ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1983, p. 42–45
0066-4804/83/010042-04$02.00/0

Vol. 23, No. 1

Comparison of Microtiter Broth Dilution and Agar Dilution Methods for Susceptibility Testing of *Eikenella corrodens*

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Eikenella corrodens is a slow-growing, capnophilic, gram-negative rod which often grows poorly in liquid media. Consequently, the agar dilution technique is the method of choice for susceptibility testing of *E. corrodens*. We report a new microtiter broth dilution method for susceptibility testing of *E. corrodens* which compared favorably with results obtained by the agar dilution technique. Minimal bactericidal concentrations correlated well with minimal inhibitory concentrations.

There is an increasing trend toward using the microtiter broth dilution technique for determining antimicrobial susceptibility (6, 9, 14). Various microtiter systems have been standardized for many commonly encountered organisms but not for less frequently encountered isolates or those with special growth requirements (1, 4, 6, 10, 11).

Eikenella corrodens is a slow-growing, fastidious gram-negative rod that is increasingly recognized as a human pathogen (2, 3, 15). It requires blood or hemin for growth in an aerobic atmosphere; growth is enhanced by the addition of 5% CO_2 to the atmosphere of incubation (3). Because *E. corrodens* grows poorly in liquid media as utilized in the macrodilution method and the disk diffusion method is inapplicable, the agar dilution technique is the method of choice for susceptibility testing of *E. corrodens* (2, 3, 5, 14, 15). The addition of whole or laked blood or a similar supplement to broth media causes a sediment to form in the bottom of the wells of microtiter plates, making the reading of minimal inhibitory concentrations (MICs) difficult. Recently, the microtiter technique has been adapted for susceptibility testing of *Streptococcus pneumoniae*, but it depends on the differentiation of various precipitated buttons of whole blood (13).

To apply the microtiter technique to *E. corrodens*, we developed a microtiter broth dilution method using a filtrate of laked sheep blood and compared it to the standard agar dilution technique. In addition, since sparse data are available on minimal bactericidal concentrations (MBCs) of *E. corrodens*, we then determined MBCs against *E. corrodens* of seven beta-lactam agents.

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MATERIALS AND METHODS

A total of 20 strains of *E. corrodens* were studied. Isolates were identified by standard criteria (2, 3) and had typical colonial morphology, were oxidase positive, and reduced nitrate to nitrite. The sources of isolates were: wounds, 6; abscesses, 3; clenched-fist injuries, 3; transtracheal aspirates, 2; sputum, 2; bile, 1; urine, 1; sinus, 1; and gingiva, 1. Organisms were stored at −70°C for various periods of time and removed and transferred twice to assure purity. Final subcultures were on Trypticase soy agar supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and were incubated in a 5% CO_2 enviroment for 48 h at 37°C. Growth was transferred to tubes containing Mueller-Hinton broth plus 5% Fildes enrichment, a peptic digest of sheep blood (BBL), and incubated for 24 to 48 h in a 5% CO_2 environment. Turbidity of growth was adjusted to one-half McFarland standard no. 1, corresponding to approximately 10^8 colony-forming units per ml.

The agar dilution technique was performed by a previously published method (7, 8). Mueller-Hinton agar plates supplemented with 5% sheep blood and the various antimicrobial concentrations, to yield 128 to 0.06 μg/ml, were inoculated with a Steers replicator. Antibiotic solutions were freshly prepared for each test, with sterilized distilled water as a solvent. Control plates containing no antibiotics were inoculated before and after each series of antibiotic-containing plates. All plates were incubated in a 5% CO_2 environment at 37°C for 48 h and then examined.

The microtiter broth dilution technique was performed by using the Dynatech MIC 2000 inoculator (Dynatech Laboratories, Alexandria, Va.). Isolates were prepared as with the agar dilution technique, except that the Mueller-Hinton broth supplemented with 5% lysed sheep blood (MHLSB) had been allowed to stand overnight until sedimented, and the supernatant was then passed through a 0.45-μm Nalgene membrane filter assembly (Sydrom Corp., Roch-

ester, N.Y.) before use. The filter was changed every 15 min to prevent clogging. A volume of 50 μl of serial twofold dilutions of the various antimicrobial agents was then added to wells already containing 50 μl of MHLSB broth. Then 1.5 μl of broth containing 10^7 colony-forming units per ml was added to each well. This yielded a final inoculum of approximately 10^6 colony-forming units per ml. Positive and negative controls were present on each microtiter plate.

Plates were incubated at 37°C in a 5% CO_2 atmosphere for 72 h and examined at 48 and 72 h in a reflective mirror. The MIC was defined as the lowest concentration of antibiotic that resulted in no bacterial growth, as indicated by the absence of diffuse clouding of the media as observed from below at 48 to 72 h. Only several of the strains were so slow growing as to require 72 h of incubation to achieve turbidity. (Prolonged incubation can be a problem in a microtiter system, since the plates will dry out unless special care is taken to seal them and to humidify the incubator.)

MBCs were determined with the Dynatech MIC 2000 inoculator by transferring 1.5 μl of inoculum from each well after 72 h of incubation onto a 150-mm Trypticase soy agar plate supplemented with 5% sheep blood and incubated at 37°C in a 5% CO_2 atmosphere. The MBC was defined as the lowest concentration of drug yielding no growth after subculture and 48 h of incubation. Since prolonged incubation tends to dry out microtiter plates, our plates were nested one on top of another with a blank plate covering them and with humidification of the incubator air.

Laboratory standard powders were kindly supplied by the following: cefoxitin and *N*-formimidoyl thienamycin, Merck Sharp & Dohme, West Point, Pa.; cefotaxime, Hoechst-Roussel, Somerville, N.J., cefoperazone, Pfizer Inc., New York, N.Y.; ampicillin, Beecham Laboratories, Bristol, Tenn.; cefazolin and moxalactam, Eli Lilly & Co., Indianapolis, Ind.

RESULTS

The ATCC control organisms (*Streptococcus faecalis* 33186, *Escherichia coli* 25922, and *Staphylococcus aureus* 25923) were run in parallel with the *E. corrodens* strain to assure standardization. The microtiter assay was run in triplicate to determine reproducibility.

The activity of the antimicrobial agents tested by both agar dilution and microtiter broth dilution methods against 20 strains of *E. corrodens* is shown in Table 1. All strains were uniformly susceptible to ampicillin, cefoxitin, cefotaxime, moxalactam, and *N*-formimidoyl thienamycin. Cefazolin and cefoperazone were the least active compounds, with more than 50% of strains requiring greater than 1 μg/ml for inhibition. Agar dilution and microtiter broth dilution MIC results were generally similar for all agents tested against *E. corrodens* strains and ATCC control strains. The MICs obtained by the microtiter broth technique were usually one to two dilutions lower than those obtained by the agar dilution method. The only antimicrobial agent tested for which a few strains of *E. corrodens* had agar dilution and microtiter broth dilution MICs that were three dilutions apart was cefoperazone.

Filtration of the MHLSB broth markedly decreased the amount of sediment. We had initially attempted to use whole sheep blood, laked blood, or Fildes supplementation of the Mueller-Hinton broth but found interpretation of growth and hence determination of MICs difficult. We then attempted to let the Mueller-Hinton broth with blood sediment overnight and filter the supernatant. This solution was difficult to pass through the filter but yielded good growth of *E. corrodens*. The MHLSB broth was slowly passed through the filter, with the filter being changed frequently. MICs could best be determined after 72 h of incubation, although many strains could be read at 48 h.

MBC results were at most one dilution higher than the MICs obtained by the microtiter broth dilution method, and in 60% of instances they were identical. When compared to MICs determined by the agar dilution method, the broth MBCs were usually one or two dilutions lower, except for those of cefoperazone. None of the strains tested showed tolerance (MBC/MIC > 32).

DISCUSSION

In the United States, the disk diffusion technique is the most commonly used method for determining antimicrobial susceptibility (9). This method is not applicable to slow-growing or capnophilic bacteria such as *E. corrodens* (5, 9). The agar dilution technique is the method of choice for susceptibility testing of *E. corrodens*, but it is impractical for laboratories testing only a few strains, and MBCs cannot be determined. The microtiter broth dilution technique has become increasingly popular with laboratories performing both large and small numbers of tests, especially those desiring quantitative results (MICs) or MBC determinations (9). Although this method is relatively inexpensive and reliable, it has not been adapted for slow-growing or fastidious organisms or those requiring supplementation of the broth media.

Recently, Tarpay et al. (13) described a microtiter broth method using the Dynatech MIC 2000 for susceptibility testing of *S. pneumoniae*. They used Mueller-Hinton broth supplemented with 5% defibrinated whole sheep blood and defined the MIC as "the lowest concentration of antibiotic that resulted in no bacterial growth as indicated by the absence of hemolysis of erythrocytes." Furthermore, Tarpay et al. (13) noted "cell buttons without growth were bright red, whereas those in wells containing subinhibitory antibiotic concentrations showed a greenish-black discoloration." *E. corrodens* does not lyse erythrocytes or cause discoloration of the but-

TABLE 1. Comparative susceptibility of 20 strains of *E. corrodens* to antimicrobial agents by agar dilution and microtiter broth dilution methods

Antimicrobial agent	Method[a]	MIC[b] (μg/ml)			MBC_{90}[c] (μg/ml)
		Range	50%	90%	
Ampicillin	AD	0.5–4	1	4	
	MD	0.125–1	0.5	1	1
Cefazolin	AD	4–32	8	16	
	MD	2–8	4	8	8
Cefoxitin	AD	0.25–1	0.5	1	
	MD	0.06–0.5	0.125	0.25	0.5
Cefotaxime	AD	0.06–0.5	0.06	0.5	
	MD	≤0.01–0.125	0.03	0.06	0.125
Cefoperazone	AD	0.5–4	2	4	
	MD	0.03–0.5	0.125	0.5	0.5
Moxalactam	AD	0.06–1	0.06	0.5	
	MD	0.06–0.25	0.125	0.125	0.25
N-Formimidoyl thienamycin	AD	0.125–0.25	0.125	0.25	
	MD	0.06–0.25	0.125	0.25	0.25

[a] AD, Agar dilution; MD, microtiter broth dilution.
[b] 50 and 90% MIC, MIC for 50 and 90% of strains, respectively.
[c] MBC_{90}, MBC for 90% of strains.

ton. Therefore, this type of differentiation cannot be made, and Tarpay's technique cannot be utilized for *E. corrodens*.

We were able to eliminate the precipitate by using lysed sheep erythrocyte-supplemented Mueller-Hinton broth and allowing overnight sedimentation, with filtration of the supernatant. This medium supported growth of *E. corrodens* strains moderately well. Our technique showed good correlations with MICs obtained by the standard agar dilution method. Half the time, the microtiter broth dilution and agar dilution MICs were within one dilution; in 80% of tests, results were within two dilutions. MICs obtained by microtiter broth dilution were always lower than those obtained by the agar dilution method. The greatest disparity existed for cefoperazone. For *N*-formimidoyl thienamycin, the results were nearly identical.

Sparse data exist on the MBCs of antibiotics against *E. corrodens*. The only English language report with such information was written by Robinson and James (12). They used brain heart infusion broth supplemented with meat granules and incubated for 4 days before subculture to determine MBCs. Although they recommended the inclusion of CO_2 in the atmosphere of incubation for *E. corrodens*, they did not determine MBCs in its presence. Tests done in an aerobic environment showed a close correlation of MICs and MBCs with ampicillin, penicillin G, nalidixic acid, lincomycin, clindamycin, gentamicin, and metronidazole but not with chloramphenicol, demethylchlortetracycline, or erythromycin. Although we did not test all of the same agents, our MBCs, obtained by the microtiter method, showed good correlation with MICs obtained by the microtiter method for all beta-lactam antimicrobial agents tested. MICs and MBCs were within one dilution in all but one instance. When MBCs by the microtiter method were compared to the MICs obtained by the agar dilution method, all MBCs were within one to two dilutions of the MICs, except those of cefoperazone. Tolerance, defined as an MBC/MIC ratio >32, was not observed in any of the strains tested.

The tests were run three times, with excellent correlation among the different runs. Because some of the strains were slow growing and required 72 h of incubation, care had to be taken to prevent the drying out of the microtiter wells. MBC determinations were easy to perform and the problem of drying out the microtiter tray and wells did not occur. The microtiter method might be recommended for future studies.

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