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Comparison of Five Methods, Including the PDM Epsilometer Test (E Test), for Antimicrobial Susceptibility Testing of *Pseudomonas aeruginosa*

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The antimicrobial susceptibilities of 100 clinical isolates of *Pseudomonas aeruginosa* to six antipseudomonal antibiotics were tested by five methods: the National Committee for Clinical Laboratory Standards (NCCLS) methods for broth microdilution, agar dilution, and agar disk diffusion; the Vitek Automicrobic System method (Vitek Systems, Hazelwood, Mo.); and the PDM Epsilometer test (E test) (AB Biodisk, Solna, Sweden). The E test results showed excellent correlation with agar dilution results, with over 90% agreement within 1 doubling dilution between the E test and reference agar dilution MICs for all antimicrobial agents tested. The E test results also showed good correlation with the results from the reference agar disk diffusion method, with 90 to 99% complete agreement and 100% essential agreement on categories for all antibiotics tested (essential agreement is the agreement obtained when minor discrepancies are ignored). Comparison of categories with the E test and broth microdilution methods, using the broth microdilution method as the reference method, gave only 59% complete agreement for gentamicin, with 28 minor discrepancies and 13 very major discrepancies. Some discrepancies were observed between results from the E test and broth methods for gentamicin, with the broth microdilution and Vitek methods giving higher MICs than the E test and other methods using agar. The most recent NCCLS guidelines for broth dilution testing have reduced the recommended levels of cation supplementation, which may enhance future agreement between results for the aminoglycosides and *P. aeruginosa* on broth and on agar. We found that the E test offers a simple, labor-efficient, and accurate method for MIC determination on an agar medium.

It is widely known that antimicrobial susceptibility testing of *Pseudomonas aeruginosa* to aminoglycosides and to a lesser extent to antipseudomonal penicillins is highly dependent on the method used (6, 10, 16, 18–20). Results can be influenced by inoculum size (5, 7) and medium composition, in particular the concentrations of unbound calcium and magnesium cations (1, 8, 9, 15, 17, 21). The situation is further complicated by the fact that the aminoglycoside MICs for a significant proportion of *P. aeruginosa* isolates are very close to the susceptible breakpoint concentrations (2). As a consequence of these and other factors, results from methods using agar do not always correlate well with results from methods using broth.

In this study, the susceptibilities of 100 isolates of *P. aeruginosa* to six antipseudomonal antibiotics were determined by the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution, agar dilution, and agar disk diffusion methods, the Vitek Automicrobic System (AMS) method (Vitek Systems, Hazelwood, Mo.), and the PDM Epsilometer test (E test) (AB Biodisk, Solna, Sweden). The E test is a recently developed commercial system which uses a predefined antibiotic gradient immobilized on a test strip to provide discrete MICs by agar diffusion testing (3).

MATERIALS AND METHODS

Organisms. A total of 100 clinical isolates of *P. aeruginosa* were collected from patients at St. Vincent's Hospital, Royal Children's Hospital, Alfred Hospital, and Prince Henry's Hospital in Melbourne, Australia. These included 21 mucoid isolates from adults and children with cystic fibrosis, in addition to other strains known to have high-level resistance to the test antimicrobial agents. Repeat isolates from patients were excluded. Isolates were identified as *P. aeruginosa* by positive oxidase reaction, pyocyanin production, and the Vitek Gram Negative Identification Card (Vitek Systems). All isolates were stored at −70°C in Protect cryogenic storage vials (Technical Service Consultants Ltd., Bury, Lancashire, United Kingdom) and subcultured twice onto Columbia agar (Oxoid Australia Ltd., Melbourne, Victoria, Australia) supplemented with 5% defibrinated horse blood before testing.

Antimicrobial agents. Antibiotic powders of known potency were obtained for testing as follows: gentamicin sulfate, David Bull Laboratories, Melbourne, Victoria, Australia; piperacillin monohydrate, Lederle Laboratories, Sydney, New South Wales, Australia; ticarcillin disodium, SmithKline Beecham, Melbourne, Victoria, Australia; amikacin, Bristol Myers Pty. Ltd., Sydney, New South Wales, Australia; tobramycin, Eli Lilly Australia Pty. Ltd., Sydney, New South Wales, Australia; and ceftazidime pentahydrate, Glaxo Australia Pty. Ltd., Melbourne, Victoria, Australia. For the NCCLS agar disk diffusion method, antimicrobial disks (BBL Microbiology Systems, Cockeysville, Md.) were used. E test strips (AB Biodisk) were obtained for gentamicin, amikacin, piperacillin, and ceftazidime. At the time of testing, E test strips were not available for ticarcillin and tobramycin.

Antimicrobial susceptibility testing methods. Five methods were used to determine the antimicrobial susceptibilities of the 100 *P. aeruginosa* isolates. Broth microdilution, agar dilution, and agar disk diffusion methods were all performed by using the procedures outlined by the NCCLS (11, 13) and Gold Label Mueller-Hinton II broth and Mueller-Hinton II

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TABLE 1. Susceptibility testing of 100 isolates of *P. aeruginosa*

Method	% of isolates per interpretative category[a] for antibiotic:																	
	Gentamicin			Amikacin			Tobramycin			Ticarcillin			Piperacillin			Ceftazidime		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Broth microdilution[b]	43	23	34	73	14	13	90	5	5	77	17	6	88	2	10	90	1	9
Vitek AMS	62	5	33	76	2	22	94	1	5	90	8	2	91	5	4	91	3	6
Agar dilution[b]	71	9	20	82	10	8	94	2	4	84	9	7	90	1	9	89	2	9
PDM Epsilometer	76	9	15	84	8	8	NT[c]	NT	NT	NT	NT	NT	89	0	11	92	1	7
Agar disk diffusion[b]	79	4	17	81	7	12	95	1	4	83	7	10	89	1	10	92	2	6

[a] S, susceptible; I, intermediate; R, resistant.
[b] NCCLS methodology.
[c] NT, not tested.

agar (BBL). Broth microdilution trays were prepared in-house and stored at −70°C until use. Doubling dilutions (0.12 to 256 μg/ml) of antibiotic stock solutions used to prepare the microdilution trays were also used to prepare the agar dilution plates.

The Vitek AMS (Vitek Systems) was used with software version AMS P3.19. The pseudomonas susceptibility cards (GNS-PA) were loaded and filled in accordance with manufacturer's instructions.

The PDM Epsilometer test (E test) (AB Biodisk) was performed by using the same batch of Mueller-Hinton agar that was used for the agar dilution and agar disk diffusion methods. A saline suspension of each isolate was adjusted to a McFarland standard of 0.5 and inoculated over the surface of a single large agar plate (140-mm diameter), using a sterile swab to produce an even inoculum. After the four E test strips were applied to the plates, the plates were incubated for 18 h at 35°C and the MIC was the point where the elliptical zone of growth inhibition intersected the MIC scale on the E test strip. Discrete MICs were recorded; however, for the determination of categories, MICs were rounded up to the next highest doubling dilution (e.g., a gentamicin MIC of 6 μg/ml was rounded up to 8 μg/ml and interpreted as intermediate).

All susceptibility testing was performed from 18- to 24-h-old subcultures on defibrinated horse blood agar. A single inoculum adjusted to a McFarland standard of 0.5 was used for the disk diffusion and E-test methods and diluted appropriately for the NCCLS broth and agar dilution methods. A separate inoculum adjusted to a McFarland standard of 1 was prepared and diluted in 0.5 N saline for loading the Vitek cards.

The NCCLS breakpoints given in tentative standard M7-T2 (12) were used to determine categories for all the methods which provided MICs. The NCCLS agar disk diffusion zone diameters were interpreted by using NCCLS tentative standard M2-T4 (13).

Quality control. *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were included with each run of each method as control organisms, and all results fell within accepted ranges (12, 13). The total concentrations of calcium and magnesium in Mueller-Hinton broth were determined by atomic absorption spectroscopy and found to be within the reference range (11).

Statistical analysis. For statistical analysis of categories, each result was converted to a numerical value (1 for susceptible, 2 for intermediate, and 3 for resistant) and the significance of the differences between the results for two methods was determined by the Student *t* test (Minitab Data Analysis software was used). A *P* value of less than 0.05 was considered to represent a statistically significant difference between the results of the two methods compared. Minitab Data Analysis software was used to determine the MICs (geometric means). The MICs were converted to $\log_2+9$, averaged, and reconverted to MICs. MIC results of >256 μg/ml and ≤0.12 μg/ml were excluded from the analysis.

RESULTS

The percentages of isolates susceptible, intermediate, and resistant to each antimicrobial agent by each test method are shown in Table 1. For gentamicin and amikacin, the broth microdilution and Vitek AMS methods reported higher percentages of resistant and intermediate isolates than the three agar methods, which all gave similar results. The greatest differences in the results from the different methods were with gentamicin, where the broth microdilution and Vitek methods gave 33 to 34% resistance compared with only 15 to 20% resistance by the agar methods. There was little variation between the four test methods for tobramycin, with the broth microdilution and Vitek methods reporting 5% resistance and the agar dilution and agar disk diffusion methods reporting 4% resistance. For ticarcillin and piperacillin, the Vitek method reported lower percentages of resistance, with 2 and 4%, respectively, compared with 6 to 10% ticarcillin resistance and 9 to 11% piperacillin resistance by the other methods. All methods yielded similar results for ceftazidime.

The statistical differences between pairs of category results for each isolate were calculated. No significant differences between the results for the five methods were found for tobramycin, amikacin, piperacillin, and ceftazidime. However, highly significant differences ($P < 0.001$) between the data from the broth microdilution method and all other methods except the Vitek method were observed for gentamicin. Although the results of the Vitek method correlated well with broth microdilution results for gentamicin, there were highly significant differences ($P < 0.001$) when gentamicin results by the Vitek method were compared with those by the agar dilution, E test, and agar disk diffusion methods. For ticarcillin, significant differences ($P < 0.05$) were observed between the data from the Vitek and broth microdilution methods and also between the data from the Vitek and agar disk diffusion methods.

A comparison of broth microdilution, agar dilution, Vitek, and discrete E test gentamicin MICs, expressed as a frequency distribution of MICs, is shown in Fig. 1. The E test gave a modal MIC of 1.5 μg/ml, and the agar dilution method gave similar results, with a modal MIC of 2 μg/ml. Note that if the E test MICs were rounded up to the next highest doubling dilution (as they were for the category compari-

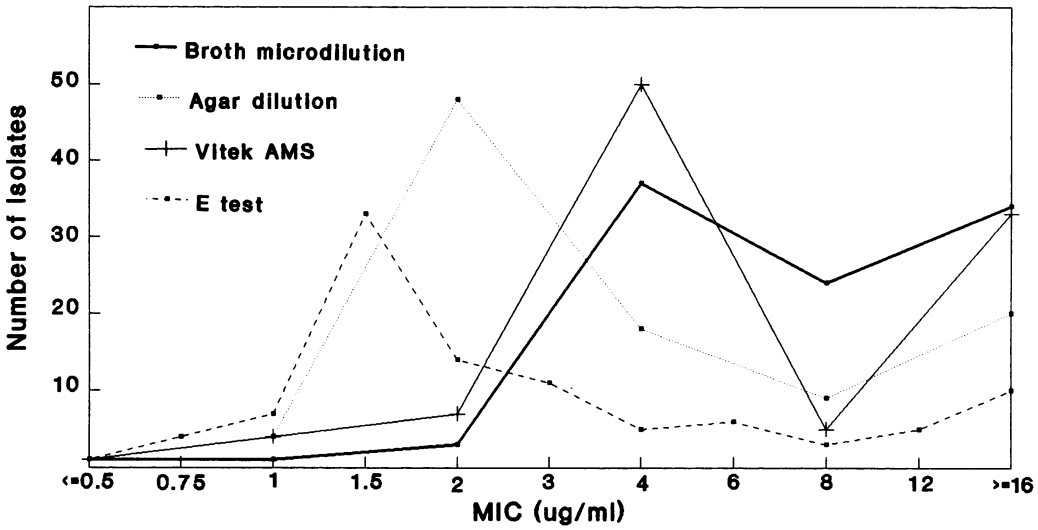

FIG. 1. Frequency of distribution of gentamicin MICs.

sons), the modal MIC would have been the same as the agar dilution MIC (2 μg/ml). The Vitek and broth microdilution methods both gave modal MICs of 4 μg/ml, 1 doubling dilution higher than for the agar methods. Twenty-five percent of isolates required an intermediate MIC of 8 μg/ml when tested by the broth microdilution method compared with less than 10% by each of the other methods.

The MICs (geometric means) by the broth microdilution, agar dilution, and E-test methods are shown in Table 2. For gentamicin, the MIC (geometric mean) by the broth microdilution method was 9.3 μg/ml compared with 4.5 μg/ml by the agar dilution method and 3.7 μg/ml by the E test. A similar trend was observed for the other antimicrobial agents tested, with the broth microdilution method giving higher MICs than the agar dilution method and the E test. The Vitek results are not included in the comparison, because Vitek MICs were not over the same range as the results from the other methods, which all produced MICs ranging from 0.12 to 256 μg/ml. The MICs (geometric means) were calculated by using only on-scale results. This was considered appropriate, because the great majority of MICs were on scale (of the 1,600 MICs analyzed, 1,545 were on scale). If the geometric means are calculated including the 55 off-scale MICs (assuming >256 to be 512 μg/ml and ≤0.12 to be 0.12 μg/ml), the same trends in geometric means are observed.

The values for percent agreement between the MICs by the E test and the MICs by the reference agar dilution method are shown in Table 3. Over 90% agreement within 1 doubling dilution was found for all antimicrobial agents tested, with excellent agreement for gentamicin (100%) and amikacin (97%). Between 95 and 100% agreement was obtained within 2 doubling dilutions of the reference method for the four antibiotics tested.

Table 4 shows a comparison of categories obtained by the E test with those by the NCCLS agar dilution method, using the agar dilution method as the reference method. The percent complete agreement between these two methods, that is, the percentage of isolates tested by the E test which gave the same category as those tested by the reference method, ranged from 88% for gentamicin to 98% for piperacillin. The percent essential agreement values, that is, the percentages of agreement obtained when minor discrepancies are ignored, were 98% for ceftazidime, 99% for piperacillin, and 100% for gentamicin and amikacin. Only two very major discrepancies involving ceftazidime and one major discrepancy for piperacillin were observed.

Table 5 shows a comparison of the categories obtained by the E test with those by the NCCLS agar disk diffusion method, using the disk diffusion method as the reference method. Complete agreement ranged from 90 to 99%, and essential agreement was 100% for all four antibiotics tested. No major or very major discrepancies were observed.

Table 6 shows a comparison of the categories obtained by the E test with those obtained by the NCCLS broth microdilution method, using the broth microdilution method as the

TABLE 2. MICs for 100 isolates of *P. aeruginosa*

Antibiotic	MIC (geometric mean) (μg/ml) by the following method:		
	Broth microdilution	Agar dilution	E test
Gentamicin	9.3	4.5	3.7
Amikacin	12.1	8.0	7.1
Tobramycin	1.8	1.0	NT[a]
Ticarcillin	24.8	18.9	NT
Piperacillin	13.5	8.9	8.9
Ceftazidime	3.9	2.5	3.1

[a] NT, not tested.

TABLE 3. Distribution of differences between the E-test and reference agar dilution MICs for 100 isolates of *P. aeruginosa*

Antibiotic	% of isolates with the following $\log_2$ dilution difference in MICs[a]:						
	≤−3	−2	−1	0	+1	+2	≥+3
Gentamicin	0	0	33	63	4	0	0
Amikacin	1	1	28	56	13	1	0
Piperacillin	4	2	6	67	19	1	1
Ceftazidime	3	2	3	46	42	3	1

[a] Zero indicates the percentage of isolates for which MICs are identical, −1 and +1 indicate ± 1 $\log_2$ dilution difference, etc.

TABLE 4. Comparison of categories for 100 isolates of *P. aeruginosa* by the E test and reference agar dilution method

Antibiotic	No. of discrepancies			% Agreement	
	Very major	Major	Minor	Complete	Essential
Gentamicin	0	0	12	88	100
Amikacin	0	0	8	92	100
Piperacillin	0	1	1	98	99
Ceftazidime	2	0	3	95	98

TABLE 6. Comparison of categories for 100 isolates of *P. aeruginosa* by the E test and reference broth microdilution method

Antibiotic	No. of discrepancies			% Agreement	
	Very major	Major	Minor	Complete	Essential
Gentamicin	13	0	28	59	87
Amikacin	1	0	13	86	99
Piperacillin	0	0	2	98	100
Ceftazidime	2	0	2	96	98

reference method. The percent complete agreement ranged from 59% for gentamicin to 98% for piperacillin, while essential agreement was 87 to 100%. Most of the discrepancies were minor, but there were 13 very major discrepancies for gentamicin.

DISCUSSION

When the susceptibility of *P. aeruginosa* to the aminoglycosides is tested, MICs can be increased by the presence of cations in the test medium. Mueller-Hinton agar may contribute various levels of unbound calcium and magnesium, whereas Mueller-Hinton broth is essentially free of these cations. To minimize discrepancies between test methods, Reller et al. (15) suggested that Mueller-Hinton broth be supplemented with calcium (50 mg/liter) and magnesium (25 mg/liter). These concentrations of cation supplements were recommended by the NCCLS for broth dilution testing (11) and were the levels present in the Gold Label Mueller-Hinton broth supplied for this study. Several studies using Mueller-Hinton broth with these cation concentrations have reported higher MICs of aminoglycosides in broth medium than in agar (1, 6, 16). Our results demonstrate the same trend, particularly with gentamicin and to a lesser extent with amikacin.

Barry et al. (1) have shown that for netilmicin tested against *P. aeruginosa*, broth microdilution and agar dilution results were most comparable when half the previously stated recommended cation concentrations were added to Mueller-Hinton broth. The more recent NCCLS guidelines for broth dilution testing (12, 14) have changed the recommended levels of cation supplementation to 25 mg/liter for calcium and 12.5 mg/liter for magnesium. This may enhance future agreement results for aminoglycosides and *P. aeruginosa* on broth versus agar.

In our study, the beta-lactam antibiotic MICs (geometric means) obtained by the broth microdilution method were greater than those obtained by the agar dilution and E-test methods. Unlike the aminoglycosides, however, there have been no reports that cation levels influence the MICs of these antibiotics.

TABLE 5. Comparison of categories for 100 isolates of *P. aeruginosa* by the E test and reference agar disk diffusion method

Antibiotic	No. of discrepancies			% Agreement	
	Very major	Major	Minor	Complete	Essential
Gentamicin	0	0	9	91	100
Amikacin	0	0	10	90	100
Piperacillin	0	0	1	99	100
Ceftazidime	0	0	1	99	100

Our study showed excellent agreement between the three agar methods. This may be due in part to the common batch of Mueller-Hinton agar, which was used for all methods and in part to the common inoculum. Staneck et al. (16) reported poor correlation between NCCLS agar dilution and disk diffusion results for gentamicin and amikacin; however, different brands of Mueller-Hinton agar were used for the two methods in their study. Discrepancies between methods using agar for testing pseudomonas against the aminoglycosides may in part be due to difficulties in adequately controlling the total cation levels in agar.

The MICs obtained with the E test have been shown by regression analysis to be directly proportional to the MICs obtained with a reference agar dilution method (4). Bolmström and Karlsson (4) tested 36 antibiotics with 200 aerobic bacteria and showed that ≥90% of E test MICs were within 1 doubling dilution of the reference agar dilution MIC. Our E test MICs also showed good correlation with the reference agar dilution MICs, giving between 91 and 100% agreement within 1 doubling dilution for the four antimicrobial agents tested.

The Vitek AMS and the broth microdilution method gave similar results for most antimicrobial agents tested. This is to be expected, as the Vitek method is a broth method which has been calibrated to correlate with the NCCLS broth microdilution method. The Vitek results for ticarcillin and piperacillin, however, showed some discrepancies compared with the broth microdilution method, with the Vitek method reporting less resistance to both antibiotics. A recent NCCLS update of the breakpoints for dilution methods (14) has removed the intermediate category for both ticarcillin and piperacillin, so that isolates requiring a MIC of 128 μg/ml would be reported as resistant, instead of intermediate. If we apply these new breakpoints to our results, the Vitek method would give 10% ticarcillin resistance and the broth microdilution method would give 23% resistance. The change in breakpoints has therefore not improved the correlation between the Vitek and broth microdilution results for ticarcillin in this study. For piperacillin, however, the new breakpoints result in 9% resistance by the Vitek method and 12% resistance by the broth microdilution method, which does give better agreement. Changes to the Vitek AMS software for the organism-drug combinations *P. aeruginosa*-ticarcillin and *P. aeruginosa*-piperacillin have been made in version 5.01, which may enhance future correlation.

Our results confirm that susceptibility testing of *P. aeruginosa* is dependent on the method used, such that a laboratory using the broth microdilution method or Vitek method can be expected to report significantly more gentamicin and amikacin resistance and intermediate results for *P. aeruginosa* than a laboratory employing an agar method and Mueller-Hinton agar. It is therefore important for laboratories to be aware of the inherent differences between testing

methods, in particular when comparing data between laboratories or when changing methodology. The question of whether MICs from broth or agar methods have more relevance to clinical outcome is yet to be answered.

The E test package insert states that E test MICs may not correlate well with MICs from broth microdilution or automated methods because of "method inherent characteristics." Our results support this observation, with some discrepancies noted between the E test and the broth microdilution and Vitek methods. The E test, however, showed excellent correlation with NCCLS agar dilution and agar disk diffusion methods and is a simple, labor-efficient, and accurate method of MIC determination on an agar medium.

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