

Evaluation of Different Methods To Detect Methicillin Resistance in Small-Colony Variants of *Staphylococcus aureus*

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To evaluate different methods for their abilities to detect methicillin resistance in small-colony variants (SCVs) of *Staphylococcus aureus*, 11 different methicillin-resistant *S. aureus* (MRSA) clones with the SCV phenotype were used in this study. The slow growth of SCVs often makes testing by disk diffusion or by automated methods invalid. Only detection of the *mecA* gene by PCR and the MRSA-Screen latex agglutination test using a higher colony number were shown to be reliable methods to rapidly detect methicillin resistance in these variants.

Considering the increasing incidence of infections due to methicillin-resistant *Staphylococcus aureus* (MRSA), reliable, accurate, and rapid testing for methicillin resistance is essential for both antibiotic therapy and infection control regimens (1, 12). However, staphylococci may have mechanisms for resisting therapy that extend beyond the classical mechanisms. The discovery and characterization of *S. aureus* small-colony variants (SCVs) have provided new insight into the understanding of the pathogenesis associated with staphylococcal diseases (14, 15). These variants are increasingly reported for antibiotic-refractory, recurrent, and/or persistent infections (7, 8, 13, 16, 18, 23, 24). SCVs gain a survival advantage from their ability to hide within host cells, which protects this naturally occurring subpopulation of *S. aureus* from host defenses and decreases exposure to antibiotics (7, 15, 22, 23, 25). The generation time for *S. aureus* SCVs is six- to ninefold longer than that for metabolically normal *S. aureus*, resulting in tiny colonies that are frequently not visible before 48 to 72 h of incubation. Consequently, correct identification and susceptibility testing for clinical laboratories become complicated (15, 18, 23), which may result in diagnostic underestimation and therapeutic failures (13, 14, 16, 17, 23, 24). A particular problem for therapy as well as for diagnosis is the combination of the SCV phenotype and methicillin resistance. Within a period of 6 years, we collected 11 different MRSA clones with the SCV phenotype in our laboratory (approximately 0.5 to 2% of the MRSA strains collected at our institution show the SCV phenotype).

The aims of this study were (i) to evaluate the influence of SCV phenotype on the detection of methicillin resistance and (ii) to compare different methods for their abilities to detect methicillin resistance in *S. aureus* SCVs by using a well-defined strain collection of methicillin-resistant SCVs and their clonal identical parent strains.

S. aureus isolates were identified and characterized as SCVs on the basis of the following: pinpoint colonies (incubation on Columbia agar, brain heart infusion plus 5% NaCl, and Schae-

pler agar for 48 to 72 h), decreased pigment formation, reduced hemolytic activity, and low coagulase activity. Very small colonies on Columbia agar were suspected as SCVs if they grew nearly normally on Schaedler agar (13). Identification as *S. aureus* was based on conventional criteria, including the coagulase tube test and the Api-Staph system (ATB32 Staph; bioMérieux, Marcy-l'Étoile, France). In addition, all strains with the SCV phenotype were confirmed as *S. aureus* by testing the *S. aureus*-specific *nuc* gene (2). To determine the clonal relationship of the strain pairs (SCV and normal phenotype isolated from a single patient) and to compare strains between different patients, *Sma*I digests of total bacterial DNA were resolved by pulsed-field gel electrophoresis as described previously (7, 24).

In total, 11 different clones of MRSA SCVs collected from 10 patients with persistent and recurrent infections were included in this study. In addition, eight isolates with the normal phenotype (clonally identical to the corresponding SCVs), which were recovered in the same (or subsequent) clinical specimens as were the SCVs, were also included in the study. From two patients, no strain with a normal phenotype was isolated. From one patient, two different clonal SCVs were isolated, and therefore both strains were included (strains 6a and 6b). Auxotrophy of SCVs for hemin, menadione, and thymidine was tested as described previously (7, 23).

The following susceptibility testing methods were evaluated for their abilities to detect methicillin resistance in SCVs in comparison with normal-phenotype strains: disk diffusion test on Mueller-Hinton agar, supplemented with 2% NaCl (30°C) with 5- μ g oxacillin disks; Etest (AB Biodisk) on Mueller-Hinton agar, supplemented with 2% NaCl (30°C); microdilution method with Mueller-Hinton medium, supplemented with 2% NaCl (30°C); MRSA-Screen latex agglutination test (Denka Seiken Co. Ltd., Tokyo, Japan); and determination of MICs by Vitek 2 (bioMérieux). Determination of the presence of the *mecA* gene was performed as described elsewhere (11).

Apart from prolonged incubation periods (up to 72 h) and an increased number of colonies required for the MRSA-Screen latex agglutination test, the techniques were applied according to the instructions of the manufacturers and the recommendations of the Deutsches Institut für Normung (5).

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TABLE 1. Comparison of different methods for detection of methicillin resistance in SCVs

Strain	Auxotrophy ^a	Agglutination of PBP 2a ^b		Vitek 2 MIC (μg/ml) ^c	Disk diffusion ^{d,e}		Etest ^e (μg/ml)		Microdilution (μg/ml)	<i>mecA</i> PCR result ^b
		1–2 colonies	100–200 colonies ^f		24 h	48 h	24 h	48 h		
1	M	–	+	MC	NG	NG	NG	NG	>32	+
2	H	+	+	>8	NG	R	NG	>256	>32	+
3	H	–	+	>8	R	R	>256	>256	>32	+
4	H	+	+	>8	S	S	0.064	0.064	0.25	+
5	H	–	+	>8	R	R	>256	>256	>32	+
6a	H	+	+	<0.5	S	S	0.064	0.064	0.5	+
6b	M, H, T	–	+	>8	S	S	2	3	>32	+
7	H	–	+	MC	NG	NG	NG	NG	>32	+
8	H	+	+	>8	NG	R	NG	>256	>32	+
9	M, T	–	+	MC	NG	NG	NG	NG	>32	+
10	H	–	+	MC	NG	NG	NG	NG	>32	+

^a H, hemin; M, menadione; T, thymidine.

^b +, positive; –, negative.

^c MC, measurement canceled.

^d R, resistant; S, susceptible.

^e NG, no growth.

^f Corresponding to a large loopful.

The results for the detection of methicillin resistance in SCVs are summarized in Table 1. While the number of isolates tested is limited, it should be stressed that 11 different clonal lineages of methicillin-resistant strains with the SCV phenotype were included in this study. A correlation between type of auxotrophy and detection of methicillin resistance in SCVs was not observed.

In the testing of our collection of *S. aureus* SCVs, the disk diffusion test detected only two strains as methicillin resistant following 24 h of incubation. After 48 h, another two strains were determined to be methicillin resistant. Prolonged incubation times up to 72 h did not reveal additional methicillin-resistant strains. Three strains appeared to be susceptible after 48 h of incubation, with zone diameters of ≥ 16 mm (5). Four strains did not show any growth following 72 h of incubation. Testing the SCVs for oxacillin resistance by Etest showed results similar to those of the disk diffusion test. Both the disk diffusion test and Etest failed to detect resistance in the same strains (Table 1). Only one further MRSA strain was detected after 24 h of incubation. Determination of MICs by Vitek 2 indicated six MRSA SCVs. One strain was misidentified as methicillin susceptible, with the MIC being 0.5 μg/ml, and in four strains (the same strains which did not show any growth in the disk diffusion test and Etest) measurement was canceled by the Vitek 2 system.

By use of the microdilution test, nine strains were found to be methicillin resistant, including all of the four strains that were misidentified as methicillin susceptible due to a lack of growth in the previously described susceptibility tests. Using an inoculum of one or two colonies according to the manufacturer's recommendations, only four MRSA SCVs were detected in the MRSA-Screen latex agglutination test. If the number of colonies used for this test was increased drastically (approximately a loopful with 100 to 200 SCV colonies, corresponding to approximately one or two colonies with normal phenotype), all SCVs were identified as methicillin resistant.

All *S. aureus* strains with the normal phenotype were identified as methicillin resistant by all test methods used. Only by

PCR targeting *mecA* were all strains tested—irrespective of their phenotype—confirmed as methicillin resistant.

The SCV phenotype has a significant impact on the sensitivity of the conventional methods used for the detection of methicillin resistance in staphylococci. Tests including the disk diffusion test, Etest, and microdilution failed to detect the methicillin resistance phenotype in SCVs accurately, except for the recently introduced MRSA-Screen latex agglutination test. Most evaluations of this latex agglutination test report a sensitivity for detection of resistant strains of $\geq 97\%$, but none of them included *S. aureus* SCVs (3, 4, 6, 9, 10, 19–21). Only 4 of 11 tested strains were correctly identified as MRSA when we used the MRSA-Screen latex agglutination test to identify PBP 2a (*mecA* product) according to the manufacturer's instructions. With the use of a drastically increased number of colonies, the latex agglutination test showed 100% correlation with *mecA* PCR. In view of increasing infections due to MRSA and to *S. aureus* SCVs, it seems advisable—following recovery of *S. aureus* SCVs from clinical specimens—to use susceptibility testing methods which are also able to detect methicillin resistance in SCVs (13, 17, 23). Furthermore, the evaluation of new susceptibility testing methods for *S. aureus* should include variant phenotypes and morphotypes.

In summary, results of susceptibility testing by the use of disk diffusion or automated methods are often invalid, since the colonies may be too small to be seen on agar or to be detected by optical density measurements in automated systems (15). Notably, a rejection by the Vitek 2 system should alert the clinical microbiologist to search for SCVs. Compared with conventional testing methods, only detection of the *mecA* gene by PCR and the MRSA-Screen latex agglutination test is able rapidly and reliably to detect methicillin resistance in *S. aureus* SCVs. With the use of drastically increased numbers of colonies, identification of methicillin resistance in *S. aureus* SCVs by applying the MRSA-Screen test is a reliable alternative for clinical laboratories where PCR or DNA hybridization methods for the *mecA* gene are not readily available.

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