacturers. The reagents may be stored frozen or lyophilized, require only a small amount of storage space, and can be removed from storage as needed. A modification of this technique has been developed by Alamar Biosciences, Inc., Sacramento, Calif. This manufacturer has used the conventional broth microdilution method and added a color indicator to enhance the detection of growth within the microdilution wells. The colorimetric growth indicator is based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction indicator that changes color in response to chemical reduction of growth medium resulting from bacterial growth. The indicator can be read visually or with a fluorometer. Other manufacturers have included fluorogenic compounds (3, 16, 21) for detection of growth in a 5- to 10-h period, but the results must be read with a fluorometer. The results of the Alamar method, on the other hand, can be read visually, and the indicator used makes reading easy because the change from blue (no growth) to red (growth) is obvious, even for wells with reduced or small amounts of growth. This overcomes problems frequently encountered in conventional broth microdilution tests, such as inoculum sedimentation or very scant or transparent growth, which occur with some species of bacteria.

This study assessed the reliability of results obtained by the Alamar colorimetric method, compared with agar dilution results, by using a well-characterized set of difficult-to-test, multiresistant, gram-negative bacteria (the Centers for Disease Control and Prevention challenge set [6]). Special attention was given to strains with borderline resistance (25), to antimicrobial agents for which there are few resistant strains, and to the method of calculating interpretative categorical errors (16).

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MATERIALS AND METHODS

Antimicrobial agents. Standard antimicrobial powders were obtained from various manufacturers for agar dilution testing; Alamar supplied the prepared antimicrobial agents with the colorimetric growth indicator in dehydrated microdilution trays. The antimicrobial agents tested were amikacin, amoxicillin-clavulanic acid, ampicillin, ampicillin-sulbactam, aztreonam, carbenicillin, cefamandole, cefazolin, cefmetazole, cefonicid, cefoperazone, cefotaxime, cefotetan, cefoxitin, ceftazidime, ceftizoxime, ceftriaxone, cefuroxime, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, mezlocillin, nitrofurantoin, norfloxacin, ofloxacin, piperacillin, tetracycline, ticarcillin, ticarcillin-clavulanic acid, tobramycin, and trimethoprim-sulfamethoxazole. There were eight dilutions for each antimicrobial agent, and the dilution scheme varied for each agent.

Bacterial strains. We tested 186 multiresistant, gram-negative strains in duplicate, including the following: 8 *Acinetobacter* species strains (2 formerly identified as *Acinetobacter anitratus*, 4 *A. anitratus* subsp. *calcoaceticus* strains, and 2 *A. anitratus* subsp. *lwoffi* strains), 2 *Aeromonas hydrophilia* strains, 1 *Citrobacter freundii* strain, 16 *Enterobacter aerogenes* strains, 2 *E. agglomerans* strains, 19 *E. cloacae* strains, 22 *Escherichia coli* strains, 1 *Hafnia alvei* strain, 6 *Klebsiella oxytoca* strains, 1 *K. ozaenae* strain, 13 *K. pneumoniae* strains, 3 *Morganella morganii* strains, 15 *Proteus mirabilis* strains, 5 *P. vulgaris* strains, 6 *Providencia rettgeri* strains, 2 *P. alcalifaciens* strains, 5 *P. stuartii* strains, 34 *Pseudomonas aeruginosa* strains, 3 *Serratia liquefaciens* strains, 18 *S. marcescens* strains, 1 *S. odorifera* biogroup 2 strain, 1 *Shigella sonnei* strain, and 2 *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*) strains. The bacteria were identified at the Centers for Disease Control and Prevention by conventional methodology (14). These strains make up the Centers for Disease Control and Prevention

challenge set of gram-negative bacteria (6). They are multiresistant and have various resistance mechanisms, and the MICs for many of them are at or near the breakpoints for resistance. The strains were stored in defibrinated rabbit blood at ≤120°C in a liquid nitrogen freezer. Control strains used in this study were *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922 and ATCC 35218, and *P. aeruginosa* ATCC 27853.

Antimicrobial susceptibility testing. Isolates were removed from storage, streaked onto a Trypticase soy agar plate supplemented with 5% sheep blood (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and incubated for 18 to 24 h at 35°C. One isolated colony was picked from the plate, streaked onto a new Trypticase soy agar plate containing 5% sheep blood, and incubated for 18 to 20 h. A growth suspension was prepared in 5 ml of Mueller-Hinton broth for agar dilution tests and in 5 ml of saline diluent for Alamar dilution tests (1). These suspensions were adjusted to equal a 0.5 barium sulfate (McFarland) standard for susceptibility testing.

For agar dilution tests, antimicrobial agents were prepared as described by the NCCLS (17, 18), in 10-times-concentrated dilutions (eight twofold dilutions per agent). Nine milliliters of molten (48°C) Mueller-Hinton agar was added to each milliliter of the diluted antimicrobial agent. Stable antimicrobial agents were stored for no more than 2 weeks at 4°C before use. Labile antimicrobial agents (e.g., imipenem) were prepared on the day of use. Antimicrobial plates were removed from storage, warmed to room temperature, and dried for approximately 20 min in a biological safety cabinet. The standardized inoculum in Mueller-Hinton broth was diluted 1:10 and added to the seed tray of a Steers replicator (23). The agar dilution plates were inoculated, dried for approximately 10 min, and incubated for 16 to 18 h at 35°C in ambient air. The final inoculum was approximately 10^4 CFU per spot. MICs were read at the lowest concentration at which no visible growth or no more than one colony occurred.

For Alamar broth microdilution tests, 25 μl of the standardized saline inoculum was added to 25 ml of Alamar Inoculum Broth and vortexed. The diluted inoculum was placed in the inoculum reservoir, and 100 μl of this diluted inoculum was added to each well of the microdilution tray with a multichannel pipettor. The final inoculum was approximately 5×10^5 CFU/ml. The inoculated microdilution trays were covered and incubated for 18 h at 35°C in ambient air. The MIC was read as the lowest concentration of an antimicrobial agent at which no color change occurred (red indicates growth, and blue indicates no growth). Both agar dilution and Alamar tests were performed on the same day for each set of test isolates and control strains. Duplicate testing was performed on the following day for each set of test strains and controls.

Repeat testing was performed on all strains in duplicate with ampicillin, ampicillin-sulbactam, gentamicin, and imipenem by using the Alamar system, agar dilution, and broth microdilution (17, 18) because the manufacturing processes were changed for these four agents. Changes for ampicillin, ampicillin-sulbactam, and imipenem were required because a stability problem with these agents was recognized, while the process for gentamicin was modified to correct a pH problem with one of the reagents. Broth microdilution was added as a reference method to determine whether it produced results more closely approximating those of the Alamar method than the agar dilution method for these four agents. During repeat testing, the antimicrobial agent solutions for the reference agar and broth microdilution tests were the same, so that any MIC difference observed between these two reference tests was due to the difference in methodology and not to a difference in antimicrobial agent concentration, as could be the case when comparing the Alamar system to either of the reference methods.

Statistical analysis. All tests were performed in duplicate. For comparison and statistical analysis, each of these tests was treated as an individual observation; therefore, 372 observations were obtained for each antimicrobial agent.

To measure the degree of agreement between the Alamar and agar dilution results, the distribution of differences in the $\log_2$ dilution MIC results was examined and the percentage of isolates that yielded identical results within the accuracy limits of the standard test (± 1 $\log_2$ dilution) was calculated. Also, to determine if the Alamar method tended to produce significantly lower or higher results than the standard method, we performed a Wilcoxon signed-rank test (12) on the $\log_2$ MIC results of the two tests. MICs within ± 1 $\log_2$ dilution were regarded as identical for this hypothesis test. Comparison of interpretative categorical results (susceptible, intermediate, and resistant) was done by calculating minor, major, very major, and essential errors (major errors plus very major errors) and their rates (24). Since a major error is a categorical change from susceptible to resistant for an isolate determined by the test method, the error rate (percent) was obtained by using the number of susceptible strains determined by the reference method as the denominator. For very major errors, the denominator equaled the number of resistant strains, and for essential errors, the denominator equaled the number of susceptible strains plus the number of resistant strains. For the details of these calculations, see the footnotes to Tables 3 and 4.

RESULTS

We compared the agar dilution MICs of 33 antimicrobial agents to the results of the Alamar microdilution method for 186 stock strains of gram-negative bacteria, the Centers for Disease Control and Prevention challenge set (6). The distribution of differences in $\log_2$ MICs, the percent agreement, and the *P* values from the Wilcoxon signed-rank test are shown in Table 1. Overall agreement at ± 1 $\log_2$ dilution was 91.0% and ranged from 69.1% for gentamicin to 97.9% for ciprofloxacin. For the penicillin- and cephalosporin-susceptible isolates, the MICs of the β-lactam antimicrobial agents tended to be lower in the Alamar system than by agar dilution; the MICs for penicillinase- and cephalosporinase-producing isolates tended to be higher in the Alamar system than on agar (data not shown). The MICs of gentamicin, tobramycin, and trimethoprim-sulfamethoxazole were also on average 1 to 2 dilutions lower in the Alamar system than by agar dilution. These trends occurred with most of the species tested.

Tests for ampicillin, ampicillin-sulbactam, gentamicin, and imipenem with all isolates were repeated with the Alamar method, agar dilution, and broth microdilution. The same statistical analysis was performed on the repeat results, and the findings are shown in Table 2. Agreement improved dramatically for ampicillin and ampicillin-sulbactam, but agreement was nearly the same for gentamicin and imipenem. However, for gentamicin, the Alamar results versus the broth microdilution results gave better agreement than either Alamar or broth microdilution versus agar dilution. This is in contrast to the results obtained for imipenem, which showed less agreement when Alamar results were compared with broth microdilution results.

MICs for the Alamar and agar dilution methods were converted to interpretative categories of susceptible, intermediate, and resistant (17, 18). Table 3 shows the number of susceptible, intermediate, and resistant strains; minor, major,

TABLE 1. Distribution of differences in the MICs of 33 antimicrobial agents for 186 gram-negative bacteria determined by Alamar versus agar dilution

Antimicrobial agent(s)	No. (%) of isolates with MIC[a] difference of:							% Agreement[b] ± SE	P value[c]
	<−2	−2	−1	0	+1	+2	>+2		
Amikacin	0 (0.0)	5 (1.3)	80 (21.5)	221 (59.4)	57 (15.3)	6 (1.6)	3 (0.9)	96.2 ± 1.0	0.140
Amoxicillin-clavulanic acid	3 (0.8)	4 (1.1)	15 (4.0)	243 (65.3)	88 (23.7)	12 (3.2)	7 (1.9)	93.0 ± 1.3	0.009
Ampicillin	4 (1.0)	29 (7.8)	55 (14.8)	274 (73.7)	4 (1.1)	4 (1.1)	2 (0.5)	89.5 ± 1.6	<0.001
Ampicillin-sulbactam	10 (2.6)	59 (15.9)	142 (38.1)	155 (41.7)	4 (1.1)	1 (0.3)	1 (0.3)	80.9 ± 2.0	<0.001
Aztreonam	2 (0.5)	0 (0.0)	125 (33.6)	201 (54.0)	31 (8.3)	4 (1.1)	9 (2.5)	96.0 ± 1.0	0.002
Carbenicillin	0 (0.0)	6 (1.6)	38 (10.2)	171 (46.0)	110 (29.6)	29 (7.8)	18 (4.8)	85.8 ± 1.8	<0.001
Cefamandole	5 (1.3)	3 (0.8)	26 (7.0)	226 (60.8)	66 (17.7)	25 (6.7)	21 (5.7)	85.5 ± 1.8	<0.001
Cefazolin	7 (1.9)	7 (1.9)	15 (4.0)	269 (72.3)	48 (12.9)	19 (5.1)	7 (1.9)	89.3 ± 1.6	0.031
Cefmetazole	2 (0.5)	3 (0.8)	20 (5.4)	234 (62.9)	81 (21.8)	23 (6.2)	9 (2.4)	90.1 ± 1.6	<0.001
Cefonicid	4 (1.1)	12 (3.2)	29 (7.8)	276 (74.2)	30 (8.1)	10 (2.7)	11 (2.9)	90.1 ± 1.6	0.193
Cefoperazone	2 (0.6)	7 (1.9)	34 (9.1)	230 (61.8)	75 (20.2)	15 (4.0)	9 (2.4)	91.1 ± 1.5	0.004
Cefotaxime	2 (0.5)	5 (1.3)	20 (5.4)	262 (70.4)	64 (17.2)	13 (3.5)	6 (1.7)	93.0 ± 1.3	0.009
Cefotetan	2 (0.6)	8 (2.2)	18 (4.8)	270 (72.6)	47 (12.6)	16 (4.3)	11 (2.9)	90.1 ± 1.6	0.002
Cefoxitin	1 (0.3)	3 (0.8)	18 (4.8)	238 (64.0)	90 (24.2)	16 (4.3)	6 (1.6)	93.0 ± 1.3	<0.001
Ceftazidime	1 (0.3)	2 (0.5)	6 (1.6)	256 (68.8)	79 (21.2)	15 (4.0)	13 (3.6)	91.7 ± 1.4	<0.001
Ceftizoxime	2 (0.5)	3 (0.8)	39 (10.5)	274 (73.7)	38 (10.2)	10 (2.7)	6 (1.6)	94.4 ± 1.2	0.008
Ceftriaxone	4 (1.1)	3 (0.8)	34 (9.1)	261 (70.2)	44 (11.8)	16 (4.3)	10 (2.7)	91.1 ± 1.5	0.001
Cefuroxime	2 (0.6)	3 (0.8)	15 (4.0)	256 (68.8)	67 (18.0)	18 (4.8)	11 (3.0)	90.9 ± 1.5	<0.001
Cephalothin	3 (0.8)	8 (2.2)	24 (6.5)	284 (76.3)	43 (11.6)	4 (1.1)	6 (1.5)	94.4 ± 1.2	0.422
Chloramphenicol	1 (0.3)	6 (1.6)	66 (17.7)	234 (62.9)	58 (15.6)	4 (1.1)	3 (0.8)	96.2 ± 1.0	0.496
Ciprofloxacin	1 (0.3)	2 (0.5)	5 (1.3)	257 (69.1)	102 (27.4)	5 (1.3)	0 (0.0)	97.9 ± 0.8	0.241
Gentamicin	7 (1.9)	107 (28.8)	153 (41.1)	97 (26.1)	7 (1.9)	0 (0.0)	1 (0.2)	69.1 ± 2.4	<0.001
Imipenem	2 (0.5)	9 (2.4)	37 (9.9)	159 (42.7)	117 (31.5)	36 (9.7)	12 (3.3)	84.1 ± 1.9	<0.001
Mezlocillin	5 (1.3)	11 (3.0)	73 (19.6)	237 (63.7)	38 (10.2)	6 (1.6)	2 (0.6)	93.6 ± 1.3	0.051
Nitrofurantoin	0 (0.0)	1 (0.3)	37 (9.9)	253 (68.0)	74 (19.9)	6 (1.6)	1 (0.3)	97.9 ± 0.8	0.017
Norfloxacin	1 (0.3)	2 (0.5)	13 (3.5)	279 (75.0)	68 (18.3)	8 (2.2)	1 (0.2)	96.8 ± 0.9	0.042
Ofloxacin	1 (0.3)	1 (0.3)	36 (9.7)	252 (67.7)	74 (19.9)	6 (1.6)	2 (0.5)	97.3 ± 0.8	0.029
Piperacillin	7 (1.9)	8 (2.2)	76 (20.4)	220 (59.1)	45 (12.1)	11 (3.0)	5 (1.3)	91.7 ± 1.4	0.434
Tetracycline	1 (0.3)	1 (0.3)	9 (2.4)	167 (44.9)	169 (45.4)	18 (4.8)	7 (1.9)	92.7 ± 1.4	<0.001
Ticarcillin	2 (0.6)	3 (0.8)	79 (21.2)	227 (61.0)	30 (8.1)	13 (3.5)	18 (4.8)	90.3 ± 1.5	<0.001
Ticarcillin-clavulanic acid	3 (0.8)	13 (3.5)	58 (15.6)	207 (55.6)	67 (18.0)	15 (4.0)	9 (2.5)	89.3 ± 1.6	0.097
Tobramycin	3 (0.8)	32 (8.6)	188 (50.5)	141 (37.9)	7 (1.9)	0 (0.0)	1 (0.3)	90.3 ± 1.5	<0.001
Trimethoprim-sulfamethoxazole	5 (1.4)	25 (6.7)	61 (16.4)	269 (72.5)	7 (1.9)	3 (0.8)	1 (0.3)	90.8 ± 1.5	<0.001
Overall	99 (0.8)	402 (3.2)	1,724 (13.6)	7,837 (62.0)	1,953 (15.4)	396 (3.1)	236 (1.9)	91.0 ± 0.3	<0.001

[a] Zero indicates the number and percentage of isolates for which MICs were identical; −1, +1, etc. indicate $\pm\log_2$ dilution differences.
[b] Percentage of isolates within the accuracy limits of the test (± 1 $\log_2$ dilution).
[c] *P* values were obtained with the Wilcoxon signed-rank test.

very major, and essential errors; and category agreement between Alamar and agar dilution results. Absolute category agreement ranged from 78.2% for ampicillin-sulbactam to 98.1% for amikacin. With the agar dilution reference method, the percentage of resistant strains tested against an antimicrobial agent ranged from a low of 2.4% for imipenem to a high of 79% for ampicillin. Minor errors were lowest for amikacin (1.6%) and highest for ampicillin-sulbactam (16.4%). Major errors ranged from 0.3% for five agents to 9.0% for cephalothin. Eight antimicrobial agents had no very major errors; however, ampicillin-sulbactam had an 8.8% rate of very major errors.

Table 4 shows the category agreement for ampicillin, ampicillin-sulbactam, gentamicin, and imipenem after the tests were repeated. There was significant improvement in the interpretative agreement for ampicillin and ampicillin-sulbactam after the manufacturer made some alterations in the production method; very major errors for ampicillin-sulbactam decreased to 0.9%. Very major errors for gentamicin increased after the manufacturing changes when Alamar results were compared with agar results, but minor and very major errors decreased for gentamicin when Alamar results were compared with broth microdilution results. In contrast, the rate of very major errors for Alamar with imipenem increased on repeat testing compared with both broth and agar. This may be due, in part, to the change in the number of strains categorized as resistant by the reference methods (10 by agar dilution and 14 by broth microdilution), which indicates that even the reference methods had difficulty in accurately predicting imipenem resistance in this group of organisms.

DISCUSSION

Recently, Jorgensen (9) reviewed the issue of selection criteria for antimicrobial susceptibility testing systems and included a discussion of the method for calculating error rates for susceptibility tests described by Murray et al. (16). The latter method, which uses only the resistant subpopulation of strains to determine rates of very major errors and only the susceptible subpopulation of strains to calculate rates of major

TABLE 2. Distribution of differences in MICs of four antimicrobial agents for 186 gram-negative bacteria on repeat testing

Comparison and antimicrobial agent(s)	No. (%) of isolates with MIC[a] difference of:							% Agreement[b] ± SE	P[c] value
	< −2	−2	−1	0	+1	+2	> +2		
Alamar vs agar									
Ampicillin	4 (1.1)	2 (0.5)	32 (8.6)	302 (81.2)	26 (7.0)	4 (1.1)	2 (0.5)	96.8 ± 0.9	0.496
Ampicillin-sulbactam	0 (0.0)	11 (3.0)	54 (14.5)	256 (68.8)	46 (12.4)	5 (1.3)	0 (0.0)	95.7 ± 1.1	0.067
Gentamicin	36 (9.7)	126 (33.9)	126 (33.9)	82 (22.0)	2 (0.5)	0 (0.0)	0 (0.0)	56.5 ± 2.6	<0.001
Imipenem	10 (2.7)	36 (9.7)	88 (23.7)	141 (37.9)	75 (20.1)	13 (3.5)	9 (2.4)	81.7 ± 2.0	0.002
Alamar vs broth									
Ampicillin	1 (0.3)	7 (1.9)	26 (7.0)	291 (78.2)	39 (10.5)	8 (2.1)	0 (0.0)	95.7 ± 1.1	0.498
Ampicillin-sulbactam	2 (0.5)	4 (1.1)	50 (13.5)	257 (69.1)	48 (12.9)	9 (2.4)	2 (0.5)	95.4 ± 1.1	0.114
Gentamicin	1 (0.3)	30 (8.1)	128 (34.4)	162 (43.5)	47 (12.6)	3 (0.8)	1 (0.3)	90.6 ± 1.5	<0.001
Imipenem	22 (5.9)	38 (10.2)	37 (9.9)	106 (28.5)	124 (33.4)	36 (9.7)	9 (2.4)	71.8 ± 2.3	0.057
Broth vs agar									
Ampicillin	3 (0.8)	5 (1.3)	40 (10.8)	294 (79.0)	24 (6.5)	3 (0.8)	3 (0.8)	96.2 ± 1.0	0.298
Ampicillin-sulbactam	2 (0.5)	7 (1.9)	57 (15.3)	267 (71.8)	32 (8.6)	4 (1.1)	3 (0.8)	95.7 ± 1.1	0.312
Gentamicin	6 (1.6)	85 (22.9)	166 (44.6)	108 (29.0)	6 (1.6)	1 (0.3)	0 (0.0)	75.3 ± 2.2	<0.001
Imipenem	5 (1.3)	28 (7.5)	118 (31.7)	154 (41.4)	49 (13.2)	18 (4.9)	0 (0.0)	86.3 ± 1.8	0.016

[a] Zero indicates the number and percentage of isolates for which MICs were identical; −1, +1, etc. indicate $\pm\log_2$ dilution differences.
[b] Percentage of isolates within the accuracy limits of the test (± 1 $\log_2$ dilution).
[c] *P* values were obtained with the Wilcoxon signed-rank test.

errors, differs from the more familiar method of Thornsberry and Gavan, which uses the total number of strains tested to calculate all error rates (24). The differences between rates of very major errors when the rates are calculated by the method of Thornsberry and Gavan and that of Murray et al. can be striking, e.g., <1.5% versus 20% for the same set of strains (9). In our study, 14 antimicrobial agents showed very major error rates of >1.5% (a cutoff value suggested by Sherris and Ryan [20]) with the method of Murray et al. while only 6 would have similar error rates with the method of Thornsberry and Gavan. Similarly, 10 antimicrobial agents showed major error rates of >3.0% with the method of Murray et al., but none would have error rates of that magnitude with the method of Thornsberry and Gavan. Since the method of Murray et al. considers only strains resistant by the reference method for very major error rates and only strains susceptible by the reference method for major error rates, it is a more accurate way to compare new susceptibility testing methods to traditional reference methods. Manufacturers of antimicrobial susceptibility testing systems must use the method of Murray et al. to calculate error rates when submitting products to the Food and Drug Administration for clearance.

We evaluated the new Alamar susceptibility testing method by using the method of Murray et al. to calculate major and very major errors and a nontraditional way to calculate essential errors. These calculation methods were applied to a set of organisms for which the MICs of many antimicrobial agents are at or near the breakpoints for resistance. Since this serves as a stress test for the method, the Alamar system will likely perform better during routine clinical use. Stress tests are important, however, because they highlight deficiencies in new methods and reference methods, as was observed in this study with imipenem. The results of our study led us to conclude that the Alamar method is an acceptable means of performing antimicrobial susceptibility testing. After manufacturing changes, all antimicrobial agents had essential error rates (major plus very major errors divided by susceptible plus resistant strains) of <5% and essential agreement rates (absolute agreement plus minor errors) of >95%. At first glance, the results may not look as favorable as previous evaluations of other systems; however, there are three major differences between our study and previously reported studies: (i) we used the method of Murray et al. to calculate errors, (ii) the challenge organisms used in the study have resistance mechanisms that are more difficult to detect than those of most routine clinical isolates, and (iii) we used agar dilution instead of broth microdilution as the reference method. The results of our study confirm earlier reports that broth microdilution and agar dilution do not always produce the same MIC results with the same set of organisms (2). Interestingly, the overall agreement between Alamar, a broth-based system, and agar dilution in this study was 91.0%, while a previous comparison of an in-house broth microdilution method and agar dilution for nine antimicrobial agents reported an overall agreement of 90.8% (2). The major differences between the current Alamar evaluation and the previous comparison of agar and broth dilution tests were in the gentamicin results. Agreement was much better in the previous study (95.0%) than in the current evaluation (69.1%); however, the error rates for the Alamar results for gentamicin improved dramatically when broth microdilution was used as the reference method in repeat testing (90.6%). Other investigators have reported significant differences for gentamicin when agar methods were compared to broth dilution methods (4, 11, 22, 26), although many of the latter studies focused on the calcium-and-magnesium content of the medium and its effect on *P. aeruginosa*. Our data for gentamicin indicate consistently higher MICs with agar dilution than with the Alamar broth dilution method. The shift was more apparent for members of the family *Enterobacteriaceae* (2 $\log_2$ dilutions) than for *Pseudomonas* and *Acinetobacter* species (only 1 $\log_2$ dilution). Although the reason for the shift, especially with members of the family *Enterobacteriaceae*, is unclear, the cation content of Mueller-Hinton broth may have been a factor. It was less in our current study, which used cation-adjusted broth, than in many of the previously cited studies that used cation-supplemented broth. Also, the quality control results for gentamicin obtained with *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were all within the NCCLS guideline limits for both the Alamar and reference methods in both phases of the current study, but the Alamar

TABLE 3. Overall results by interpretative categories[a] for agar dilution method and errors and agreement between Alamar and agar dilution

Antimicrobial agent(s)	No. (%) of strains[b]			No. (%) of errors[c]				No. (%) with agreement	
	Susceptible	Intermediate	Resistant	Minor[d]	Major[e]	Very major[f]	Essential[g]	Absolute[h]	Essential[i]
Amikacin	340 (91.4)	6 (1.6)	26 (7.0)	6 (1.6)	1 (0.3)	0 (0.0)	1 (0.3)	365 (98.1)	371 (99.7)
Amoxicillin-clavulanic acid	100 (26.9)	22 (5.9)	250 (67.2)	29 (7.8)	8 (8.0)	1 (0.4)	9 (2.6)	334 (89.9)	363 (97.6)
Ampicillin	62 (16.7)	16 (4.3)	294 (79.0)	27 (7.3)	4 (6.5)	10 (3.4)	14 (3.9)	331 (89.0)	358 (96.2)
Ampicillin-sulbactam	110 (29.6)	47 (12.6)	215 (57.8)	61 (16.4)	1 (0.9)	19 (8.8)	20 (6.2)	291 (78.2)	352 (94.6)
Aztreonam	279 (75.0)	17 (4.6)	76 (20.4)	11 (3.0)	4 (1.4)	0 (0.0)	4 (1.1)	357 (96.0)	368 (98.9)
Carbenicillin	185 (49.7)	22 (5.9)	165 (44.4)	35 (9.4)	13 (7.0)	0 (0.0)	13 (3.7)	324 (87.1)	359 (96.5)
Cefamandole	158 (42.5)	14 (3.8)	200 (53.7)	29 (7.8)	10 (6.3)	6 (3.0)	16 (4.5)	327 (87.9)	356 (95.7)
Cefazolin	97 (26.1)	20 (5.4)	255 (68.5)	30 (8.1)	4 (4.1)	5 (2.0)	9 (2.6)	333 (89.5)	363 (97.6)
Cefmetazole	164 (44.0)	27 (7.3)	181 (48.7)	25 (6.7)	5 (3.0)	1 (0.6)	6 (1.7)	341 (91.7)	366 (98.4)
Cefonicid	126 (33.9)	29 (7.8)	217 (58.3)	24 (6.5)	8 (6.3)	4 (1.8)	12 (3.5)	336 (90.3)	360 (96.8)
Cefoperazone	266 (71.5)	29 (7.8)	77 (20.7)	22 (5.9)	3 (1.1)	3 (3.9)	6 (1.7)	344 (92.5)	366 (98.4)
Cefotaxime	257 (69.1)	48 (12.9)	67 (18.0)	29 (7.8)	3 (1.2)	1 (1.5)	4 (1.2)	339 (91.1)	368 (98.9)
Cefotetan	226 (60.8)	12 (3.2)	134 (36.0)	20 (5.4)	2 (0.9)	2 (1.5)	4 (1.1)	348 (93.5)	368 (98.9)
Cefoxitin	138 (37.1)	20 (5.4)	214 (57.5)	29 (7.8)	6 (4.3)	1 (0.5)	7 (2.0)	336 (90.3)	365 (98.1)
Ceftazidime	301 (80.9)	5 (1.3)	66 (17.8)	12 (3.2)	8 (2.7)	2 (3.0)	10 (2.7)	350 (94.1)	362 (97.3)
Ceftizoxime	253 (68.0)	50 (13.4)	69 (18.6)	20 (5.4)	3 (1.2)	1 (1.4)	4 (1.2)	348 (93.5)	368 (98.9)
Ceftriaxone	258 (69.4)	45 (12.1)	69 (18.5)	36 (9.7)	4 (1.6)	2 (2.9)	6 (1.8)	330 (88.7)	366 (98.4)
Cefuroxime	155 (41.7)	17 (4.6)	200 (53.7)	29 (7.8)	7 (4.5)	2 (1.0)	9 (2.5)	334 (89.8)	363 (97.6)
Cephalothin	67 (18.0)	28 (7.5)	277 (74.5)	34 (9.1)	6 (9.0)	3 (1.1)	9 (2.6)	329 (88.4)	363 (97.6)
Chloramphenicol	180 (48.4)	29 (7.8)	163 (43.8)	42 (11.3)	5 (2.8)	1 (0.6)	6 (1.7)	324 (87.1)	366 (98.4)
Ciprofloxacin	319 (85.8)	19 (5.1)	34 (9.1)	12 (3.2)	1 (0.3)	0 (0.0)	1 (0.3)	359 (96.5)	371 (99.7)
Gentamicin	286 (76.9)	13 (3.5)	73 (19.6)	24 (6.5)	1 (0.3)	0 (0.0)	1 (0.3)	347 (93.3)	371 (99.7)
Imipenem	359 (96.5)	4 (1.1)	9 (2.4)	12 (3.2)	2 (0.6)	0 (0.0)	2 (0.5)	358 (96.2)	370 (99.5)
Mezlocillin	221 (59.4)	31 (8.3)	120 (32.3)	35 (9.4)	1 (0.5)	7 (5.8)	8 (2.3)	329 (88.4)	364 (97.8)
Nitrofurantoin	85 (22.9)	63 (16.9)	224 (60.2)	52 (14.0)	1 (1.2)	0 (0.0)	1 (0.3)	319 (85.8)	371 (99.7)
Norfloxacin	320 (86.0)	12 (3.2)	40 (10.8)	8 (2.2)	1 (0.3)	0 (0.0)	1 (0.3)	363 (97.6)	371 (99.7)
Ofloxacin	296 (79.6)	20 (5.4)	56 (15.0)	28 (7.5)	0 (0.0)	1 (1.8)	1 (0.3)	343 (92.2)	371 (99.7)
Piperacillin	240 (64.5)	31 (8.3)	101 (27.2)	23 (6.2)	3 (1.3)	8 (7.9)	11 (3.2)	338 (90.9)	361 (97.0)
Tetracycline	160 (43.0)	20 (5.4)	192 (51.6)	31 (8.3)	6 (3.8)	1 (0.5)	7 (2.0)	334 (89.8)	365 (98.1)
Ticarcillin	199 (53.5)	19 (5.1)	154 (41.4)	35 (9.4)	4 (2.0)	5 (3.2)	9 (2.5)	328 (88.2)	363 (97.6)
Ticarcillin-clavulanic acid	204 (54.9)	57 (15.3)	111 (29.8)	45 (12.1)	2 (1.0)	5 (4.5)	7 (2.2)	320 (86.0)	365 (98.1)
Tobramycin	293 (78.8)	14 (3.7)	65 (17.5)	19 (5.1)	1 (0.3)	1 (1.5)	2 (0.6)	351 (94.4)	370 (99.5)
Trimethoprim-sulfamethoxazole	266 (71.7)		105 (28.3)	0 (0.0)	2 (0.8)	16 (15.1)	18 (4.9)	353 (95.1)	353 (95.1)
Overall	6,970 (56.8)	806 (6.6)	4,499 (36.6)	874 (7.1)	130 (1.9)	108 (2.4)	238 (2.1)	11,163 (90.9)	12,037 (98.1)

[a] Categories of susceptibility as defined by NCCLS standards M7-A2 and M100-S4.
[b] Number and percentage of strains interpreted as susceptible, intermediate, or resistant by the reference method.
[c] Error classes as defined by Thornsberry and Gavan (21).
[d] Minor interpretative differences between methods. Percentage calculated with susceptible plus intermediate plus resistant strains as the denominator.
[e] Strains interpreted as falsely resistant by the test method. Percentage calculated with susceptible strains as the denominator.
[f] Strains interpreted as falsely susceptible by the test method. Percentage calculated with resistant strains as the denominator.
[g] Strains with major and very major errors. Percentage calculated with susceptible plus resistant strains as the denominator.
[h] Strains with complete interpretative agreement. Percentage calculated with susceptible plus intermediate plus resistant strains as the denominator.
[i] Strains with absolute agreement plus strains with minor errors. Percentage calculated with susceptible plus intermediate plus resistant strains as the denominator.

and broth microdilution results were in the lower range of acceptable limits and the agar dilution results were in the upper range of acceptable limits. This difference of approximately 2 dilutions may also explain the poor agreement between agar and broth results observed in this study.

The quinolones, chloramphenicol, and nitrofurantoin had very good MIC agreement between methods and did not appear to be affected by methodology. Beta-lactam antimicrobial agents, on the other hand, had a dichotomous shift in dilution differences, depending upon whether or not the antimicrobial agent was susceptible to penicillinase or cephalosporinase activity. The Alamar method tended to produce lower MICs than did agar dilution if the organism was very susceptible to the β-lactam agent but higher MICs if the organism produced an inducible or extended-spectrum β-lactamase. Other investigators have reported this dichotomous effect when agar dilution and broth dilution were compared (5, 7, 10, 13, 19). Our additional testing of ampicillin, ampicillin-sulbactam, gentamicin, and imipenem suggests that we might have obtained better agreement of results between the Alamar system and the reference method if we had chosen broth microdilution as the reference method for all of the agents except imipenem. Very major and minor errors increased with imipenem, in part because of the change in the manufacturing process for imipenem and the comparison with broth microdilution (the Alamar-determined MICs averaged 1 dilution lower in the repeat study than in the original study). However, all of the errors occurred with strains of *P. aeruginosa* for which the MICs were near the breakpoints. As previously mentioned, the MICs of imipenem and other β-lactams are usually higher by broth microdilution than by agar dilution. These two factors, and the small number of imipenem-resistant strains available, made it difficult to assess the accuracy of imipenem testing with the Alamar method in this study.

TABLE 4. Overall results by interpretative categories[a] for reference methods and errors and agreement between reference and test methods on repeat testing

Comparison and antimicrobial agent(s)	No. (%) of strains[b]			No. (%) of errors[c]				No. (%) with agreement	
	Susceptible	Intermediate	Resistant	Minor[d]	Major[e]	Very major[f]	Essential[g]	Absolute[h]	Essential[i]
Alamar vs agar									
Ampicillin	66 (17.7)	12 (3.2)	294 (79.0)	16 (4.3)	3 (4.5)	3 (1.0)	6 (1.7)	350 (94.1)	366 (98.4)
Ampicillin-sulbactam	120 (32.3)	41 (11.0)	211 (56.7)	60 (16.1)	2 (1.7)	2 (0.9)	4 (1.2)	308 (82.8)	368 (98.9)
Gentamicin	284 (76.3)	18 (4.8)	70 (18.8)	25 (6.7)	0 (0.0)	9 (12.8)	9 (2.5)	338 (90.9)	363 (97.6)
Imipenem	350 (94.1)	12 (3.2)	10 (2.7)	15 (4.0)	0 (0.0)	2 (20.0)	2 (0.6)	355 (95.4)	370 (99.5)
Alamar vs broth									
Ampicillin	62 (16.7)	22 (5.9)	288 (77.4)	20 (5.4)	1 (1.6)	0 (0.0)	1 (0.3)	351 (94.4)	371 (99.7)
Ampicillin-sulbactam	113 (30.4)	48 (12.9)	211 (56.7)	51 (13.7)	4 (3.5)	2 (0.9)	6 (1.9)	315 (84.7)	366 (98.4)
Gentamicin	301 (80.9)	14 (3.8)	57 (15.3)	15 (4.0)	0 (0.0)	1 (1.8)	1 (0.3)	356 (95.7)	371 (99.7)
Imipenem	347 (93.3)	11 (3.0)	14 (3.8)	14 (3.8)	0 (0.0)	6 (42.9)	6 (1.7)	352 (94.6)	366 (98.4)
Broth vs agar									
Ampicillin	66 (17.7)	12 (3.2)	294 (79.0)	18 (4.8)	4 (6.1)	3 (1.0)	7 (1.9)	347 (93.3)	365 (98.1)
Ampicillin-sulbactam	120 (32.3)	41 (11.0)	211 (56.7)	33 (8.9)	4 (3.3)	4 (1.9)	8 (2.4)	331 (89.0)	364 (97.8)
Gentamicin	284 (76.3)	18 (4.8)	70 (18.8)	32 (8.6)	0 (0.0)	0 (0.0)	0 (0.0)	340 (91.4)	372 (100.0)
Imipenem	350 (94.1)	12 (3.2)	10 (2.7)	15 (4.0)	1 (0.2)	0 (0.0)	1 (0.3)	355 (95.4)	370 (99.5)

[a] Categories of susceptibility as defined by NCCLS standards M7-A2 and M100-S4.
[b] Number and percentage of strains interpreted as susceptible, intermediate, or resistant by the reference method.
[c] Error classes as defined by Thornsberry and Gavan (21).
[d] Minor interpretative differences between methods. Percentage calculated with susceptible plus intermediate plus resistant strains as the denominator.
[e] Strains interpreted as falsely resistant by the test method. Percentage calculated with susceptible strains as the denominator.
[f] Strains interpreted as falsely susceptible by the test method. Percentage calculated with resistant strains as the denominator.
[g] Strains with major and very major errors. Percentage calculated with susceptible plus resistant strains as the denominator.
[h] Strains with complete interpretative agreement. Percentage calculated with susceptible plus intermediate plus resistant strains as the denominator.
[i] Strains with absolute agreement plus strains with minor errors. Percentage calculated with susceptible plus intermediate plus resistant strains as the denominator.

The change in the method of calculation makes many of the results of our study fall out of the acceptable limits described by Thornsberry and Gavan (24); i.e., very major plus major errors (essential errors) should not exceed 5%, and complete agreement for interpretive criteria should be ≥90%. Ampicillin-sulbactam, chloramphenicol, nitrofurantoin, and ticarcillin-clavulanic acid, in particular, showed >10% minor errors, in large part because of the number of organisms tested for which the MICs of these antimicrobial agents were at or near the interpretative breakpoints. Major interpretative errors occurred at >3% with nine β-lactam antimicrobial agents, all of which are susceptible to the action of β-lactamase. Tetracycline's major error rate of 3.8% also can be attributed to methodology (8). Very major interpretative errors occurred at >1.5% (20) with 12 β-lactam antimicrobial agents, ofloxacin, and trimethoprim-sulfamethoxazole. Trimethoprim-sulfamethoxazole, however, has no intermediate category, which causes a difference of 1 $\log_2$ dilution at the interpretative breakpoint to categorize all errors as major or very major. Essential category agreement (agreement plus minor errors) was very good (>95%) for all of the agents tested.

Antimicrobial susceptibility testing methods using fluorochromes have been developed previously and are currently being marketed as methods that decrease the initial reading time for susceptibility tests (3, 15). However, these test methods must be read with a fluorometer and cannot be interpreted visually. In contrast, the Alamar system uses a fluorochrome that is easily read with a fluorometer or visually. In this evaluation of the Alamar MIC system, all test results were read visually. The only difficulty in interpreting test results occurred with nitrofurantoin at the higher concentrations because of the intense yellow color of the antimicrobial agent. There also was slight discoloration of the indicator with imipenem and some strains of *Serratia*, *Providencia*, and *Proteus* spp. for several dilutions past the endpoint (trailing endpoints); however, there was no difficulty in reading imipenem endpoints for most susceptible strains. In the smaller repeat series, trailing endpoints were not as prominent for the previously mentioned strains with imipenem. Trailing endpoints were observed, however, with the reference methods, particularly with *P. mirabilis* in the repeat series of tests.

We also encountered some difficulties in reading the MICs of β-lactams for *P. mirabilis* because with both the agar and broth methods there was a very light haze of growth that was apparent only when the plates were examined carefully. For the original test results, this haze of growth was ignored, as suggested in the M7-A2 guidelines for agar dilution testing (17). In the repeat testing, however, the reference tests were interpreted with the haze of growth first as positive and then as negative (standard method) to determine the effect on the categorical interpretations. Reading the haze as positive in the reference tests shifted the MICs higher and, consequently, closer to the MIC results obtained with the Alamar system, which has an indicator system that more readily detects small amounts of growth. This shift accounts for the differences observed between the Alamar system and agar dilution for many of the β-lactam drugs. Higher sensitivity of fluorochrome indicators for detection of growth also has been reported by Staneck et al. (21).

In conclusion, the Alamar colorimetric MIC determination system demonstrated approximately the same degree of agreement as the broth microdilution reference method when compared to agar dilution by distribution of $\log_2$ differences, the Wilcoxon signed-rank test, and the rate of interpretative category agreement (2). Thus, with the possible exception of imipenem, which was difficult to interpret in this study, we found the results of the Alamar method to be comparable to the results of reference methods. However, our study highlights the fact that the reference method used to study susceptibility testing methods must be chosen carefully and should

resemble, as closely as possible, the susceptibility testing method under evaluation.

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