

Evaluation of New Vitek 2 Card and Disk Diffusion Method for Determining Susceptibility of *Staphylococcus aureus* to Oxacillin[∇]

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Detection of methicillin resistance in *Staphylococcus aureus* is a challenge, especially low-level resistance, which is often misdiagnosed. The aim of this study was to compare the diagnostic accuracies of the automated Vitek 2 system and disk diffusion tests, using cefoxitin and moxalactam, for the detection of methicillin resistance in *S. aureus* strains. Four sets of genotypically diverse isolates were selected from a national reference collection, including *mecA*-negative *S. aureus* isolates ($n = 56$), hospital-acquired ($n = 88$) and community-acquired ($n = 40$) *S. aureus* isolates, and heterogeneous methicillin-resistant *S. aureus* isolates ($n = 29$). Oxacillin susceptibility was tested by the Vitek 2 system with the AST P549 card and by disk diffusion methods using 10, 30, and 60 μg cefoxitin and 30 μg moxalactam. Oxacillin resistance was confirmed by PCR for the *mecA* gene. The overall sensitivities for oxacillin resistance detection were 97.5% for the Vitek 2 automated system, 98.7% for 60- μg cefoxitin and moxalactam disk diffusion, and 99.6% for 10- and 30- μg cefoxitin disks, respectively. Methicillin-susceptible *S. aureus* isolates were correctly reported as susceptible by all methods. The median times for methicillin testing were 7 h for the Vitek 2 system versus 24 h for disk diffusion methods. In conclusion, the cefoxitin and moxalactam disk diffusion methods and the Vitek 2 automated system are highly accurate methods for methicillin resistance detection, including a range of representative Belgian methicillin-resistant *S. aureus* strains and unusual strains exhibiting cryptic or low-level oxacillin resistance.

Staphylococcus aureus causes serious community-acquired (CA) and nosocomial infections and is a major pathogen implicated in various infections, such as bacteremia, pneumonia, skin infections, and soft tissue and osteoarticular infections (11). Another potential issue is the development of methicillin-resistant *S. aureus* (MRSA). The prevalence of MRSA has increased and has become a worldwide problem inside and outside the hospital. Furthermore, MRSA strains cause further therapeutic problems because of their ability to develop resistance to other classes of antibiotics, including glycopeptides (6).

In MRSA strains, the *mecA* gene encodes the low-affinity penicillin-binding protein PBP2a, which confers resistance to all beta-lactams. MRSA strains differ in their levels of expression of methicillin resistance, which allows their categorization into four phenotypic classes described by Tomasz et al. based on population analysis of growth at increasing methicillin MICs (20). Strains belonging to classes 1 to 3 are heterogeneously resistant to methicillin, showing subpopulation MICs ranging from 1.5 to 200 mg/liter, respectively. Strains belonging to class 4 are homogeneous and composed of uniformly and highly resistant strains (MIC \geq 800 mg/liter) (20).

Previously, phenotypic tests used oxacillin to identify methicillin resistance in *S. aureus*. Unfortunately, oxacillin susceptibility tests often failed to detect low-level-heterogeneous MRSA populations. Cefoxitin, a cephamycin antibiotic that is a strong inducer of the *mecA* regulatory system, was shown to

be superior to oxacillin for detection of methicillin resistance, especially in low-level-resistant *S. aureus* strains. For this reason, the method has been recommended by the CLSI (Clinical and Laboratory Standards Institute) since 2005 (9, 14–19).

Automated antimicrobial susceptibility testing systems are widely used in clinical laboratories and provide results with shorter incubation times than disk diffusion testing (8).

Recently, a new antimicrobial susceptibility testing card, AST-P549 (bioMérieux, Marcy l'Etoile, France), has been developed for staphylococcus testing by Vitek 2. In this card, the oxacillin screen has been replaced by a cefoxitin screen. The objectives of this study were to evaluate the performance of the new Vitek 2 AST-P549 card and the disk diffusion methods using cefoxitin Neo-Sensitabs (10 μg , 30 μg , and 60 μg) and paper disks of cefoxitin (30 μg) and moxalactam (30 μg) to detect methicillin resistance using a well-defined collection of MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates.

MATERIALS AND METHODS

Bacterial collection. *S. aureus* strains were selected from a national reference collection composed of systematic survey samples and atypical strains, including a borderline beta-lactam resistance phenotype.

All strains were previously categorized by triplex PCR for 16S rRNA, *mecA*, and *nuc* genes (12) and genotyped by SmaI macrorestriction analysis of genomic DNA resolved by pulsed-field gel electrophoresis (PFGE). Genotypes were classified as previously described into (i) a PFGE group, designated by a capital letter, that included patterns showing a \leq 6-DNA-fragment difference, equivalent to \geq 65% similarity, and (ii) a PFGE type, designated by the group letter followed by an Arabic numeral suffix, that included PFGE patterns showing a \leq 3-DNA-fragment difference, equivalent to \geq 80% similarity (4).

The MIC for oxacillin was determined by the agar dilution method using Mueller-Hinton II agar (Becton Dickinson, Heidelberg, Germany) according to CLSI recommendations (2).

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TABLE 1. Distribution of cefoxitin and moxalactam inhibition zone diameters by disk diffusion testing of 213 isolates of *S. aureus* by resistance genotype (*mecA* PCR results)

Cefoxitin disk												Moxalactam 30- μ g disk (Oxoid)		
10 μ g (Rosco)			30 μ g (Rosco)			60 μ g (Rosco)			30 μ g (Oxoid)			Inhibition zone diam (mm)	No. of results <i>mecA</i> positive	No. of results <i>mecA</i> negative
Inhibition zone diam (mm)	No. of results <i>mecA</i> positive	No. of results <i>mecA</i> negative	Inhibition zone diam (mm)	No. of results <i>mecA</i> positive	No. of results <i>mecA</i> negative	Inhibition zone diam (mm)	No. of results <i>mecA</i> positive	No. of results <i>mecA</i> negative	Inhibition zone diam (mm)	No. of results <i>mecA</i> positive	No. of results <i>mecA</i> negative			
≤ 11	151		≤ 14	148		≤ 19	124		≤ 15	146		≤ 13	102	
12	2		15	2		20	9		16	5		14	18	
13			16	2		21	14		17	3		15	12	
14	2		17	1		22	7		18			16	15	
15			18	2		23			19	1		17	2	
16^a	1		19	1		24	1		20			18	2	
17		4	20			25			21			19	2	
18	1 ^b	3	21			26			22	1 ^b		20	1	
19		6	22			27			23			21	1	
20		8	23		1	28	1 ^b		24			22		
21		15	24		1	29			25		1	23		
≥ 22		20	≥ 25	1 ^b	54	≥ 30	1 ^b	56	≥ 26	1 ^b	55	≥ 24	2 ^b	56

^a Susceptible and resistant breakpoints fall between the boldface diameters.

^b Cryptic MRSA strain (MIC = 0.5 mg/liter).

Four sets of *S. aureus* strains ($n = 213$) were included in this study.

(i) **Belgian health care-associated (HA) MRSA strains.** The HA-MRSA strains ($n = 88$) were selected to represent the major epidemic genotypes found in a national MRSA survey conducted in 2003 in Belgian hospitals. They included the genotypes (number of isolates) PFGE type B2-sequence type (ST) 45-staphylococcal chromosomal cassette *mec* (45-SCC*mec*) type IV ($n = 11$), A20-ST8-SCC*mec* type IV ($n = 12$), A21-ST8-SCC*mec* type IV ($n = 7$), G10-ST5-SCC*mec* type II ($n = 7$), A1-ST247-SCC*mec* type I ($n = 8$), C3-ST5-SCC*mec* type IV ($n = 5$), D8-ST228-SCC*mec* type I ($n = 2$), and L1-ST22-SCC*mec* type IV ($n = 7$), as well as sporadic PFGE types ($n = 27$) and the reference strains *S. aureus* ATCC 33592 and *S. aureus* ATCC 43300 (10). For HA-MRSA strains, the oxacillin MIC ranged from 8 to >256 mg/liter, with a median of 64 mg/liter.

(ii) **Low-level-resistant MRSA strains ($n = 29$).** Low-level-resistant MRSA strains were referred to the reference laboratory between 1995 and 2005 to confirm oxacillin resistance. They were all *mecA* positive and exhibited low-level MICs to oxacillin ranging from 0.5 to 16 mg/liter, with a median of 8 mg/liter, by the agar dilution method (4, 5, 7). Of the last strains, five *mecA*-positive isolates were phenotypically categorized as oxacillin susceptible by CLSI breakpoints, with oxacillin MICs of ≤ 2 mg/liter and were therefore defined as "cryptic MRSA strains."

(iii) **Panton-Valentine leucocidin-positive CA-MRSA strains ($n = 40$).** The Panton-Valentine leucocidin-positive MRSA strains were collected in Belgium from 2002 to 2005 (3). They belonged by molecular typing to PFGE type X-ST80-SCC*mec* IV ($n = 26$), PFGE type A23-ST8-SCC*mec* IV ($n = 3$), and PFGE type J-ST30-SCC*mec* IV ($n = 5$) and to other sporadic genotypes ($n = 6$). For CA-MRSA, the oxacillin MICs ranged from 4 to >256 mg/liter, with a median of 16 mg/liter.

(iv) **Nonduplicate MSSA isolates ($n = 56$).** Nonduplicate MSSA isolates were collected from patients admitted to Belgian hospitals during the national survey conducted in 2003 (10). The oxacillin MICs for these *mecA*-negative strains ranged between 0.25 and 1 mg/liter, with a median of 0.5 mg/liter.

Susceptibility testing. All strains stored at -80°C were subcultured on two consecutive days on Columbia blood agar before blind testing.

A bacterial suspension equivalent to a 0.5 McFarland standard was prepared with isolated colonies after 18 to 24 h of incubation on a Columbia blood plate. Vitek 2 AST-P549 cards (bioMérieux, Marcy l'Etoile, France) were inoculated according to the manufacturer's instructions. In parallel, Mueller-Hinton II agar plates (Becton-Dickinson, Heidelberg, Germany) were streaked with the same bacterial suspension, and the following disks were applied on the surface: 10- μ g, 30- μ g, and 60- μ g cefoxitin tablets (CFOX-10, CFOX-30, and CFOX-60) (Neo-Sensitabs; Rosco, Taastrup, Denmark); 30 μ g cefoxitin (FOX-30); and 30 μ g moxalactam (MOX-30) (Oxoid, Basingstoke, United Kingdom). The agar plates were incubated for 18 to 24 h in ambient air at 35°C . The reference strains *S. aureus* ATCC 29213 and ATCC 25922 (oxacillin susceptible) and ATCC 43300 and ATCC 33592 (oxacillin resistant) were included in each run.

Interpretation criteria. The inhibition zone diameters were measured with a ruler after 24 h of incubation. Crude and appraised (Advanced Expert System [AES]) susceptibility results with the AST-P549 Vitek card were recorded.

According to the CLSI guidelines, the following zone diameter interpretative criteria were used: CFOX-60, resistant (R), ≤ 24 mm, and susceptible (S), ≥ 25 mm; CFOX-30 and FOX-30, R, ≤ 19 mm, and S, ≥ 20 mm; MOX-30, R, ≤ 14 mm, and S, ≥ 23 mm. For CFOX-10, we used the interpretative criteria proposed by Skov et al.: R, ≤ 16 mm, and S, ≥ 17 mm (16).

In case of discrepancy, the disk diffusion method and Vitek 2 susceptibility testing were repeated. The gold standard test used for categorization as MRSA was detection of the *mecA* gene by PCR (12).

Statistical method. Statistical differences between methods were analyzed by McNemar's test for paired samples, with a P value of <0.05 considered significant.

RESULTS

Disk diffusion tests. MRSA and MSSA isolates showed distinct distributions of inhibition zone diameters with all disks included in the study (Table 1). Only a few MRSA and MSSA isolates showed inhibition zone diameters near the cutoff (Table 1). All MSSA isolates were correctly categorized irrespective of antimicrobial and disk contents, thereby demonstrating 100% specificity of the disk diffusion assays. Only two "cryptic MRSA" strains (MIC = 0.5 mg/liter) were incorrectly classified as susceptible by some disk diffusion tests (a very major error) (Table 1). One "cryptic MRSA" strain was classified as oxacillin susceptible by all disks, whereas another "cryptic MRSA" strain was not detected with CFOX-60, FOX-30, and MOX-30 but was correctly categorized as MRSA by CFOX-10 and CFOX-30. Thus, the sensitivity of the disk diffusion method ranged from 98.7% for CFOX-60, FOX-30, and MOX-30 to 99.6% for CFOX-10 and CFOX-30, respectively ($P > 0.05$) (Table 2).

Vitek 2 automated system. All MSSA isolates were cefoxitin screen negative and showed oxacillin MICs of ≤ 1 mg/liter with the Vitek 2 system. Four (2.5%) MRSA strains were misclassified as oxacillin susceptible. These strains had both a negative cefoxitin screen and Vitek-based oxacillin MICs of ≤ 2 mg/liter.

TABLE 2. Sensitivities and specificities of disk diffusion and Vitek 2 susceptibility testing methods for detection of methicillin resistance in *S. aureus* by category of phenotypic resistance expression

Method	Sensitivity (%)			Specificity (%) for MSSA (n = 56)
	All MRSA (n = 157)	Low-level MRSA (n = 29)	High-level MRSA (n = 128)	
Vitek 2 AST-P549	97.5	89.65	99.2	100
Disk diffusion				
Cefoxitin				
10 µg (Rosco)	99.6	96.4	100	100
30 µg (Rosco)	99.6	96.4	100	100
60 µg (Rosco)	98.7	92.9	100	100
30 µg (Oxoid)	98.7	92.9	100	100
Moxalactam 30 µg (Oxoid)	98.7	92.9	100	100

The same results were obtained after repeat testing. These strains were two cryptic MRSA strains with MICs of 0.5 and 1 mg/liter, one low-level MRSA strain with a MIC of 4 mg/liter, and one CA-MRSA strain with a MIC of 32 mg/liter by the agar dilution method. Five other MRSA strains were accurately categorized by the AES, including four strains showing Vitek-based oxacillin MICs of ≤ 2 mg/liter but a positive cefoxitin screen test and one strain (a sporadic clone) showing a negative cefoxitin screen test but an oxacillin MIC of ≥ 4 mg/liter. These strains were three cryptic or low-level MRSA strains with MICs of 0.5, 2, and 8 mg/liter; one CA-MRSA strain with a MIC of 8 mg/liter; and one MRSA sporadic clone with a MIC of 128 mg/liter by the agar dilution method. The overall sensitivities and specificities of the Vitek 2 system were 97.5% and 100%, respectively (Table 2). The cefoxitin screen showed higher sensitivity (96.8%) than the oxacillin MIC test (94.9%) ($P > 0.05$). The diagnostic performance for oxacillin resistance detection showed a nonsignificant difference from the disk diffusion method ($P > 0.05$).

The median time to final susceptibility reporting based on the Vitek 2 system was 7 h (range, 6 h 15 min to 12 h 30 min) and showed no difference between MSSA strains (median time, 6 h 45 min) and MRSA strains (median time, 7 h).

DISCUSSION

The gold standard method for determination of methicillin resistance in *S. aureus* is *mecA* detection by PCR assay (1, 12). Detection of PBP2a by latex agglutination is a valid alternative method. However, PCR testing requires a trained staff and special equipment, and the latex test is relatively costly, thereby limiting the use of these assays in routine testing by clinical microbiology laboratories. Previously recommended phenotypic MRSA detection methods based on oxacillin disk diffusion or oxacillin screen agar testing were found to fail to detect MRSA isolates with very heterogeneous subpopulations (1, 9). Because cefoxitin is a strong inducer of the *mecA* gene that appears to be less affected than oxacillin by penicillinase-hyperproducing isolates, it is more reliable for MRSA detection and is currently recommended in the CLSI guidelines (9, 18, 19).

In our evaluation, the different assays, antimicrobials, and

concentrations tested showed accurate and comparable performances for categorization of MRSA and MSSA isolates. MRSA strains belonging to endemic CA and hospital-acquired genotypes were accurately detected by both the disk diffusion methods and the Vitek 2 automated system. MRSA strains misclassified as susceptible were restricted to atypical strains that contained the *mecA* gene but that were categorized as oxacillin susceptible by the reference MIC level ("cryptic MRSA" strains) of 0.5 mg/liter. Interestingly, CFOX-10 and CFOX-30 disks were able to correctly identify one of these challenge strains. We also confirmed the results obtained by Skov et al. concerning the excellent sensitivity of the 10-µg cefoxitin disk (17). With these low-content disks (10 and 30 µg), the interpretative zone diameters for S (≥ 17 mm) and R (≤ 16 mm) better discriminate between MRSA and MSSA isolates than with higher-content disks. The smaller inhibition zones observed with both concentrations also interfered less with the inhibition zones of other antimicrobials. As reported by Felten et al., the moxalactam disk test also performed well for routine detection of MRSA, showing a sensitivity of 98.7% and a specificity of 100% with our test collection (9).

The Vitek 2 system combines an oxacillin screen and MICs to detect methicillin resistance in staphylococci. A previous study of a first-generation test card conducted in our laboratory showed a sensitivity of 87.8% for all MRSA strains and a sensitivity of 73.1% for the heteroresistant strains (13). The new card tested in the present study, which included a cefoxitin screen (6 µg/liter) in addition to oxacillin MIC testing, performed better, with a sensitivity and a specificity of 97.5% and 100%, respectively. Comparable results were found by Swenson et al. using the Phoenix automated system (18). The Vitek 2 AES failed to detect one CA-MRSA isolate and three low-level-resistant strains. Amplification of the *mecA* gene and detection of PBP2a were the only reliable techniques to detect MRSA isolates with oxacillin MICs of 0.5 mg/liter. Five other MRSA strains were accurately categorized by the AES but showed discrepant results between cefoxitin screening and oxacillin MIC values. One MRSA (a sporadic clone) was cefoxitin screen negative with an oxacillin MIC of ≤ 4 mg/liter, whereas four other isolates were only cefoxitin screen positive and oxacillin susceptible (≤ 2 mg/liter). One of these MRSA isolates was a CA-MRSA isolate, and the other three were low-level MRSA isolates. The cefoxitin screen was marginally more sensitive (96.9%) than the Vitek 2 oxacillin MIC (94.9%) for detection of methicillin resistance in *S. aureus* isolates, and the combination of the cefoxitin screen and oxacillin testing results increased the sensitivity to 97.5% for the new card.

Vitek 2 results showed no difference in time to response between MSSA strains and MRSA strains. The Vitek 2 system allowed a response within working hours (≤ 8 h) for 88.7% of *S. aureus* isolates, whereas the disk diffusion method required 24 h of incubation, as recommended by the CLSI (2).

In conclusion, as described previously, this study confirmed the reliability and the robustness of cefoxitin and moxalactam disk testing for detection of MRSA, including atypical strains showing cryptic or low-level-heterogeneous resistance. Its use also enhances the performance of MRSA detection in automated systems, such as Vitek 2 (16).

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