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Evaluation of Differential Inoculum Disk Diffusion Method and Vitek GPS-SA Card for Detection of Oxacillin-Resistant Staphylococci

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This study was conducted in order to compare the accuracy of detection of oxacillin-resistant staphylococci, defined by microdilution MICs, population analyses, and *mec* gene hybridization, with the Vitek GPS-SA Susceptibility Card with that of the standard inoculum (10^7 CFU) and high-inoculum (10^9 CFU) disk diffusion tests. By the standard inoculum disk diffusion test, 10 of 67 (15%) isolates of oxacillin-resistant *Staphylococcus aureus* and 3 of 47 (6%) isolates of *Staphylococcus epidermidis* were falsely susceptible after 24 h of incubation at 35°C. By the high-inoculum disk diffusion test (10^9 CFU), 4 of the 10 isolates of *S. aureus* remained falsely susceptible, whereas none of the isolates of *S. epidermidis* was falsely susceptible. Of the 10 isolates of *S. aureus* falsely susceptible by the standard disk test, only one remained falsely susceptible after an additional 24 h of incubation at 22°C. All four isolates of *S. aureus* that were falsely susceptible by the high-inoculum disk diffusion test after 24 h of incubation at 35°C became resistant after an additional 24 h of incubation at 22°C. Thus, extended incubation of both the standard and high-inoculum disk diffusion tests increased their accuracy in detecting oxacillin resistance. All isolates of oxacillin-resistant staphylococci were accurately detected with the Vitek software upgrades (6.1 and 7.1) of the GPS-SA card.

The accurate detection of methicillin-resistant staphylococci, particularly those expressing heterogeneous resistance, continues to pose a problem for the clinical laboratory. Many variables are known to affect the detection of heterogeneously resistant strains (1). Variable results have been reported regarding the accuracy of detection of methicillin resistance with commercially available susceptibility testing devices, in part because of (i) variations in the reference methodology used, (ii) changes in software over time, and (iii) poor definition as to the relative distribution of isolates expressing homogeneous and various degrees of heterogeneous resistance.

Prior studies using the Vitek system (bioMérieux Vitek, Inc., Hazelwood, Mo.) have raised concerns over its accuracy in detecting methicillin resistance (3, 5, 6, 10). The purpose of our study was to test this system with recently upgraded software against a well-characterized population of staphylococci and to compare its accuracy with that of the reference broth microdilution test (8), the oxacillin agar screen (9), and the standard inoculum disk diffusion test (7) and the high-inoculum disk diffusion test described by de Lencastre et al. (2).

MATERIALS AND METHODS

Organisms tested. Sixty seven clinical isolates of *Staphylococcus aureus* and 47 clinical isolates of *Staphylococcus epidermidis* were determined to be oxacillin resistant by the standard broth microdilution method (8), by population analysis (4), and by the oxacillin agar screen method (9). In addition, all of the isolates of *S. aureus* and *S. epidermidis* were tested and found to be positive for the *mec* gene at either The Rockefeller University (courtesy of H. de Lencastre and A. Tomasz), New York, N.Y., or San Francisco General Hospital (courtesy of H. F. Chambers). All of the clinical isolates were also classified as resistant on the basis of population analyses.

Strains used for control purposes are listed in Table 1. Strains CDC-1 and NYHB-3 were generously provided by Alexander Tomasz, The Rockefeller University. Strains CDC-6, ATCC 43300, and ATCC 43387 were generously provided by Carolyn Baker, Centers for Disease Control and Prevention, Atlanta, Ga. Strains CCF-6 and CCF-10 were clinical isolates of *S. epidermidis* from The Cleveland Clinic Foundation, Cleveland, Ohio. *S. aureus* ATCC 29213 was used for quality control purposes as recommended by the National Committee for Clinical Laboratory Standards (7, 8).

Determination of MICs. Each isolate was tested by the standard broth microdilution test (8) with an inoculum of approximately 5×10^5 CFU/ml in salt-supplemented (2%) Mueller-Hinton broth. Each isolate was also tested with the Vitek GPS-SA card, and the results were interpreted with programs 4.2 and 6.1. Results were also analyzed with program 7.1.

Population analysis. Population analysis profiles for each isolate were determined at either The Rockefeller University (courtesy of H. de Lencastre and A. Tomasz) or The Cleveland Clinic Foundation according to methods described by Hartman and Tomasz (4) and Tomasz et al. (12). Briefly, the proportion of heterogeneous cells in the overnight broth culture of a particular isolate was calculated on the basis of the ratio of colony-forming units growing on agar plates containing a range of concentrations (usually 0.5 to 200 μg/ml) of methicillin to the number of colony-forming units growing on methicillin-free agar plates following 48 to 72 h of incubation. By plotting the number of colony-forming

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TABLE 1. Results of tests of control strains of *S. aureus* and *S. epidermidis*[a]

Organism and strain	Characteristics	MIC (μg/ml)		Zone diam (mm)[b]	
		MD	Vitek	10^7 CFU	10^9 CFU
S. aureus					
ATCC 29213	$Meth^s$, β-lac^-	≤0.5	≤2	22	22
ATCC 25923	$Meth^s$, β-lac^-	≤0.5	≤2	22	22
ATCC 43387	$Meth^s$, hyper-β-lac^+, *mec* mutant	2	≤2	19	15
CDC-1	$Meth^r$, mec^+ class 1	8	>8	12	9
NYHB-3	$Meth^r$, mec^+ class 2	16	>8	16	6
CDC-6	$Meth^r$, *mec* mutant, MOD	≥4	>8	6	6
ATCC 43300	$Meth^r$, mec^+, heteroresistant	4	4	16[c]	16[c]
S. epidermidis					
CCF-6	$Meth^s$, β-lac^-	≤0.5	≤2	31	26
CCF-10	$Meth^s$, β-lac^-	≤0.5	≤2	31	27

[a] Abbreviations: MD, microdilution; $Meth^s$, methicillin susceptible; $Meth^r$, methicillin resistant; β-lac, β-lactamase; MOD, modified penicillin-binding protein.
[b] Results are by the disk diffusion test using standard (10^7 CFU) and high (10^9 CFU) inocula after 24 h of incubation at 35°C.
[c] Zone diameters of inhibition were 6 mm after additional incubation for 24 h at 22°C.

units at each concentration tested, it is possible to classify isolates as belonging to four expression classes, depending upon the degrees of heterogeneity or homogeneity of methicillin-resistant cells in the population (12).

Differential inoculum disk diffusion testing. The differential inoculum disk diffusion tests were performed according to the methods described by de Lencastre et al. (2) in which zone diameters of inhibition obtained with inocula of 10^7 and 10^9 CFU are compared following 24 h of incubation at 35°C. The method used with an inoculum of 10^7 CFU was the standard disk diffusion test recommended by the National Committee for Clinical Laboratory Standards (7). Zone diameters of inhibition of both tests were measured after 24 h of incubation at 35°C and again after an additional 24 h of incubation at 22°C.

RESULTS

The results obtained with each of the control strains are presented in Table 1. All oxacillin-susceptible strains, as defined by microdilution testing, agar screen, and population analyses, required MICs of ≤2 μg of oxacillin per ml when tested with the Vitek GPS-SA card and yielded zone diameters of inhibition ranging from 19 to 31 mm by the standard disk diffusion test. By the high-inoculum disk diffusion test, these strains had zone diameters of inhibition ranging from 15 to 27 mm. All oxacillin-resistant isolates, defined on the basis of *mec* gene analysis and population analysis, were correctly classified as resistant on the basis of MICs obtained with the GPS-SA card. With the exception of a single isolate of *S. epidermidis*, which had previously been found to be inhibited on Mueller-Hinton agar containing 4% NaCl, all *mec* gene-positive staphylococci grew on the oxacillin agar screen test, although two isolates required 48 h of incubation for growth to appear. Strain CDC-1, which was an expression class 1 strain, yielded an intermediate (12-mm) zone diameter of inhibition by the standard disk diffusion test; however, by the high-inoculum disk diffusion test, the zone diameter of inhibition was 9 mm. With strain NYHB-3, an expression class 2 strain, the standard disk diffusion test yielded a susceptible (16-mm zone diameter of inhibition) result, whereas by the high-inoculum test the zone diameter of inhibition was 6 mm. With strain CDC-6, which was a methicillin-resistant, *mec*-negative strain with modified penicillin-binding proteins (11), the zone diameter of inhibition was 6 mm by broth disk diffusion techniques. Finally, with ATCC 43300, a *mec*-positive, heteroresistant strain requiring a Vitek MIC of 4 μg of oxacillin per ml, the zone diameters of inhibition after 24 h of incubation were 16 mm by both disk diffusion techniques; however, after an additional period of incubation of the plates for 24 h at 22°C, there was growth in both instances, although it was heavier with the larger inoculum, all the way to the disk margins.

Clinical isolates of oxacillin-resistant staphylococci were defined on the basis of MICs determined by the reference broth microdilution method, the agar screen, the presence of the *mec* gene, and population analysis studies. All isolates were in addition subjected to testing with the Vitek GPS-SA card and by the standard and high-inoculum disk diffusion tests.

A comparison of the results of tests with clinical isolates by the standard and high-inoculum disk diffusion tests with the results of the reference tests are shown in Table 2. By the standard disk diffusion test (inoculum size, 10^7 CFU), 10 of 67 (15%) isolates of oxacillin-resistant *S. aureus* were falsely susceptible and two were intermediate. All 10 falsely susceptible isolates belonged to heterogeneously resistant expression class 1 or 2. Following additional incubation of these isolates at 22°C, all but two of these isolates became resistant (Table 2). Of the 10 isolates falsely susceptible by the standard disk diffusion test, 2 became resistant, 4 became intermediate, and 4 remained falsely susceptible when tested by the high-inoculum (10^9 CFU) disk diffusion test.

TABLE 2. Comparison of reference methods with oxacillin standard and high-inoculum disk diffusion methods

Oxacillin-resistant strain	No. of isolates	No. of isolates[a] with the following inoculum (CFU)/plate:					
		10^7			10^9		
		S	I	R	S	I	R
S. aureus	67	10 (1)	2 (1)	55 (65)	4 (0)	4 (2)	59 (65)
S. epidermidis	47	3 (3)	3 (2)	41 (42)	0	0	47

[a] Numbers in front of parentheses represent results after 24 h of incubation at 35°C. Numbers in parentheses represent results after an additional 24 h of incubation at 22°C. S, susceptible; I, intermediate; R, resistant.

TABLE 3. Comparison of reference methods and Vitek GPS-SA card[a]

Oxacillin-resistant strain	No. of isolates	No. of isolates by the following software update(s):			
		4.2		6.1 and 7.1	
		S	R	S	R
S. aureus	67	0	66	0	66
S. epidermidis	47	1	46	0	47

[a] One isolate yielded no growth in the GPS-SA card. S, susceptible; R, resistant.

Both of the isolates intermediate by the standard disk diffusion test became resistant when tested by the high-inoculum disk diffusion test. Thus, 4 (6%) of the 67 oxacillin-resistant isolates of *S. aureus* remained falsely susceptible at the higher inoculum. All but two of these isolates of *S. aureus* became resistant following the additional incubation at 22°C (Table 2).

Of the 47 isolates of oxacillin-resistant *S. epidermidis*, 3 (6%) were falsely susceptible and 3 were intermediate by the standard disk diffusion test (Table 2). The three falsely susceptible isolates belonged to heteroresistant expression class 1 or 2 and remained falsely susceptible after the additional 24 h of incubation at 22°C. Of the three intermediate isolates, two remained unchanged and one became resistant following the additional 24 h of incubation at 22°C. No isolates of *S. epidermidis* were falsely susceptible or intermediate when tested with an inoculum of 10^9 CFU.

On the basis of these data, complete agreement between the standard disk diffusion test and the reference methods was 83%, whereas complete agreement between the high-inoculum disk diffusion test and the reference methods was 93%.

The comparison of the Vitek GPS-SA card and the reference methods with the clinical isolates is shown in Table 3. Of the 67 isolates of oxacillin-resistant *S. aureus*, 1 isolate failed to grow in the GPS-SA card, and there were no falsely susceptible isolates in the 4.2, 6.1, and 7.1 software updates of the GPS-SA card. Of 47 isolates of oxacillin-resistant *S. epidermidis*, there was 1 falsely susceptible isolate with the 4.2 software program; however, all isolates were resistant with the 6.1 and 7.1 software updates. Thus, there was 100% complete agreement between the 6.1 and 7.1 software updates and the reference methods for all staphylococci tested.

DISCUSSION

This study was performed in order to compare the accuracy of detection of oxacillin-resistant staphylococci as defined by microdilution, the agar screen, *mec* gene analysis and population analyses with the Vitek GPS-SA card and the standard (10^7 CFU) (7) and high- (10^9 CFU) (2) inoculum disk diffusion tests. Control strains expressing various levels of heterogeneous resistance required oxacillin MICs of ≥4 μg/ml by both the microdilution and GPS-SA methods. The control oxacillin-susceptible strains were fully susceptible by both MIC methods, and they exhibited zone diameters in the susceptible range by both the standard and high-inoculum disk diffusion tests. All control strains exhibiting low-level and class 1 or 2 heterogeneous resistance yielded zone diameters in the resistant category by the high-inoculum disk diffusion test; however, by the standard disk test two expression class 2 strains were susceptible and another was intermediate. Two of these strains exhibited resistance by the high-inoculum test. Among clinical isolates of *S. aureus*, 15% were falsely susceptible to oxacillin by the standard disk test. Only 6% of the clinical isolates of *S. epidermidis* were falsely susceptible by the standard disk test.

De Lencastre et al. (2) described an 18 to 20% discrepancy between the results of the standard disk diffusion test and those of a *mec*-specific DNA probe and suggested that the use of a high-inoculum disk test would more accurately detect heteroresistant strains, particularly those belonging to expression class 1 or 2. Our results with the high-inoculum disk test showed that 6% of oxacillin-resistant clinical isolates of *S. aureus* remained falsely susceptible after the first 24 h of incubation, and an additional 24 h of incubation at 22°C was required for all isolates of *S. aureus* to become resistant. None of the isolates of *S. epidermidis* was falsely susceptible by the high-inoculum disk test. All of the isolates yielding falsely susceptible results belonged to heteroresistant expression class 1 or 2.

Several studies have been performed in order to examine the accuracy of the Vitek GPS-SA card (3, 5, 6, 10) with various results. In the most recent of these studies, Skulnick et al. (10) compared results obtained by reference methods, including the broth microdilution test and the *mec* gene probe, with those obtained by the Vitek and MicroScan systems and found 14.2 and 6.7% very major errors, respectively. It was not clear, however, what GPS-SA software program they used in their study. Our reference methodology was comparable to theirs, but we used recent software upgrades (6.1 and 7.1), which might have accounted for the difference in results. A more recent upgrade (8.1) did not alter the parameters for defining oxacillin resistance and therefore has not altered our observations. Although the number of oxacillin-susceptible strains that were subjected to *mec* gene testing and population analysis in addition to microdilution and Vitek susceptibility testing was small, the problem with older software programs in the Vitek system has always been one of accurate detection of oxacillin resistance and not one of false resistance to oxacillin. More problematic perhaps with any currently available susceptibility testing system, however, is the presence of isolates of staphylococci with either decreased susceptibilities or low-level resistance to oxacillin on the basis of either excessive β-lactamase production or decreased affinity of oxacillin for normal penicillin-binding proteins. Only two such strains (ATCC 43387 and CDC-6; Table 1) were included in our study.

In conclusion, we have confirmed the earlier observations of de Lencastre et al. (2) regarding the frequency of false susceptibility of heteroresistant isolates of staphylococci, particularly among those with expression class 1 or 2 resistance, when tested by the standard disk diffusion test. Most, but not all, of these false-susceptible results were corrected by the high-inoculum test; however, additional incubation at 22°C was required in order to minimize very major errors. We did not experience any difficulty with the current Vitek GPS-SA card.

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