JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1983, p. 1122–1126
0095-1137/83/111122-05$02.00/0

Vol. 18, No. 5

Reliability of In Vitro Susceptibility Tests for Detecting Coagulase-Negative Staphylococcal Resistance to Penicillinase-Resistant Semisynthetic Penicillins

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The reliabilities of five in vitro susceptibility tests (agar dilution, broth microdilution, automated MS-2, Kirby-Bauer disk diffusion, and ability to grow on methicillin-containing agar) to predict the susceptibility of 204 coagulase-negative staphylococcal isolates to penicillinase-resistant semisynthetic penicillins were compared. There was wide variation in susceptibility, with results ranging from 86.3% susceptible by MS-2 to 38.2% by growth on methicillin-containing agar. The results of the broth dilution techniques, including the MS-2, were significantly different ($P < 0.02$) from the remaining tests. Nafcillin disks were less effective ($P < 0.02$) than oxacillin disks in predicting resistance. Kirby-Bauer oxacillin disks and the ability to grow on methicillin-containing agar were the most reliable predictors of resistance. The MS-2 did not reliably predict resistance.

Considerable variability has been reported among tests used for detecting staphylococcal susceptibility to penicillinase-resistant semisynthetic penicillins (PRSPs) (2, 4, 7). Most of these studies have been performed with *Staphylococcus aureus*, in part because of the recent increased frequency of methicillin-resistant *S. aureus* infections reported in the United States (10). Coagulase-negative staphylococci have also been increasingly recognized as important nosocomial pathogens (5, 12). The frequency of resistance to PRSPs among these clinical isolates has been reported to range from 63 to 87%, far higher than the frequency of resistance reported for isolates of *S. aureus* (1, 10, 12). Despite this, comparable comparative susceptibility studies with coagulase-negative staphylococci have not been performed. The purpose of the present study was to compare the reliability of five standard in vitro susceptibility tests for detecting resistance to PRSPs.

(The results of this study were presented in part at the 22nd Interscience Conference on Antimicrobial Agents and Chemotherapy, Miami Beach, Fla., abstr. no. 382, 1982.)

MATERIALS AND METHODS

Bacterial strains. A total of 204 catalase-positive, coagulase-negative laboratory isolates with positive anaerobic glucose and negative mannitol fermentation tests were collected and stored at −70°C in Mueller-Hinton broth. The strains were identified to species by the Staph-Ident staphylococcal system according to the recommendations of the manufacturer (Analytab Products, Plainview, N.Y.) (13).

The 204 strains were identified as follows: *Staphylococcus epidermidis*, 118; *Staphylococcus haemolyticus*, 33; *Staphylococcus hominis*, 20; *Staphylococcus simulans*, 20; *Staphylococcus warneri*, 5; *Staphylococcus cohnii*, 3; *Staphylococcus saprophyticus*, 3; *Staphylococcus sciuri*, 1; and *Staphylococcus xylosus*, 1.

Susceptibility testing. All studies were performed at 35°C with bacteria that had been grown overnight in Mueller-Hinton broth at 35°C. The *S. aureus* control strain ATCC 25923 was included in all assays. Five methods were compared: agar dilution, broth microdilution, disk diffusion, an automated system using an optical determination of bacterial growth rates, and bacterial growth on methicillin-containing agar.

Agar dilution minimal inhibitory concentrations (MICs) against methicillin were performed with a Steers replicator (Craft Machine, Inc., Chester, Pa.) (18, 19). Mueller-Hinton agar plates containing serial twofold dilutions of methicillin (range, 128 to 0.5 μg/ml) were prepared and used within 24 h. An MIC of ≤4 μg/ml was interpreted as sensitive. The MIC was interpreted after 24 and 48 h of incubation.

Broth microdilution MICs were performed with the Sceptor System (BBL Microbiology Systems, Cockeysville, Md.) for gram-positive bacteria (11). Susceptibility to methicillin was determined (range, 8 to 0.12 μg/ml), with an MIC ≤4 μg/ml interpreted as susceptible.

Kirby-Bauer disk diffusion tests were performed by standard technique with methicillin (5 μg), nafcillin (1

TABLE 1. Percent susceptibility of 204 coagulase-negative staphylococci to PRSPs based on the particular method used

Test	% Susceptible	Test	% Susceptible
Agar dilution MIC (M)[a]	54.4	Growth on methicillin agar (20 μg/ml)	38.2
Microdilution MIC (M)	75.5	Kirby-Bauer (M)	45.6
MS-2 (M)	80.4	Kirby-Bauer (N)	58.3
MS-2 (O)	86.3	Kirby-Bauer (O)	38.7

[a] M, Methicillin; O, oxacillin; N, nafcillin.

μg), and oxacillin (1 μg) disks as outlined by the National Committee for Clinical Laboratory Standards (3, 15). The determinations were performed in duplicate and the results averaged. There was minimal variation between the two disks. Zone sizes were measured after 18 h of incubation at 35°C.

The MS-2 system (Abbott Diagnostics, Division of Abbott Laboratories, Dallas, Tex.) was the automated method used in this study (4). The system is based on an optical determination of bacterial growth rates and utilizes a computer program to measure antibiotic susceptibility. The test was performed according to the recommendations of the manufacturer. Susceptibility to methicillin and oxacillin (provided by Abbott Diagnostics) was determined by using both the standard computer program and a research program which measures growth rates for 18 to 24 h.

The ability of coagulase-negative staphylococci to grow on Mueller-Hinton agar containing methicillin (20 μg/ml) was the final test used. The procedure, adopted from Archer (1), was to add 0.1 ml of the overnight bacterial culture onto the surface of methicillin-containing agar plates. The plates were incubated for 72 h at 35°C and then inspected for growth. Those strains demonstrating any growth were considered resistant.

Statistical evaluation of the sensitivity of the 5 tests in predicting resistance to PRSPs was performed by Cochrans Q test (17). For this analysis, results were recorded as either susceptible or resistant. This analysis was followed with a program directed at performing multiple comparisons to establish whether there were pairwise differences. The results were also compared by using an analysis of variance and a correlation matrix. In this analysis, all intermediate results from the Kirby-Bauer disk diffusion tests were recorded as resistant.

RESULTS

The percent susceptibility to PRSPs showed marked variability depending on the particular method used (Table 1). The results ranged from 38.2% susceptible with the methicillin-containing agar method to 86.3% with the MS-2 oxacillin test. There was total agreement among all tests with 85 isolates (42.0%), 61 sensitive and 24 resistant. Agar dilution MICs for the 24 resistant isolates were uniformly ≥128 μg/ml. The highest percent resistance was found with the methicillin-containing agar and the Kirby-Bauer oxacillin disk results. Variable susceptibility among the disks used for the Kirby-Bauer disk diffusion tests was also noted, with 38.7% susceptible to the oxacillin disk compared with 58.3% susceptible to the nafcillin disks.

The relative correlation among tests is demonstrated in Table 2. Correlation among the three broth dilution techniques was high; however, these tests showed poor correlation with the agar dilution and disk diffusion techniques. The highest correlation was between the Kirby-Bauer oxacillin disk and methicillin-containing agar assays (0.80). Changing the cutoff point for susceptibility to methicillin from ≤4 μg/ml to <4 μg/ml in the agar dilution and microtiter MIC tests had only limited effect on the correlation of these tests with the others. The most notable change was a further decrease in correlation with the MS-2 data. Correlation of the MS-2 data with the agar dilution results went from 0.51 to 0.34.

Statistical analysis of the results by Cochrans

TABLE 2. Correlation in susceptibility to PRSPs among the different tests used for 204 laboratory isolates[a]

Test	M-20[b]	Kirby-Bauer (O)[c]	Kirby-Bauer (M)	Kirby-Bauer (N)	Agar dilution MIC (M)	Microdilution MIC (M)	MS-2 (M)	MS-2 (O)
M-20	1.00							
Kirby-Bauer (O)	0.80	1.00						
Kirby-Bauer (M)	0.64	0.72	1.00					
Kirby-Bauer (N)	0.44	0.59	0.71	1.00				
Agar dilution MIC (M)	0.64	0.67	0.66	0.64	1.00			
Microdilution MIC (M)	0.42	0.43	0.52	0.58	0.53	1.00		
MS-2 (M)	0.36	0.37	0.45	0.56	0.51	0.78	1.00	
MS-2 (O)	0.31	0.32	0.36	0.47	0.44	0.67	0.72	1.00

[a] Results were recorded as either sensitive (1) or resistant (0) for this comparison (intermediate results were coded as resistant).

[b] M-20, ability to grow on methicillin-containing agar.

[c] M, Methicillin; N, nafcillin; O, oxacillin.

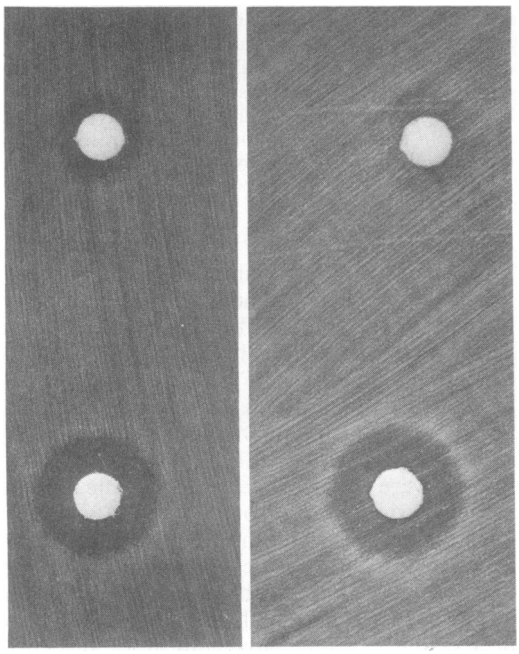

FIG. 1. Photograph of Kirby-Bauer susceptibility test for strain no. 32 after 24-h (left) and 48-h (right) incubation at 35°C. The disks are oxacillin (top) and nafcillin (bottom). An inner zone of growth is visible around the nafcillin disk at 48 h.

Q test demonstrated a significant difference ($P < 0.02$) in sensitivity between the broth dilution and the other tests. The differences between the Kirby-Bauer nafcillin and oxacillin disk results were also significant ($P < 0.02$).

Reincubation of the agar dilution MIC plates for an additional 24 h at 35°C altered the susceptibility results of eight isolates.

Because of the different results obtained with the oxacillin and nafcillin disks in the Kirby-Bauer tests, additional studies were performed. Ten nafcillin-susceptible and oxacillin-resistant isolates were selected for comparison with 10 isolates susceptible to both antibiotics. Both sets were incubated for 48 h at 35°C with nafcillin disks. All 10 oxacillin-resistant isolates showed resistance to nafcillin with an inner zone of growth visible only after 48 h of incubation (Fig. 1). No change in susceptibility was noted with the control strains.

A similar study was carried out with the broth microdilution MIC trays. Eleven strains which were methicillin susceptible by the broth microdilution test and resistant by Kirby-Bauer tests were reincubated for an additional 24 h at 35°C. After 48 h, six strains were resistant. Nine strains susceptible to methicillin by both tests remained susceptible after 48 h of incubation.

Growth curves (24 h) from the MS-2 studies demonstrated a total of 40 (22 with oxacillin, 18 with methicillin) strains with growth between 5 and 24 h (Fig. 2). The routine susceptibility results for the MS-2 are determined within 5 h. An additional 19 strains showed a slight outgrowth by 24 h. Of the 40 isolates, 24 were resistant to PRSPs by Kirby-Bauer oxacillin disk or agar dilution MICs (median MIC, 16 μg/ml), whereas 12 were susceptible (median MIC, 2 μg/ml). Seven of the latter strains were sensitive to PRSPs by all other tests.

The possibility that delayed outgrowth was a result of the slower growth rate of the heteroresistant subpopulation was investigated. Reincubation in methicillin of a methicillin-susceptible strain (by MS-2) which demonstrated delayed outgrowth in the methicillin-containing cartridge following a standard 18-h incubation resulted in the demonstration of resistance within 5 h.

DISCUSSION

Coagulase-negative staphylococci are now recognized as important nosocomial pathogens, particularly in infections associated with pros-

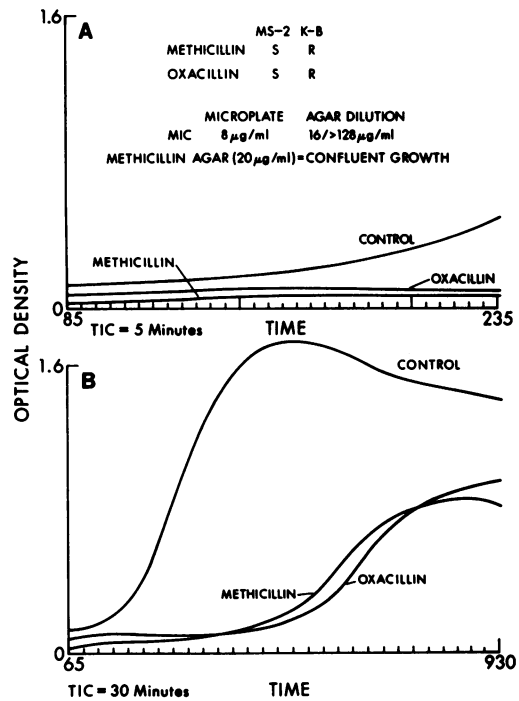

FIG. 2. Results of a susceptibility test with the MS-2. The growth of strain no. 204 without antibiotic (control) and in the presence of methicillin and oxacillin over 235 min, the standard test period, are displayed as well as the results of the other susceptibility tests (A). Delayed outgrowth in the presence of both methicillin and oxacillin occurring after 5 h of incubation is demonstrated by the research system (B).

thetic valves (12), orthopedic devices (9), and intravenous catheters (5). A high percentage of these clinical isolates are methicillin resistant (1, 12). The increased clinical importance of these staphylococcal isolates has made the ability to reliably detect resistance to PRSPs of critical importance in selecting appropriate antimicrobial therapy.

Documentation of PRSP resistance among staphylococcal isolates has been complicated by the heteroresistant nature of this type of resistance. This has made not only laboratory recognition of these strains difficult but has made even a standard definition of PRSP resistance uncertain. The heteroresistant subpopulation represents only a small proportion of the total bacterial population, and because these highly resistant bacteria grow at a relatively slower rate, detection at 24 h is difficult. For coagulase-negative staphylococci, the proportion of highly resistant bacteria is lower than for *S. aureus*, making the problems of detection worse (16).

Previous studies have investigated the reliability of a number of in vitro susceptibility tests in predicting PRSP resistance among *S. aureus* isolates and have found great variability (2, 4, 7). In particular, broth dilution techniques such as the microdilution or the automated, computerized systems have proven unreliable (2, 4, 6).

The strains collected for the present study were similar to those reported in previous studies, with *S. epidermidis* being the predominant species (6, 8, 14). The results were similar to those reported for *S. aureus* (2, 4, 7). The broth dilution tests were poor predictors of PRSP resistance. It is uncertain whether the results obtained with the broth microdilution system used in the present study are applicable to other similar systems because of the modified Mueller-Hinton broth used in this assay (11). As reported by Barry and Badal (2) with *S. aureus*, incubation of microtiter trays for an additional 24 h improved the reliability of the results. The results with either the oxacillin or methicillin cartridges in the MS-2 were unreliable. This was in part due to the short incubation time (1 to 5 h) used for these tests, although many strains failed to grow even after 18 h of incubation. In addition, even among those strains demonstrating delayed outgrowth, there were seven strains which were sensitive to PRSPs by all other tests. This suggests that antibiotic concentration as well as incubation time contribute to this problem.

The Kirby-Bauer disk diffusion tests predicted PRSPs more reliably than the broth dilution methods, although there was a significant difference in sensitivity between the nafcillin and oxacillin disk. Reports by Drew et al. (7) of the poor stability of the methicillin disks resulted in the recommendation that nafcillin or oxacillin be used as the class disks for PRSPs. Our results suggest that nafcillin disks do not predict resistance to PRSPs as reliably as oxacillin disks. Reincubation of nafcillin-susceptible, oxacillin-resistant isolates demonstrated an inner zone of growth around the nafcillin disks. There was a strong correlation between the Kirby-Bauer oxacillin disks and the methicillin-containing agar technique. The latter test predicted the highest percent resistance, although it also required a 72-h incubation time. In 10 instances, strains resistant by Kirby-Bauer disks did not grow on methicillin-containing agar. It is possible that using a slightly lower concentration of methicillin such as 12.5 μg/ml would be more effective.

The agar dilution MICs were not as effective as the Kirby-Bauer oxacillin- or methicillin-containing agar assay in predicting resistance, although these differences were not statistically significant. Incubation of the plates for an additional 24 h or changing the MIC cutoff for susceptibility had only limited effect.

These studies demonstrate considerable variability in susceptibility to PRSPs depending on the test used. The automated MS-2 was unreliable, as was the broth microdilution MIC at 24 h. The oxacillin disk at 24 h was the most reliable of the Kirby-Bauer disks and correlated closely with growth on methicillin-containing agar after 72 h. Either of these two tests appears to be the most reliable method for screening isolates for PRSP susceptibility.

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