JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2001, p. 3781–3784 Vol. 39, No. 10
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.10.3781–3784.2001

Optimal Inoculation Methods and Quality Control for the NCCLS Oxacillin Agar Screen Test for Detection of Oxacillin Resistance in *Staphylococcus aureus*

JANA M. SWENSON,[1]* JEAN SPARGO,[2] FRED C. TENOVER,[1] AND MARY JANE FERRARO[2]

Nosocomial Pathogens Laboratory Branch, Centers for Disease Control and Prevention, Atlanta, Georgia 30333,[1] and Department of Microbiology, Massachusetts General Hospital, Boston, Massachusetts 02114[2]

Received 5 April 2001/Returned for modification 1 June 2001/Accepted 18 July 2001

To define more precisely the inoculation methods to be used in the oxacillin screen test for *Staphylococcus aureus*, we tested agar screen plates prepared in house with 6 μg of oxacillin/ml and 4% NaCl using the four different inoculation methods that would most likely be used by clinical laboratories. The organisms selected for testing were 19 heteroresistant *mecA*-producing strains and 41 non-*mecA*-producing strains for which oxacillin MICs were near the susceptible breakpoint. The inoculation method that was preferred by all four readers and that resulted in the best combination of sensitivity and specificity was a 1-μl loopful of a 0.5 McFarland suspension. A second objective of the study was to then use this method to inoculate plates from five different manufacturers of commercially prepared media. Although all commercial media performed with acceptable sensitivity compared to the reference lot, one of the commercial lots demonstrated a lack of specificity. Those lots of oxacillin screen medium that fail to grow heteroresistant strains can be detected by using *S. aureus* ATCC 43300 as a positive control in the test and by using transmitted light to carefully examine the plates for any growth. However, lack of specificity with commercial lots may be difficult to detect using any of the current quality control organisms.

The oxacillin agar screen test for detection of oxacillin resistance in *Staphylococcus aureus* was first included in the NCCLS dilution methods document, M7, in 1990 (11), although neither that edition nor the current edition (14) provides details on how the test should be inoculated. Furthermore, a variety of inoculation methods are recommended on the product labels of commercially prepared media. Despite this, many studies have shown that the oxacillin screen test performs well for detection of *S. aureus* strains that contain the *mecA* gene, which mediates oxacillin resistance (1–4, 6–8, 15–17, 20). However, when studies have included strains whose resistance is heterogeneous, the test has been shown to perform less well (2, 15).

Recommendations for quality control of the test were not included in NCCLS documents until 1997 (13), when *S. aureus* ATCC 43300, a *mecA*-positive strain that is very heterogeneous in its expression of oxacillin resistance (10), was suggested as the quality control strain. The reason for recommending a strain that was difficult to detect was that, when adequate growth was obtained, the user would be assured that the test could detect heterogeneously resistant strains. Recent comments addressed to the NCCLS Subcommittee on Antimicrobial Susceptibility Testing (14) have questioned the appropriateness of this strain for this procedure. Other references (9) and package inserts from commercial plates have recommended the use of *S. aureus* ATCC 33591, a strain which expresses homogeneous resistance.

The present study was undertaken both to clarify the best inoculation methods for the NCCLS oxacillin agar screen test and to verify that *S. aureus* ATCC 43300 is adequate for use as a positive quality control strain for the test.

Study design. The study was conducted in two laboratories, the Centers for Disease Control and Prevention (CDC) and Massachusetts General Hospital (MGH), in three phases. Phase 1 evaluated several inoculation methods in order to select a method that could be used in the subsequent phases. Phase 2 was undertaken to confirm that commercial lots of agar performed well with the chosen inoculation method. Phase 3 was performed to document the specificity of the test using a set of more commonly encountered susceptible organisms (see below).

Organisms. In phases 1 and 2, the organisms chosen for testing represented a challenge set from the CDC culture collection. For the resistant organisms, those of a low expression class were selected (19), with oxacillin MICs ranging from 4 to 128 μg/ml; for the susceptible strains, organisms for which the oxacillin MICs were 1 to 8 μg/ml when tested by agar dilution using 4% NaCl were included. All strains used in phases 1 and 2 had been tested by PCR for the presence of the *mecA* gene and by population analysis (for oxacillin-resistant strains only) to determine the level of expression of oxacillin resistance (19). The organisms fell into four groups. Group 1 ($n = 19$) comprised *mecA* positive strains determined to be in expression class 1 or 2, i.e., they shared a high degree of heterogeneity. Group 2 ($n = 16$) comprised *mecA*-negative strains for which agar dilution MICs of oxacillin were ≥4 μg/ml when tested with 4% salt (5) but were ≤2 μg/ml with 2% salt. Group 3 ($n = 5$) comprised *mecA*-negative strains for which the oxacillin MICs were ≥4 μg/ml with both 4 and 2% salt. The five strains in group 3 were considered to have the "MOD" phenotype; two of the strains had been characterized previously and had penicillin-binding proteins (PBPs) with modified affinity to

* Corresponding author. Mailing address: CDC, Mailstop G08, 1600 Clifton Rd., Atlanta, GA 30333. Phone: (404) 639-0196. Fax: (404) 639-1381. E-mail: jswenson@cdc.gov.

TABLE 1. Phase 1 results of testing four inoculation methods in two laboratories using an in-house lot of oxacillin screen agar prepared with a reference standard lot of Mueller-Hinton agar containing 4% salt and 6 μg of oxacillin/ml

Inoculation method	Sensitivity[a]				Specificity[b]			
	CDC		MGH		CDC		MGH	
	Reader 1	Reader 2	Reader 1	Reader 2	Reader 1	Reader 2	Reader 1	Reader 2
Swab, spotted	18 (94.7)	17 (89.5)	19 (100)	19 (100)	35 (97.2)	35 (97.2)	35 (97.2)	35 (97.2)
Swab, quadrant	18 (94.7)	19 (100)	19 (100)	19 (100)	34 (94.4)	34 (94.4)	35 (97.2)	35 (97.2)
1-μl loop	19 (100)	18 (94.7)	19 (100)	19 (100)	35 (97.2)	35 (97.2)	35 (97.2)	35 (97.2)
10-μl pipette	19 (100)	19 (100)	19 (100)	19 (100)	21 (58.3)	28 (77.8)	32 (88.9)	31 (86.1)

[a] Expressed as number (percent) of 19 *mecA*-positive strains that grew.
[b] Expressed as number (percent) of 36 *mecA*-negative strains that did not grow, excluding 5 strains with the MOD resistance phenotype.

penicillin but did not contain PBP2a, the *mecA* gene product (18). Three additional strains in group 3 were classified as "MOD" only phenotypically, i.e., the oxacillin MICs for these strains were >2 μg/ml and were not lowered with the addition of clavulanic acid, and these strains did not contain *mecA*. The "MOD" strains were included to see how they would test; however, it was decided before beginning the study that because of the unknown prevalence of such strains, their unknown clinical significance, and the lack of molecular characterization of all the strains, the results of their testing would be considered separately in data analysis. The last group (group 4 [$n = 20$]) comprised *mec*A-negative strains for which the oxacillin MICs were 1 to 2 μg/ml with 4% salt.

In phase 3, additional, oxacillin-susceptible clinical isolates were tested along with a subset from phases 1 and 2. Combined, these included 64 susceptible strains: 47 strains for which the oxacillin broth microdilution MICs were ≤0.12 to 2 μg/ml, 15 susceptible crossover strains from groups 2 and 4 described above, and the 2 susceptible quality control strains (ATCC 29213 and ATCC 25923) that were blinded to the readers. Eight resistant strains were also included in phase 3: six less-challenging resistant strains (expression class 3 or 4) and the two oxacillin-resistant quality control strains (ATCC 43300 and ATCC 33591), also blinded. All strains were subcultured twice from the freezer on trypticase soy agar with 5% sheep blood (TSA-SB) before being tested.

Quality control strains. Four *S. aureus* strains were used as controls on each day of testing: ATCC 43300, a heteroresistant strain; ATCC 33591, a homogeneously resistant strain; and two oxacillin-susceptible strains, ATCC 25923 and ATCC 29213.

Oxacillin screen agar medium. For phase 1, plates containing 4% salt and 6 μg of oxacillin (Sigma, St. Louis, Mo.)/ml were prepared at CDC using the standard reference lot of Mueller-Hinton agar that is used by manufacturers to standardize their production lots (12). For phase 2, commercial plates were purchased from five different manufacturers, i.e., BD Biosciences (BBL, Cockeysville, Md.), PML Microbiologicals (Wilsonville, Oreg.), Hardy Diagnostics (Santa Maria, Calif.), Gibson Laboratories, Inc., (Lexington, Ky.), and Remel (Lenexa, Kans.); three manufacturers' plates were tested at MGH, and two manufacturers' plates were tested at CDC. In addition, for phases 2 and 3, plates were prepared and tested at CDC using Mueller-Hinton II agar (BBL). All plates were stored at 4 to 8°C for no longer than 4 weeks or until the manufacturer's expiration date. In the latter part of phase 2 and in phase 3, additional lots of commercial plates were obtained from the two most commonly used manufacturers (BBL and Remel).

Inoculation methods. Inoculum suspensions were prepared from 18- to 24-h cultures grown on TSA-SB and adjusted to equal a 0.5 McFarland standard. Four inoculation methods were studied during phase 1: (i) spotting an area 10 to 15 mm in diameter using a cotton swab that was dipped in the suspension and from which excess fluid was expressed, (ii) streaking a quadrant of the plate using a swab prepared as above, (iii) spotting an area 10 to 15 mm in diameter using a 1-μl disposable loop, and (iv) spotting 10 μl using a micropipette. Four organisms were tested per plate, with only one inoculation method used per plate to avoid biasing the reading between plates. For phases 2 and 3, testing was done by spotting an area 10 to 15 mm in diameter using a 1-μl disposable loop. All plates were incubated at 35°C and read after 24 h.

Reading. All readings were done by two independent readers using transmitted light at both sites, except for the testing of additional lots of media at the end of phase 2, where one reader only was used. All readers had had previous experience in reading oxacillin screen plates. Plates were read as positive (if confluent growth was observed), weak positive (if the growth was almost confluent but hazy or >20 colonies), the number of colonies (if <20), or negative. However, for data analysis purposes, any growth of >1 colony was considered positive.

Phase 1. In phase 1, the abilities of the four inoculation methods to detect resistant strains were essentially equivalent (Table 1), with the method that would be expected to deliver the largest inoculum (the 10-μl pipette) achieving 100% sensitivity among all readers and laboratories. All the *mecA*-positive strains were detected by the other three methods by all readers in both laboratories, except for two strains (strains 107 and 351) which were not detected by all three methods in one laboratory. For these strains oxacillin MICs were 4 μg/ml by both conventional broth microdilution (2% salt) and agar dilution testing with increased salt (4%), and these strains were determined to be among the most heterogeneous of those tested (expression class 1; data not shown). Although the 10-μl-pipette inoculation method gave the best sensitivity across laboratories and readers, it resulted in the growth of a large number of susceptible strains (i.e., false positives), which resulted in a specificity of 88.9% in the best case and as low as 58% in the worst case. The swab and loop methods performed better in terms of specificity, ranging from 94 to 97%. The best combination of sensitivity (95 to 100%) and specificity (97%) among all readers and all laboratories was obtained with the

TABLE 2. Phase 2 results of testing five commercial lots and one in-house lot of oxacillin screen agar inoculated using a 1-μl loop

Manufacturer	Testing laboratory	Sensitivity[a]		Specificity[b]	
		Reader 1	Reader 2	Reader 1	Reader 2
In house (lot 2)	CDC	17 (89.5)	16 (84.2)	36 (100)	36 (100)
BBL	CDC	19 (100)	17 (89.5)	35 (97.2)	35 (97.2)
PML	CDC	19 (100)	19 (100)	31 (86.1)	35 (97.2)
Hardy	MGH	19 (100)	19 (100)	32 (88.9)	29 (80.6)
Gibson	MGH	16 (84.2)	16 (84.2)	35 (97.2)	33 (91.7)
Remel	MGH	18 (94.7)	19 (100)	21 (58.3)	17 (47.2)

[a] Expressed as the number (percent) of 19 *mecA*-positive strains that grew.
[b] Expressed as the number (percent) of 36 *mecA*-negative strains that did not grow, excluding 5 strains with the MOD resistance phenotype.

1-μl loop. However, the performances of all methods, except the 10-μl pipette, were not significantly different ($P > 0.05$) from each other (McNemar's chi-square test).

Phase 2. Of the five commercial lots tested (Table 2), the lowest sensitivity (84%) was obtained with the Gibson medium, although the performance of the in-house lot was similar (84 to 90%). There was no statistically significant difference among the sensitivity results when readers or medium lots were compared ($P > 0.05$ by McNemar's chi-square test). If the two highly heterogeneous strains (strains 107 and 351) are excluded, all the phase 2 media detected the remaining 17 resistant strains except for the lot from Gibson, which failed to grow one of the remaining 17 resistant strains (as noted by both readers), and the in-house lot, which was read as negative for one strain by one reader only. The Gibson lot also failed to grow the resistant control strain, ATCC 43300, and therefore results from that round of testing on that medium should not have been reported in a clinical laboratory.

In phase 2, specificity was best with the BBL lot (97%) and the in-house lot (100%), but dropped to 47 to 58%, depending upon the reader, for the Remel medium (Table 2). In analyzing specificity results, statistically significant differences were found between the in-house lot and the lots from PML, Hardy, and Remel for reader 1, and between the in-house lot and the lots from Hardy and Remel for reader 2 ($P < 0.05$). When reader 1 was compared to reader 2, there were significant differences for both PML and Remel ($P < 0.05$).

To determine if the lower specificity of Remel media was related to a single lot, laboratory, or reader, we purchased and retested two additional lots from Remel and one from BBL along with a third in-house lot (data not shown). Sensitivity was very good (95 to 100%) for all additional lots tested. The specificity of the second lot from BBL was similar to that in the first testing in phase 2 (97%). Although differences could be attributed to the person performing the testing and how conservatively the test was read, one of the two additional lots from Remel performed slightly better in terms of specificity (83%); the other performed similarly (58%).

Phase 3. In order to determine how well the media would perform in a routine fashion (presumably with less-challenging strains), additional lots of both BBL and Remel media (and a new in-house lot) were tested against a group of susceptible organisms for which the oxacillin MICs did not cluster around the resistance breakpoint and a subset of the susceptible challenge organisms from phases 1 and 2. For the susceptible strains, the specificity of the Remel lot improved but still remained below 90% (range, 84 to 88%). For eight *mecA*-positive strains, the sensitivity was 100% with all three media tested (data not shown).

Testing strains with modified PBPs. Results for the MOD strains tested in phases 1 and 2 were dependent on the oxacillin MIC. Strains for which MICs were 8 μg/ml were more likely to give positive results than those for which MICs were 4 μg/ml and which displayed variable growth depending on the laboratory and reader (data not shown).

Quality control. In phase 1, both oxacillin-resistant organisms grew with all inoculation methods. On two occasions, weak growth of one of the susceptible control strains, *S. aureus* ATCC 25923 (the strain recommended for disk diffusion quality control), was recorded as positive by one of the readers in one of the laboratories when the 10-μl inoculum was used. In phase 2, one of the commercial lots failed to grow *S. aureus* ATCC 43300, and growth of ATCC 25923 was detected on the PML lot by one reader. Growth of ATCC 25923 was also detected during phase 3 in one laboratory by both readers on one of two days of testing using Remel's medium. On this day growth was noted for both the quality control strain and the blinded quality control strain.

During all 3 phases, when the 1-μl loop inoculum was used, ATCC 43300 showed adequate growth on all media except one lot of commercial medium. The susceptible control strain ATCC 29213 also performed as expected, i.e., no growth on any of the medium lots. However, the susceptible strain ATCC 25923 did grow sporadically during phases 2 and 3 on some of the commercial medium lots.

In summary, three of four inoculation methods were found to perform adequately in the oxacillin screen test. With the commercial media tested, the specificity of the medium of one of the manufacturers was inferior. However, given the challenging nature of the organisms used in phases 1 and 2, lack of specificity is understandable. When strains that would be considered more representative of those likely to be encountered in a clinical laboratory were tested, the performance of that medium improved. However, laboratories need to be aware that some current commercial medium lots may overcall resistance. Given the expectation that the oxacillin screen test is comparable to MIC reference methods in reliability (14), if it is used without further confirmation of resistance, the potential exists for falsely labeling strains as oxacillin resistant. This could lead to inappropriate treatment for patients and unnecessary infection control measures. Although the potential also exists for missing truly resistant strains of low expression classes (as happened with two of the resistant strains in one laboratory in this study), proper attention to test conditions (e.g., inoculum and time of incubation), careful examination of plates for any growth using transmitted light, and the use of *S. aureus* ATCC 43300 as a resistant control to check medium and observer performance will decrease the chances of such errors. Although *S. aureus* ATCC 25923, a susceptible control, occasionally grew, its performance was not consistent enough to recommend its use as a negative control to detect media with the potential for decreased specificity. The use of an inoculum larger than 1 μl or larger than that achieved with a swab is not recommended because of greatly decreased specificity. Package inserts that accompany commercially available

oxacillin screen agar should be revised to reflect proper inoculation procedures and use of quality control organisms.

We acknowledge Linda McDougal (CDC) and Lisa Gartland (MGH) for expert help as second readers in the study. We also thank Alaeddin Abu-Zant, a visiting fellow at CDC at the time of the study, for the population analysis studies of the *mecA*-producing strains.

REFERENCES

1. **Brakstad, O. G., J. A. Mæland, and Y. Tveten.** 1993. Multiplex polymerase chain reaction for detection of genes for *Staphylococcus aureus* thermonuclease and methicillin resistance and correlation with oxacillin resistance. APMIS **101:**681–688.
2. **Cavassini, M., A. Wenger, K. Jaton, D. S. Blanc, and J. Bille.** 1999. Evaluation of MRSA-Screen, a simple anti-PBP2a slide latex agglutination kit, for rapid detection of methicillin resistance in *Staphylococcus aureus*. J. Clin. Microbiol. **37:**1591–1594.
3. **Frebourg, N. B., D. Nouet, L. Lemée, E. Martin, and J. Lemeland.** 1998. Comparison of ATB Staph, Rapid ATB Staph, Vitek, and E-Test methods for detection of oxacillin heteroresistance in staphylococci possessing *mecA*. J. Clin. Microbiol. **36:**52–57.
4. **Gerberding, J. L., C. Miick, H. H. Liu, and H. F. Chambers.** 1991. Comparison of conventional susceptibility tests with direct detection of penicillin-binding protein 2a in borderline oxacillin-resistant strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **35:**2574–2579.
5. **Huang, M. B., T. E. Gay, C. N. Baker, S. N. Banerjee, and F. C. Tenover.** 1993. Two percent sodium chloride is required for susceptibility testing of staphylococci with oxacillin when using agar-based dilution methods. J. Clin. Microbiol. **31:**2683–2688.
6. **Knapp, C. C., M. D. Ludwig, J. A. Washington, and H. F. Chambers.** 1996. Evaluation of Vitek GPS-SA card for testing of oxacillin against borderline-susceptible staphylococci that lack *mec*. J. Clin. Microbiol. **34:**1603–1605.
7. **Kobayashi, Y., M. Kizaki, Y. Kawakami, H. Uchida, and Y. Ikeda.** 1993. Assessment of oxacillin salt agar for detection of MRSA identified by presence of the *mecA* gene. J. Hosp. Infect. **23:**279–285.
8. **Kohner, P., J. Uhl, C. Kolbert, D. Persing, and F. Cockerill.** 1999. Comparison of susceptibility testing methods with *mecA* gene analysis for determining oxacillin (methicillin) resistance in clinical isolates of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. J. Clin. Microbiol. **37:**2952–2961.
9. **Leitch, C., and S. Boonlayangoor.** 1992. Tests to detect oxacillin (methicillin)-resistant staphylococci with an oxacillin screen plate, p. 5.5.1–5.5.7. *In*: H. D. Isenberg (ed.), Clinical microbiology procedures handbook. American Society for Microbiology, Washington, D.C.
10. **Mackenzie, A. M. R., H. Richardson, R. Lannigan, and D. Wood.** 1995. Evidence that the National Committee for Clinical Laboratory Standards disk test is less sensitive than the screen plate for detection of low-expression-class methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **33:**1909–1911.
11. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 2nd ed. NCCLS document M7–A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
12. **National Committee for Clinical Laboratory Standards.** 1996. Protocols for evaluating dehydrated Mueller-Hinton agar. Approved standard, 1st ed. NCCLS document M6-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
13. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 4th ed. NCCLS document M7–A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
14. **NCCLS.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 5th ed. NCCLS document M7–A5. NCCLS, Wayne, Pa.
15. **Resende, C. A., and A. M. Figueiredo.** 1997. Discrimination of methicillin-resistant *Staphylococcus aureus* from borderline-resistant and susceptible isolates by different methods. J. Med. Microbiol. **46:**145–149.
16. **Skov, R. L., L. V. Palleson, R. L. Poulsen, and F. Espersen.** 1999. Evaluation of a new 3-h hybridization method for detecting the *mecA* gene in *Staphylococcus aureus* and comparison with existing genotypic and phenotypic susceptibility testing methods. J. Antimicrob. Chemother. **43:**467–475.
17. **Skulnick, M., A. E. Simor, D. Gregson, M. Patel, G. W. Small, B. Kreiswirth, D. Hathoway, and D. E. Low.** 1992. Evaluation of commercial and standard methodology for determination of oxacillin susceptibility in *Staphylococcus aureus*. J. Clin. Microbiol. **30:**1985–1988.
18. **Tomasz, A., H. B. Drugeon, H. M. de Lencastre, D. Jabes, L. McDougal, and J. Bille.** 1989. New mechanism for methicillin resistance in *Staphylococcus aureus*: clinical isolates that lack the PBP 2a gene and contain modified penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob. Agents Chemother. **33:**1869–1874.
19. **Tomasz, A., S. Nachman, and H. Leaf.** 1991. Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. Antimicrob. Agents Chemother. **35:**124–129.
20. **Unal, S., K. Werner, P. DeGirolami, F. Barsanti, and G. Eliopoulos.** 1994. Comparison of tests for detection of methicillin-resistant *Staphylococcus aureus* in a clinical microbiology laboratory. Antimicrob. Agents Chemother. **38:**345–347.