JOURNAL OF CLINICAL MICROBIOLOGY, May 1995, p. 1318–1321 Vol. 33, No. 5
0095-1137/95/$04.00+0

Comparison of E-test with Broth Microdilution and Disk Diffusion for Susceptibility Testing of Coryneform Bacteria

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Received 7 November 1994/Returned for modification 8 December 1994/Accepted 16 February 1995

The susceptibilities of 135 coryneform bacteria isolated from clinical samples to ampicillin (AMP), cephalothin (CR), cefoxitin (FOX), cefotaxime (CTX), erythromycin (E), ciprofloxacin (CIP), tetracycline (TE), amikacin (AK), vancomycin (VA), and rifampin (R) were determined by disk diffusion, broth microdilution, and the E-test. The following species (number of isolates in parentheses) were included: *Corynebacterium urealyticum* (30), *Corynebacterium minutissimum* (20), coryneform CDC group ANF-1 (20), *Corynebacterium striatum* (20), *Corynebacterium jeikeium* (15), coryneform CDC group I2 (8), *Listeria monocytogenes* (7), *Corynebacterium xerosis* (5), and other coryneform bacteria (10). Agreement within one twofold dilution between the E-test and broth microdilution was 31% (VA), 64% (AK), 71% (CTX), 77% (FOX and CIP), 79% (TE), 84% (AMP), 87% (E), and 88% (CR and R). For the 1,350 combinations of microorganisms and antimicrobial agents, 85 (6.3%) discrepancies in interpretive category were found (4.2% minor, 1.2% major, and 0.9% very major). Seventy (5.1%) disagreements in interpretive category were found between disk diffusion and the E-test (3.8% minor, 0.4% major, and 0.9% very major), and 85 (6.3%) disagreements were found between microdilution (reference method) and disk diffusion (4.2% minor, 0.5% major, and 1.5% very major). MICs obtained with the E-test were highly reproducible. No category discrepancy was observed for VA, despite quantitative results. Considering interpretive categories, there is a good overall agreement between the three methods studied here, but further evaluation of current methodologies for susceptibility testing is required when considering coryneform bacteria and determination of quantitative activity of antimicrobial agents.

Coryneform bacteria other than *Corynebacterium diphtheriae* have often been considered contaminant organisms, but there is increasing evidence of the clinical importance of several organisms including *Corynebacterium jeikeium*, *Corynebacterium urealyticum*, *Corynebacterium striatum*, *Arcanobacterium haemolyticum*, *Actinomyces pyogenes*, *Rhodococcus equi*, and other species (3). There are few reports on the activity of antimicrobial agents against coryneform bacteria. The majority refer to *C. jeikeium* or *C. urealyticum* (4, 12, 13) or to particular groups of antimicrobial agents (8). Both *C. jeikeium* and *C. urealyticum* are fastidious slowly growing organisms, which limits the use of traditional susceptibility testing assays. In fact, the methodology used in previous reports is not uniform, and at this moment the National Committee for Clinical Laboratory Standards (NCCLS) has not yet provided specific guidelines for the testing of these organisms. Also, most of the approaches using commercial panels for routine susceptibility testing of clinically important bacteria have not yet been evaluated for the testing of coryneform bacteria. These factors hinder the determination in the clinical laboratory of the quantitative activity of antimicrobial agents against coryneform bacteria.

The E-test provides a simple, rapid, and reliable method for determining quantitative activity of antimicrobial agents against microorganisms, including gram-negative bacilli, gram-positive cocci (1, 2), and fastidious bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (6). Moreover, it has been reported (9) that this method is cost-effective when a limited number of antimicrobial agents is studied. The E-test could be a useful method for susceptibility testing of coryneform bacteria, including *C. jeikeium*, *C. urealyticum*, and other fastidious organisms within this group.

The purpose of this study is to evaluate the activity of 10 antimicrobial agents against coryneform bacteria isolated from clinical samples by three different methods: disk diffusion, microdilution, and the E-test.

(This work was presented in part at the 34th Interscience Conference of Antimicrobial Agents and Chemotherapy in Orlando, Fla. [7]).

MATERIALS AND METHODS

Bacteria. One hundred and thirty-one coryneform bacteria isolated from clinical samples at the Hospital Universitario "Virgen Macarena," Seville, Spain, in the period 1991 to 1992, and four ATCC strains were evaluated. Bacteria were identified by the method of Hollis and Weaver (5); identification was confirmed by using the API CORYNE System. The following species were tested (number of isolates in parentheses): *C. urealyticum* (30, including strains ATCC 43042, ATCC 43043, and ATCC 43044), *Corynebacterium minutissimum* (20), coryneform CDC group ANF-1 (20), *C. striatum* (20), *C. jeikeium* (15 including strain ATCC 43734), coryneform CDC group I2 (8), *Listeria monocytogenes* (7), *Corynebacterium xerosis* (5), *Corynebacterium aquaticum* (1), coryneform CDC group A3 (3), coryneform CDC group B (2), coryneform CDC group F (2), coryneform CDC group A5 (1), and coryneform CDC group ANF-3 (1). After identification, bacteria were maintained in 10% glycerol in tryptic soy broth at −70°C until they were used. *Staphylococcus aureus* ATCC 29213 and ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains.

Microdilution assay. An in-house microdilution method was used (8). We followed the guidelines described by the NCCLS (10) with the exception that cation-supplemented Mueller-Hinton broth containing 0.5% Tween 80 (MHT80) was used. The following antimicrobial agents were used as powders of known potency: ampicillin (AMP; Smith-Kline Beecham), cephalothin (CR; Sigma), cefoxitin (FOX; Merck, Sharp, & Dohme), cefotaxime (CTX; Hoechst), erythromycin (E; Abbott), ciprofloxacin (CIP; Bayer), tetracycline (TE; Sigma), amikacin (AK; Bristol Myers), vancomycin (VA; Eli Lilly), and rifampin (R; Ciba Geigy). Twofold dilutions across a range of 0.015 to 16 μg/ml for E, CIP, and R; 0.06 to 64 μg/ml for AMP, TE, and VA; and 0.125 to 128 μg/ml for CR, FOX, CTX, and AK were used. The inoculum (5×10^4 CFU per well) was prepared in MHT80 from cultures grown on Columbia agar with 5% sheep blood for 20 to 24 h. Plates were incubated aerobically at 35°C for 18 h or in the case of *C. jeikeium* and *C. urealyticum*, for 24 h, since with both species there was no visible

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TABLE 1. Distribution of differences in MICs of ten antimicrobial agents against 135 coryneform bacteria by E-test versus microdilution reference method

Antimicrobial agent	% of isolates with the following difference in MIC:									% Agreement[a]
	<−3	−3	−2	−1	0	+1	+2	+3	>+3	
AMP	0.7	1.5	2.2	11.1	57.8	15.6	5.9	2.2	3.0	84.5
CR	0	0	0.7	1.5	66.7	20.0	5.9	2.2	3.0	88.2
FOX	1.5	1.5	8.1	9.6	57.8	9.6	7.4	0.7	3.7	77.0
CTX	3.0	5.2	5.2	8.9	56.3	5.9	6.7	4.4	4.4	71.1
E	3.0	3.0	1.5	4.4	77.8	4.4	1.5	0	4.4	86.6
CIP	0	0	1.5	5.9	51.1	20.0	13.3	3.7	4.4	77.0
TE	0	2.2	4.4	3.7	60.7	14.8	8.9	4.4	0.7	79.2
AK	0.7	0	0	2.2	46.7	15.6	16.3	8.9	9.6	64.5
VA	0	0	0.7	0.7	8.9	21.5	41.5	17.0	9.6	31.1
R	0	0.7	0.7	1.5	77.8	8.9	5.9	1.5	3.7	88.2
All agents	0.9	1.4	2.5	5.0	56.2	13.5	11.3	4.6	4.6	74.7

[a] Percentage of isolates within the accuracy limits of the reference tests (MIC ± 1 $\log_2$ dilution).

growth in the control wells at 18 h. The MIC was determined to be the lowest concentration of antimicrobial agent that inhibited visible growth.

Disk diffusion assay. Plates containing Mueller-Hinton agar supplemented with 5% sheep blood and 0.5% Tween 80 were used. The inoculum was prepared by suspending bacteria grown on Columbia agar with 5% sheep blood for 20 to 24 h in MHT80 to a concentration of about 10^8 CFU/ml. Plates were inoculated with a cotton swab according to the NCCLS guidelines for this procedure (11). Afterwards, disks containing the following antimicrobial agents (Difco) were aseptically placed on the surface of the plates: AMP (10 μg), CR (30 μg), FOX (30 μg), CTX (30 μg), E (15 μg), CIP (5 μg), TE (30 μg), AK (30 μg), VA (30 μg), and R (5 μg). Plates were incubated aerobically for 18 h or for 24 h in the case of *C. jeikeium* and *C. urealyticum.*

E-test. The conditions for disk diffusion were also followed for the E-test, except that E-test strips (Biodisk, Solna, Sweden) instead of disks containing the antimicrobial agents were applied to the surface of the plates. For each microorganism, two 150-mm-diameter plates were used, and five strips were applied to each agar surface. The MICs were read at the point of intersection between the edge of the zone of bacterial growth and the E-test strip following incubation as described above.

Control strains were used as follows: *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 for microdilution and E test and *S. aureus* ATCC 25923 for disk diffusion and E test.

The reproductibility of the E-test was assessed by performing the assay five times over 5 days with one clinical isolate each of *C. jeikeium*, *C. striatum*, *L. monocytogenes*, *C. minutissimum*, and *C. urealyticum.*

Evaluation criteria. Because of the absence of accepted breakpoints for assignment of coryneform bacteria to interpretive categories, those established for microdilution (10) and disk diffusion (11) by the NCCLS for organisms other than *Haemophilus* species and *Neisseria gonorrhoeae* were used. In the case of AMP, the breakpoint for *L. monocytogenes* (the only gram-positive rod specifically included by the NCCLS) was considered. Because studies on the clinical effectiveness of vancomycin against coryneform bacteria are lacking, the breakpoints for "other gram-positives" (not those for enterococci) were used. For comparison of MICs determined by microdilution and E-test results, the latter MICs were rounded up to the next higher twofold dilution value. Disagreements of the E-test with either microdilution or disk diffusion or of disk diffusion with microdilution (the reference method) were classified as very major (false susceptibility), major (false resistance), or minor (disagreement because of an intermediate result by either the evaluated method or the reference method and susceptibility or resistance with the other method).

RESULTS

The distribution of differences in MICs determined by reference microdilution and the E-test are presented in Table 1. Agreement within one twofold dilution between the E-test and the MIC reference method was 31.1% for VA, 64.5% for AK, 71.1% for CTX, 77.0% for FOX and CIP, 79.2% for TE, 84.5% for AMP, 86.6% for E, and 88.2% for CR and R. For the 1,350 combinations of microorganisms and antimicrobial agents, 85 (6.3%) disagreements in interpretive category were found. As shown in Table 2, 57 (4.2%) of the disagreements were minor, 16 (1.2%) were major, and 12 (0.9%) were very major discrepancies.

Discrepancies between interpretive categories determined by the E-test and the disk diffusion method are presented in Table 3. For the 1,350 combinations of microorganisms and antimicrobial agents, 70 (5.1%) disagreements in interpretive category were found: 52 (3.8%) minor, 6 (0.4%) major, and 12 (0.9%) very major. Comparatively, as shown in Table 4, 85 (6.3%) discrepancies were observed when disk diffusion and microdilution methods (microdilution as the reference test) were compared: 57 (4.2%) minor, 7 (0.5%) major, and 21 (1.5%) very major. No disagreement in interpretive categories for VA were observed when values of any of the three methods evaluated were compared.

MICs obtained with the E-test were highly reproducible. For the five tests performed on different days with five different species (250 results), we found 8 (3.2%) out of one twofold dilution from the most frequent MIC, specifically, in 2 cases with FOX and VA and in 1 case (each) with CTX, E, CIP, and TE.

DISCUSSION

There has been increasing interest on the clinical importance of coryneform bacteria in recent years, but only a few reports have dealt with their antimicrobial susceptibility testing and characteristics. Development of standard methodology for routine antimicrobial agent testing of these organisms in the clinical microbiology laboratory seems warranted.

TABLE 2. Discrepancies between MICs determined by the E-test and the microdilution reference method (n = 135)

Antimicrobial agent	% of isolates by error category			
	Very major	Major	Minor	Total
AMP	1.5	1.5	0	3.0
CR	0	1.5	1.5	3.0
FOX	0	1.5	6.7	8.2
CTX	0	1.5	9.6	11.1
E	2.2	3.7	5.2	11.1
CIP	0.7	0	8.9	9.6
TE	3.0	0	3.7	6.7
AK	0.7	0.7	2.2	3.6
VA	0	0	0	0
R	0.7	1.5	4.4	6.6
Total	0.9	1.2	4.2	6.3

TABLE 3. Discrepancies between interpretive categories determined by E-test and the disk diffusion reference method (n = 135)

Antimicrobial agent	% of isolates by error category			
	Very major	Major	Minor	Total
AMP	0.7	0.7	0	1.4
CR	0	2.2	0	2.2
FOX	0	0	5.9	5.9
CTX	0	0.7	5.2	5.9
E	3.0	0.7	10.4	14.1
CIP	0.7	0	5.9	6.6
TE	4.4	0	8.1	12.5
AK	0	0.7	0	0.7
VA	0	0	0	0
R	0	0	3.7	3.7
Total	0.9	0.4	3.8	5.1

We have previously studied the activity of several quinolones against coryneform bacteria (8) and noted that both *C. jeikeium* and *C. urealyticum* grew satisfactorily on MHT80 with a 24-h incubation period. In this study, we used MHT80 to ensure the growth of all organisms. Five percent sheep blood was added to agar plates to improve the ease of reading zones of inhibition around disks or strips, especially when testing slowly growing organisms.

Traditionally, disk diffusion and microdilution have been used for bacterial susceptibility testing. In routine MIC determination, the E-test could serve as an alternative to microdilution. To our knowledge, there are no data on the use of automated methods for susceptibility testing of coryneform bacteria, another possibility.

When interpretive categories are considered, the data of this study show good agreement between the three methods used. Discrepancies were noted in 6.3% (E-test versus microdilution), 5.1% (E-test versus disk diffusion), and 6.3% (disk diffusion versus microdilution) of comparisons. Most of these discrepancies were within the minor category (4.2, 3.8, and 4.2%, respectively). On the other hand, when the two quantitative methods are compared, the overall agreement is lower. As shown in Table 1, the agreement within one twofold dilution of the E-test with microdilution was only 31.1% for VA and 64.5% for AK. Agreement for all other antimicrobial agent values ranged from 71.1% (CTX) to 88.2% (CR and R).

TABLE 4. Discrepancies between interpretive categories determined by disk diffusion and the microdilution reference method. (n = 135)

Antimicrobial agent	% of isolates by error category			
	Very major	Major	Minor	Total
AMP	1.4	1.4	0	2.8
CR	0	0	0	0
FOX	1.4	0.7	3.7	5.8
CTX	2.2	0.7	11.1	14.0
E	4.4	0.7	7.0	12.1
CIP	0.7	0.7	8.8	10.2
TE	3.0	0	6.7	9.7
AK	0.7	0.7	1.4	2.8
VA	0	0	0	0
R	1.4	0.4	3.7	5.5
Total	1.5	0.5	4.2	6.3

The MICs of VA (determined by microdilution) against 127 of the 131 strains we tested ranged from 0.06 to 0.5 μg/ml. This narrow range of variation has been shown to reduce the correlation coefficient between microdilution and the E-test for other gram-positive bacteria (1).

The actual causes of the discrepancies observed between the E-test and either broth microdilution or disk diffusion are not known. The actual causes of the differences observed between the E-test and broth microdilution could be dependent on the different nature of assays, but this explanation seems unlikely for the discrepancies between the E-test and disk diffusion, since the same laboratory conditions were used for both methods. The data of Table 4 suggest that the number of discrepancies between disk diffusion and broth microdilution is similar to that of the E-test with either method.

The E-test is a simple and easy to interpret method, which does not require special equipment. Its versatility allows the choice of particular antimicrobial agents. This feature, less likely available with use of commercial panels containing predetermined agents, may be advantageous when a limited number of antibiotics are to be tested (9). Data in this study show that although quantitative results obtained with the E-test (especially for VA and to a lesser extent AK) are not in complete agreement with microdilution results, the translation of MICs to interpretive categories highly correlates with those of either microdilution or disk diffusion. In our opinion, the E-test is as reliable as broth microdilution or disk diffusion to study the susceptibility testing of coryneform bacteria.

Very major errors were more frequently encountered when comparing disk diffusion with microdilution than when comparing the E-test with either method (Tables 2, 3, and 4), which suggests methodological problems in susceptibility testing of coryneform bacteria. Further studies considering the most appropriate medium, incubation conditions, and breakpoint definition for interpretive categories are necessary in order to advance our knowledge of the activity of antimicrobial agents against coryneform bacteria.

ACKNOWLEDGMENT

We thank P. Hidalgo for excellent secretarial assistance.

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