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Comparison of PCR Detection of *mecA* with Standard Susceptibility Testing Methods To Determine Methicillin Resistance in Coagulase-Negative Staphylococci

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Approximately 75% of coagulase-negative staphylococci are resistant to methicillin, but it is suspected that even more resistance exists that is not detected by standard susceptibility assays. To determine the most accurate assay for measuring resistance, we compared the detection of *mecA* by PCR with detection by National Committee for Clinical Laboratory Standards methods using oxacillin as the class drug. Strains from 11 species of coagulase-negative staphylococci were selected such that 84% were susceptible by the broth microdilution method. Of 45 *mecA*-positive strains, 1 strain was unable to express the *mecA* gene product after induction and was not included in further analyses. For microdilution with 2% NaCl, the disk test without salt, and agar screen containing 4% NaCl plus 6 μg of oxacillin per ml, the sensitivities in detecting the 44 *mecA*-positive strains were 50, 84, and 70%, respectively, at 24 h and 77, 82, and 100%, respectively, at 48 h. The specificities of microdilution, disk, and agar screen in detecting the 97 strains lacking *mecA* were 100, 89, and 100%, respectively, at 24 h. Only the disk test proved to be less specific at 48 h (81%). Furthermore, for 10 of the *mecA*-positive strains plus an additional 8 strains subsequently added to the analyses, the MICs were 2 μg/ml at 24 h by the broth microdilution method; all 18 strains were positive for *mecA* by PCR. Thus, an oxacillin MIC of ≥2 μg/ml indicated resistance and is probably a more appropriate breakpoint than the current National Committee for Clinical Laboratory Standards breakpoint of 4 μg/ml for coagulase-negative staphylococci. Strains for which MICs are <2 μg/ml may be methicillin resistant and should be verified as susceptible by oxacillin agar screening with incubation for 48 h.

Coagulase-negative staphylococci are a leading cause of nosocomial infections, especially in neonates, immunocompromised individuals, and patients with internal prosthetic devices. Although approximately 75% of hospital strains are resistant to methicillin, treatment with methicillin or other penicillinase-resistant penicillins, when the strains are susceptible to these drugs, is preferred to treatment with vancomycin because β-lactam drugs are more easily absorbed into body fluids and tissues, cause fewer complications from treatment, and do not select for vancomycin-resistant organisms. Use of oxacillin, nafcillin, and methicillin, as well as those cephalosporins indicated for the treatment of coagulase-negative staphylococci can reduce costs in the treatment of hospitalized patients. Yet, there has been concern that the available susceptibility test systems are not accurate in detecting resistance to methicillin (1, 8, 11, 15), leaving physicians no option but to use vancomycin therapy.

The *mecA* gene, which encodes penicillin-binding protein PBP 2a, correlates with methicillin resistance in coagulase-negative staphylococci (3, 10, 12, 16, 17, 19, 22). In an effort to improve the detection of resistance, methods to easily and rapidly detect the *mecA* gene have been recommended for routine laboratory use (1, 2, 5, 17, 21, 23). Although these methods are feasible for some laboratories, most clinical laboratories do not have the resources to efficiently develop and perform PCR or DNA probe techniques routinely. In addition, performing only these techniques can lead to results of false resistance, because not all strains that possess *mecA* are able to express the *mecA* gene product (5, 6, 19). False-susceptible results have also been reported by some DNA probe methods which were able to detect *mecA* only after exposure of cultures to methicillin (15, 17). Furthermore, testing by probe methods alone fails to detect resistance mechanisms that are not mediated by *mecA* (20).

To detect methicillin resistance, the National Committee for Clinical Laboratory Standards (NCCLS) recommends broth dilution, agar dilution, salt agar screen, and disk diffusion with oxacillin as the class drug (13, 14). Several reports indicate that the standard disk test is comparable in sensitivity to *mecA* detection (7, 11, 15), but very major errors have been reported by this technique (8), and interpretations of intermediate resistance must be resolved by another method. NCCLS recommends resolving discrepant results with growth on oxacillin salt agar, but no extensive study comparing the results obtained by this method and the detection of *mecA* have been reported for coagulase-negative staphylococci.

We compared the NCCLS methods for broth microdilution, salt agar screen, and disk diffusion with *mecA* detection by PCR on selected strains of coagulase-negative staphylococci with emphasis on strains considered oxacillin susceptible by broth microdilution. Our goal was to define which NCCLS assays could accurately predict methicillin resistance, providing a cost-effective alternative to the PCR assay for the detection of *mecA*.

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MATERIALS AND METHODS

Strains. For initial testing, 142 strains of coagulase-negative staphylococci were retrieved from stocks stored at −70°C in tryptic soy broth with 20%

glycerol. Strains were subcultured once onto sheep blood agar for susceptibility testing and onto Mueller-Hinton agar for PCR. Strains had been isolated from clinical specimens and were selected with a bias toward those which had been categorized previously as susceptible to oxacillin either by the broth or by the disk test. The isolates were from anatomic sites, as follows: urine (9%), blood (54%), cerebrospinal fluid (11%), and bone, other tissues, fluids, and wounds (26%). For the latter part of the study, 28 isolates (9 from blood, 7 from cerebrospinal fluid, and 12 from other sites) were selected for which MICs were ≤2 μg/ml.

Identification to species level. Strains were identified to the species level with the Staph-Ident system (bioMérieux Vitek, Inc., Hazelwood, Mo.) by following the manufacturer's instructions. Additional biochemical tests (nitrate, Voges-Proskauer, *o*-nitrophenyl-β-D-galactopyranoside, novobiocin susceptibility, lactose and xylose fermentation) were used when necessary.

MIC determinations. The MIC of oxacillin was determined by the microdilution methodology of NCCLS (14). Trays containing oxacillin in cation-adjusted Mueller-Hinton broth with 2% NaCl were frozen at −70°C until use. Inoculations were performed with a MIC-2000 inoculator (Dynatech Laboratories, Inc., Alexandria, Va.) to a final concentration of 1×10^5 to 5×10^5 CFU/ml. The trays were incubated in ambient air at 35°C and were read for turbidity with indirect light at 24 and 48 h.

Disk testing. The disk test was performed as described by NCCLS (13) with a 1-μg oxacillin disk and Mueller-Hinton agar without additional NaCl. The plates were incubated in ambient air at 35°C, and zone diameters were read with transmitted light at 24 and 48 h. Any growth, including light growth, within the 12-mm-diameter zone around the disk was considered indicative of resistance. The intermediate category was defined as zone sizes from 12 to 16 mm.

Oxacillin salt agar screen. Mueller-Hinton agar plates with 4% NaCl and 6 μg of oxacillin per ml (MHOX; Prepared Media Laboratories, Tualatin, Oreg.) were inoculated as a streak in three directions by using a cotton swab dipped into a direct colony suspension equivalent to a 0.5 McFarland standard in tryptic soy broth (14). As a control, the same medium containing 4% NaCl without oxacillin was inoculated first. Plates were incubated in ambient air at 35°C and were read at 24 and 48 h. Any growth was considered a positive test result.

PCR detection of *mecA*. Isolates were grown on Mueller-Hinton agar in ambient air at 35°C for 18 to 24 h. A direct colony suspension of the culture equivalent to a 1.0 McFarland standard was prepared in 500 μl of 10 mM Tris–1 mM EDTA (pH 8.0), vortexed, and boiled for 10 min; 5 μl of the suspension was used for each 50-μl reaction mixture. The PCR mixture contained 1× PCR buffer (Perkin-Elmer/Roche, Norwalk, Conn.) supplemented with $MgCl_2$ to a final concentration of 4 mM, 30 pmol each RSM 2647 and RSM 2648 primers (12), 200 μM dUTP, 100 μM (each) dATP, dCTP, and dGTP, and 1 U of uracil *N*-glycosylase (Perkin Elmer/Roche). The tubes were placed in a Perkin-Elmer Cetus DNA thermal cycler and were heated for 10 min at 50°C and 10 min at 95°C; this was followed by a 1-min denaturation at 94°C. Then, 2 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.) was added. This was followed by annealing for 30 s at 55°C and elongation for 30 s at 72°C. Denaturation, annealing, and elongation were repeated for a total of 35 cycles. The samples were held in the thermal cycler at 72°C until they were loaded onto a 2% agarose gel and were electrophoresed for 30 min at 125 V, stained with ethidium bromide, and viewed with UV light. The presence of a 533-bp band was considered a positive result. A positive control PCR product was confirmed by restriction enzyme digestion with *Hha*I, which produces fragments of 332 and 201 bp.

If the results of the broth, disk, or salt agar screen demonstrated the presence of oxacillin resistance and no PCR product was visualized, the PCR was repeated and the second result was used for the comparisons. To detect the gene product of *mecA* with one strain, immunoblot assays for PBP 2a were performed after each of several induction methods (courtesy of Henry Chambers, San Francisco, Calif.) (6, 19).

Calculation of sensitivity and specificity. Calculations of sensitivity were made by dividing the number of strains detected as resistant by the susceptibility test method by the total number of strains that were *mecA* positive. Specificity was calculated by dividing the number of strains detected as susceptible by the test method by the total number of strains that were *mecA* negative.

RESULTS

The *mecA* gene was found in 4 of the 11 coagulase-negative staphylococcal species: *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and *S. warneri* (Table 1). Of the 142 strains tested, 32% possessed the *mecA* gene, with >50% of the *S. epidermidis* strains being *mecA* positive. All strains were initially tested to determine the MICs of oxacillin by the standard broth microdilution method of NCCLS (Table 2). Surprisingly, for 23 of the 45 strains that were *mecA* positive by PCR, the MICs indicated that the strains were considered susceptible (≤2 μg/ml). At 48 h, several additional strains showed resistance, but 11 of the *mecA*-positive strains were still classified as susceptible. All the strains for which the MICs were 2 μg/ml at 24 h of incubation were positive for the *mecA* gene. This correlation was not observed at 48 h, MICs were 2 μg/ml after the extended incubation since for several *mecA*-negative strains. One *mecA*-positive strain for which the MIC was 0.25 μg/ml at both 24 and 48 h did not produce a *mecA* gene product even after the use of several induction methods and Western blot (immunoblot) analyses of the protein products for the detection of PBP 2a. That strain was not used in the remainder of the analyses.

TABLE 1. Distribution of *mecA* by species of *Staphylococcus*

Staphylococcus species	No. of isolates	No. (%) of *mecA*-positive isolates
S. auricularis	2	
S. capitis	6	
S. cohnii	4	
S. epidermidis	67	38 (57)
S. haemolyticus	7	2 (29)
S. hominis	17	4 (24)
S. hyicus	2	
S. saprophyticus	12	
S. simulans	5	
S. warneri	19	1 (5)
S. xylosus	1	
Total	142	45 (32)

The standard disk test with oxacillin (Table 3) showed both poor sensitivity and specificity in the detection of *mecA*-positive or -negative strains either at 24 or at 48 h. Three strains grew so poorly at 24 h that the zone sizes could not be determined. Some strains appeared to have a double zone, with less confluent growth in the inner zone, which usually went up to the disk. All of the 12 strains of *S. saprophyticus* had zone sizes that classified them in either the intermediate or the resistant category, even though *mecA* was not detected in any of the strains. If the data for *S. saprophyticus* were removed from the analysis, the specificity of the test for detecting *mecA*-negative strains increased from 84 to 94%.

None of the 97 *mecA*-negative strains grew on MHOX after either 24 or 48 h of incubation. Growth was evaluated for the 44 *mecA*-positive strains at 24 and 48 h of incubation (Table 3). All strains grew by 48 h, however, growth was barely visible for two of the strains at 48 h. To determine if the poor growth was due to prior freezing of the cultures, the tests on the two *mecA*-positive strains which grew poorly and three other *mecA*-positive strains were repeated after one and two subcultures of the frozen stocks. Consistently, each strain showed little or no growth at 48 h on the oxacillin salt agar after one subculture from the freezer, but all five strains grew well by 48 h when they were tested after two subcultures. The MICs for the five strains were identical after the first, second, and even a third subculture. Consequently, the initial poor growth of the two strains on MHOX at 48 h appears to be an effect of freezing, which had little effect on the MICs.

The sensitivities and specificities of each of the NCCLS methods for the detection of methicillin resistance were compared with PCR for detection of *mecA* (Table 4). The results indicated that when the MICs were ≥2 μg/ml at 24 h, the strains possessed the *mecA* gene. When the MICs were <2 μg/ml, growth on MHOX by 48 h correlated with resistance to methicillin and the presence of *mecA*. Conversely, a lack of growth correlated with susceptibility and a lack of *mecA*.

To verify these results, 28 strains for which oxacillin MICs were ≤2 μg/ml at the time of isolation from patient cultures were collected. At that time, when the MICs were <2 μg/ml,

TABLE 2. Comparison of presence of *mecA* with oxacillin MIC

MIC (μg/ml)	Distribution of *mecA* (no. of strains)					
	By 24-h MIC		By 48-h MIC		For fresh clinical isolates (24 h)	
	Negative	Positive	Negative	Positive	Negative	Positive
≤0.25	78	4[a]	70	1[a]	14	
0.5	11	1	11		1	
1	8	10	13	5	1	2
2		8	3	5		10
≥4		22		34		
Total	97	45[a]	97	45[a]	16	12

[a] One strain demonstrated no gene product after induction.

the isolates were tested by the salt agar screen. All 28 strains were tested for the presence of *mecA*, and the results were compared with those of the microdilution and salt agar screen tests performed previously (Table 2). The 15 strains for which MICs were <1 μg/ml did not grow on MHOX and did not have evidence of *mecA*. For three strains MICS were 1 μg/ml. Two of the three strains were *mecA* positive and grew on MHOX; one grew at 24 h and one grew at 48 h. The third strain did not grow on MHOX and was *mecA* negative. Oxacillin MICs were 2 μg/ml by the broth dilution method for 10 strains; all 10 strains possessed the *mecA* gene. The results with fresh clinical isolates confirmed that an MIC of 2 μg/ml predicts resistance to oxacillin and that growth on MHOX will detect the remaining *mecA*-positive strains that appear to be susceptible to oxacillin by the broth dilution method.

DISCUSSION

None of the NCCLS methods were accurate in the detection of methicillin resistance in the recommended 24 h of incubation (13, 14); however, both the agar screen and the microdilution test were able to accurately detect methicillin-susceptible strains. Growth or no growth on oxacillin salt agar showed an excellent correlation with the presence or absence of *mecA*, provided that the plates were read after both 24 and 48 h of incubation.

The poor sensitivities of MIC techniques for the detection of methicillin resistance have been reported previously (1, 8, 11, 15, 24). All isolates in our study for which MICs were 2 μg/ml possessed *mecA* and were resistant to oxacillin. These isolates would be called susceptible by current NCCLS MIC breakpoints. Our data indicate that strains of coagulase-negative staphylococci for which MICs are ≥2 μg/ml at 24 h should be classified as resistant to oxacillin. McDonald et al. (11) have also recommended that NCCLS consider changing the breakpoint for coagulase-negative staphylococci, but they suggest that a breakpoint of ≥1 μg/ml is more appropriate. Of the 21 strains in our study for which MICs were 1 μg/ml, 12 strains (57%) had *mecA* and grew on MHOX, but 9 strains were *mecA* negative and did not grow on MHOX. Therefore, our data do not support a breakpoint of ≥1 μg/ml.

McDonald et al. (11) indicated that the MICs for coagulase-negative staphylococci lacked a bimodal distribution, with MICs for 31% of the strains clustering around 1 or 2 μg/ml. Cumulative susceptibility data from our laboratory in 1994 for 490 strains do not corroborate their results and showed that for only 6% of strains were MICs 1 or 2 μg/ml. To account for their results, it is possible that a methicillin-resistant clone for which the MIC is 1 or 2 μg/ml was prevalent in the population studied by McDonald et al. (11). A large collection of strains from other geographic areas needs to be studied before making a final decision on the appropriate breakpoint. We suggest that the oxacillin salt agar screen be used as a standard when comparing these strains.

The strains in our study were selected because most of them (84%) were methicillin susceptible by microdilution testing. The microdilution method had a 50% sensitivity in detecting methicillin resistance in this selected population of isolates. The prevalence of methicillin-susceptible clinical isolates of coagulase-negative staphylococci in our institution is 27% by the microdilution method with NCCLS breakpoints. Extrapolating the data from our study to a population with 27% methicillin-susceptible strains predicts a sensitivity of 94% for the microdilution method in detecting methicillin resistance. This sensitivity might be considered acceptable; however, our data indicate 18% of the strains reported as susceptible are likely to be methicillin resistant. A more reliable method than microdilution testing for the detection of methicillin resistance in coagulase-negative staphylococci is essential.

The detection of methicillin resistance in coagulase-negative staphylococci poses a challenge for the clinical laboratory. The *mecA* gene is regulated by a region designated *mecR*, which reduces the overall expression of resistance by negatively regulating the synthesis of PBP 2a (10, 18). Very slow derepres-

TABLE 3. Comparison of presence with *mecA* with oxacillin disk test and growth on MHOX at 24 and 48 h of incubation[a]

mecA result	No. of isolates								
	Total	24-h disk test				48-h disk test			Growth on MHOX at:
		S	I	U	R	S	I	R	24 h	48 h
Positive	44	7	4	1	32	5	3	36	31	44
Negative	97	80	8	2	7	79	6	12	0	0
Negative[b]	85[b]	80	2	2	1	79	2	4	0	0

[a] S, susceptible; I, intermediate; U, result uninterpretable because of poor growth at 24 h; R, resistant.
[b] Data exclude those for *S. saprophyticus*.

TABLE 4. Sensitivity and specificity of detection methods for 44 *mecA*-positive and 97 *mecA*-negative strains[a]

Method	Sensitivity (%)		Specificity (%)	
	24 h	48 h	24 h	48 h
MIC, ≥2 μg/ml	68	89	100	97
MIC, ≥4 μg/ml	50	77	100	100
Disk test[b]	84	82	89	81
MHOX	70	100	100	100

[a] Sensitivity equals (number of resistant isolates/total number of *mecA*-positive isolates) × 100. Specificity equals (number of susceptible isolates/total number of *mecA*-negative isolates) × 100.

[b] NCCLS breakpoints were used; isolates with zone sizes indicating intermediate or uninterpretable susceptibility were not included in the numerator.

sion of the *mecA* gene in coagulase-negative staphylococci can result in strains that appear to be susceptible by laboratory testing. Thus, special considerations must be used to detect methicillin resistance in coagulase-negative staphylococci, including increased incubation times and lower breakpoints than those used for *S. aureus* strains.

The recommendation to alter the MIC breakpoints or to alter the incubation time for oxacillin salt agar screening does not apply for *S. aureus* strains. *S. aureus* strains for which MICs are 2 μg/ml have been found to be both *mecA* positive and *mecA* negative (8). Also *mecA*-negative strains of *S. aureus* can grow on MHOX with extended incubation times, possibly because some strains of *S. aureus* are hyperproducers of β-lactamase (6).

No strains among the 24 strains that were resistant by the broth microdilution method lacked *mecA*, but the numbers were too small to say that there are no other mechanisms of resistance other than *mecA* in our population of coagulase-negative staphylococci. Suzuki et al. (20) found that 4 of 125 strains of coagulase-negative staphylococci in Japan had a mechanism of methicillin resistance that was not associated with *mecA* or PBP 2a.

All 12 *S. saprophyticus* strains in our study were classified in the intermediate or resistant category by disk testing, but for none of the strains did the MICs indicate resistance, none of the strains grew on MHOX, and none of the strains had the *mecA* gene, as demonstrated by PCR. The discrepancy between the disk test and MIC has been reported previously for *S. saprophyticus* (4). Testing of *S. saprophyticus* for β-lactamase production has also been reported to be problematic and requires induction and then incubation with nitrocefin for up to 24 h (not the usual 2 h) for expression (9). The *mecA* gene has been found in *S. saprophyticus* (12), and the presence of PBP 2a has been demonstrated previously (19). Suzuki et al. (20) were unable to demonstrate *mecA* in two methicillin-resistant strains, suggesting that another mechanism of resistance besides *mecA* may also be present in this species. Because of these problems and because we did not find *mecA*-positive strains, we cannot recommend any test method that can be used to predict methicillin resistance in *S. saprophyticus*. Fortunately, treatment of urinary infections with *S. saprophyticus* is not problematic.

We found no evidence of *mecA* in six species of staphylococci; however, the number of isolates tested in the present study was too small to conclude that *mecA* is truly lacking in these species. Although most resistance was seen in *S. epidermidis*, 43% of the strains of this species tested were *mecA* negative. Thus, identification to the species level is not helpful in predicting methicillin resistance except to correctly evaluate susceptibility results for *S. saprophyticus*.

Disk diffusion susceptibility testing of our strains with oxacillin lacked both sensitivity and specificity, with a large number of strains reported in the intermediate category. Some results were difficult to read because of faint growth at 24 h. The problems that we experienced were not reported by the studies that showed a high degree of correlation between the disk test and the presence of *mecA* (7, 11, 15). Woods et al. (24) also showed a good correlation between the disk test and the salt agar screen, but they reported poor sensitivity with the microdilution test at 24 h of incubation, with some improvement at 48 h. Possibly, our problems with disk testing were the result of testing of frozen stock cultures. Alternatively, problematic strains may be unique to our geographic area, although Huang et al. (8) reported similar problems with strains submitted to the Centers for Disease Control and Prevention from throughout the United States.

The salt agar screen method is less costly, less labor-intensive, and as accurate as *mecA* detection by PCR. In all cases the growth on MHOX correlated with the PCR result, although PCR occasionally had to be repeated to detect the amplified product. Unfortunately, the MHOX test is not rapid. Most isolates that are resistant will produce visible colonies within 24 h, but 48 h is required to confirm negative results. Since most physicians will use vancomycin to treat serious infections caused by coagulase-negative staphylococci until a reliable oxacillin test result is received, a report that the isolate is truly susceptible to oxacillin, even though the report is delayed, will allow effective long-term therapy with a less toxic and costly antimicrobial agent.

In summary, we conclude that disk testing by the NCCLS methodology is not an accurate method for the determination of methicillin susceptibility for coagulase-negative staphylococci. Rather, we recommend that the NCCLS broth microdilution method with oxacillin in 2% salt be considered a reliable 24-h screening method, but that the breakpoint of resistance should be defined as ≥2 μg/ml. This breakpoint for resistance may not be applicable to commercial MIC systems; thus, we cannot recommend their use. Lastly, we recommend testing on oxacillin salt agar with a 48-h final reading on all isolates of coagulase-negative staphylococci for which MICs are ≤1 μg/ml. Strains that grow on the agar are truly resistant to methicillin and should be reported as such, despite the MIC result. For laboratories in which the broth microdilution method is not available, the salt agar screen with reading at 48 h should be used to determine methicillin resistance on all significant isolates of coagulase-negative staphylococci.

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