Accuracy of phenotypic methicillin susceptibility methods in the detection of *Staphylococcus aureus* isolates carrying different SCC*mec* types

Pricilla DM de Matos, Ricardo P Schuenck, Fernanda S Cavalcante, Roberta MF Caboclo, Kátia Regina N dos Santos/+

Departamento de Microbiologia Médica, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

A total of 138 isolates, 118 methicillin-resistant Staphylococcus aureus *(MRSA) isolates (staphylococcal cassette chromosome type II, 20 isolates, type III, 39 isolates and type IV, 59 isolates) and 20 methicillin-sensitive* S. aureus *isolates were evaluated by phenotypic methods: cefoxitin and oxacillin disk diffusion (DD), agar dilution (AD), latex agglutination (LA), oxacillin agar screening (OAS) and chromogenic agar detection. All methods showed 100% specificity, but only the DD tests presented 100% sensitivity. The sensitivity of the other tests ranged from 82.2% (OAS)-98.3% (AD). The LA test showed the second lowest sensitivity (86.4%). The DD test showed high accuracy in the detection of MRSA isolates, but there was low precision in the detection of type IV isolates by the other tests, indicating that the genotypic characteristics of the isolates should be considered.*

Key words: *Staphylococcus aureus* - SCC*mec* types - phenotypic methods

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major epidemiological and clinical problem over the several last decades (Aires de Sousa & de Lencastre 2004). Methicillin resistance is encoded by the *mecA* gene that is located in a mobile genetic element called the staphylococcal cassette chromosome (SCC*mec*) (Katayama et al. 2000). The analysis of SCC*mec* has revealed eight different allotypes, which are designated types I-VIII. Worldwide, most hospital-derived isolates belong to types I, II and III, most community-derived isolates belong to type IV (Chambers & Deleo 2009). In Brazil, only isolates belonging to types II, III and IV have been found in hospitals (Reinert et al. 2008, Schuenck et al. 2009, Silva-Carvalho et al. 2009).

The reliable detection of methicillin resistance in clinical *S. aureus* isolates is essential for the appropriate treatment and epidemiological control of MRSA infections. In general, conventional phenotypic methods have shown high sensitivity and specificity in the detection of MRSA isolates (Velasco et al. 2005, Baddour et al. 2007). However, the emergence of *S. aureus* isolates carrying the SCC*mec* type IV allotype, which is associated with a minimum inhibitory concentration (MIC) for methicillin near the breakpoint (Schuenck et al. 2009), could influence the detection of this resistance. Here, we present the first study to evaluate the accuracy of different phenotypic methods in the detection of MRSA strains carrying different SCC*mec* types, using isolates from hospitals in Rio de Janeiro.

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+ Corresponding author: santoskrn@micro.ufrj.br
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One hundred and thirty-eight isolates of *S. aureus*, including 118 MRSA and 20 methicillin-sensitive *S. aureus* (MSSA) isolates, were obtained from different clinical specimens (nostrils, n = 33; blood, n = 31; respiratory specimen, n = 30; other sites, n = 44) from patients in five hospitals in Rio de Janeiro between August 1999-July 2008. The isolates were identified according to Bannerman and Peacock (2007). The *mec*A gene and the SCC*mec* types were detected according to Ferreira et al. (2003) and to Oliveira and de Lencastre (2002), respectively.

The susceptibilities to cefoxitin (30 µg) and oxacillin (1 µg) (Oxoid, Basingstoke, England) were determined by the disk diffusion (DD) method on Mueller-Hinton agar (Difco Laboratories, Detroit, USA) according to the Clinical and Laboratory Standards Institute National Committee for Clinical Laboratory Standards (CLSI/NCCLS) protocol (CLSI/NCCLS 2009a, c). *S. aureus* ATCC 25923 was used as the control strain.

The MIC of oxacillin (Sigma Chemical Company, St. Louis, MO, USA) was determined by the agar dilution (AD) method (CLSI/NCCLS 2009b, c). Briefly, bacterial suspensions were adjusted to a 0.5 McFarland standard, diluted 1:10 and inoculated (10^4 colony-forming unit) on Mueller-Hinton agar (Difco) plates with 2% (wt/vol) NaCl and varying oxacillin concentrations (0.5-256 µg/mL). The plates were incubated at 35ºC for 24 h. *S. aureus* ATCC 29213 was used as the control strain.

All isolates were plated using a 0.5 McFarland bacterial suspension on Mueller-Hinton agar (Difco) with 4% (wt/vol) NaCl and oxacillin (Sigma) at either 4 or 6 µg/mL (CLSI/NCCLS 2009b). Oxacillin resistance was confirmed by the presence of bacterial growth after 24 h and 48 h of incubation at 35ºC (CLSI/NCCLS 2009c). *S. aureus* ATCC 29213 (susceptible) and *S. aureus* ATCC 33591 (resistant) were included as control strains.

Isolates were grown on chromogenic agar that is selective for MRSA, known as CHROMagar (Microbiol-

ogy, Paris, France) (Diederen et al. 2005). The composition of this medium is proprietary. A 0.5 McFarland bacterial suspension was used to inoculate the chromogenic medium using a cotton swab and the tests were read at two time points: after 24 h and 48 h of incubation at 35ºC. The growth of pink/purple-clear colonies was indicative of the presence of MRSA isolates. *S. aureus* ATCC 29213 (oxacillin susceptible) and *S. aureus* ATCC 33591 (oxacillin resistant) were included as controls.

The latex agglutination (LA) test (Slidex MRSA Detection test; bioMérieux S/A, Paris, France) was performed according to the manufacturer's instructions. A loop full of colonies obtained from blood agar was suspended in 200 µL of extraction reagent nº1 (0.1 M NaOH) and boiled for 3 min. Next, 50 µL of extraction reagent nº2 (0.5 M KH_2PO_4) was added to the suspension and the components were mixed well. This mixture was centrifuged (1,500 *g*/5 min) and 50 µL of the supernatant was placed in each of the two circles of the test slide. Anti-penicillin binding protein 2a (PBP2a) monoclonal antibody-sensitized latex (50 µL) was added to one of the circles and 50 µL of negative control latex was added to the other circle. The presence of agglutination was visually evaluated after 3 min. *S. aureus* ATCC 33591 (positive) and 25923 (negative) were included as controls in the test.

The sensitivities, specificities and positive and negative predictive values of the assays for MRSA strains with different SCC*mec* types and for MSSA isolates are presented in Table I. All the tests showed 100% specificity and positive predictive values, but only the DD test had 100% sensitivity. MRSA isolates with SCC*mec* IV showed discordant results for all other tests that were evaluated. The AD test showed 98.3% sensitivity. Oxacillin MICs ranged from 0.5 µg/mL-> 256 µg/mL for the 118 MRSA isolates evaluated. For type III isolates, the MIC values were ≥ 128 µg/mL, whereas for 80% of type II isolates, the MICs were between 64-128 µg/mL. For type IV strains, 88.1% of the isolates showed MICs between 4-32 µg/mL and two isolates had MICs of 0.5 and 2 µg/mL, values that are lower than the breakpoint values. The oxacillin agar screening (OAS) test with 6 µg/mL of oxacillin showed the lowest sensitivity (82.2%) among all of the tests evaluated; this test was not able to detect 21 MRSA isolates after 24 h of incubation. OAS with 4 µg/mL of oxacillin and CHROMagar™ showed sensitivities of 88.1% and 94%, respectively. When the

TABLE I

Sensitivities, specificities and predictive values for antimicrobial susceptibility methods in comparison with the staphylococcal cassette chromosome (SCC*mec*) types in 118 methicillin-resistant *Staphylococcus aureus* (MRSA) and 20 methicillin-sensitive *S. aureus* (MSSA) isolates evaluated in this study

	Number of isolates											
	MRSA (n = 118)											
	SCC*mec* II (n = 20)		SCC*mec* III (n = 39)		SCC*mec* IV (n = 59)		MSSA (n = 20)					
Methods	P	FN	P	FN	P	FN	N	FN	Sensitivity[a] %	Specificity[b] %	PPV[c] %	NPV[d] %
DD	20	-	39	-	59	-	20	-	100	100	100	100
AD (MIC)	20	-	39	-	57	2	20	-	98.3	100	100	90.9
LA	20	-	39	-	43	16	20	-	86.4	100	100	55.5
OAS												
4 µg/mL												
24 h[e]	20	-	39	-	45	14	20	-	88.1	100	100	58.8
48 h[e]	20	-	39	-	54	5	20	-	95.7	100	100	80
6 µg/mL												
24 h	20	-	39	-	38	21	20	-	82.2	100	100	48.8
48 h	20	-	39	-	52	7	20	-	94	100	100	74
CHROMagar												
24 h	20	-	39	-	52	7	20	-	94	100	100	74
48 h	20	-	39	-	55	4	20	-	96.6	100	100	83.3

a: number of true positive results by the test/number of MRSA isolates; *b*: number of true negative results by the test/number of MSSA isolates; *c*: positive predictive value (PPV) (number of true positive results by the test/total number of positive results by testing); *d*: negative predictive value (NPV) (number of true negative results by the test/total number of negative results by the test); *e*: incubation period; AD: agar dilution; DD: disk diffusion; F: false-negative; LA: latex agglutination; MIC: minimum inhibitory concentration; N: negative; OAS: oxacillin agar screening; P: positive.

TABLE II

Profiles presented by 31 methicillin-resistant *Staphylococcus aureus* isolates with staphylococcal cassette chromosome IV that were discordant by one or more oxacillin susceptibility phenotypic methods

Isolates n	AD (MIC)	LA	OAS (4 µg/mL)[a] Incubation 24 h	OAS (4 µg/mL)[a] Incubation 48 h	OAS (6 µg/mL)[a] Incubation 24 h	OAS (6 µg/mL)[a] Incubation 48 h	CHROMagar Incubation 24 h	CHROMagar Incubation 48 h
9	8-256 (R)	-	+	+	+	+	+	+
5	4-32 (R)	+	+	+	-	+	+	+
3	8-16 (R)	-	-	+	-	+	+	+
3	8 (R)	+	-	+	-	+	+	+
2	8 (R)	+	-	-	-	-	-	-
1	16 (R)	+	-	+	-	+	-	+
1	16 (R)	-	+	+	-	-	+	+
1	8 (R)	-	-	-	-	-	-	-
1	8 (R)	+	-	-	-	-	+	+
1	8 (R)	-	-	+	-	-	+	+
1	8 (R)	-	+	+	-	+	+	+
1	8 (R)	+	+	+	+	+	-	+
1	2 (S)	+	-	+	-	+	-	+
1	0.5 (S)	+	-	-	-	-	-	-

a: Clinical and Laboratory Standards Institute interpretation categories; AD: agar dilution; LA: latex agglutination; MIC: minimum inhibitory concentration in µg/mL; OAS: oxacilin agar screening; R: resistant; S: susceptible; +: resistance result; -: sensibility result.

incubation period was extended to 48 h, the sensitivity values for the 6 µg/mL OAS test, the 4 µg/mL OAS test and the CHROMagar™ test increased to 94%, 95.7% and 96.6%, respectively. When using a 24-h incubation, 35.6%, 23.7% and 11.8% of type IV MRSA isolates showed no growth in the 6 µg/mL OAS test, the 4 µg/mL OAS test and the CHROMagar test, respectively. After 48 h of incubation, the percentages of non-growing type IV isolates were 11.9%, 8.5% and 6.8%, respectively. Non-confluent growth was observed for 10.2%, 13.6% and 6.8% of isolates. Table II shows the results for the 31 (52.5%) type IV MRSA isolates that were discordant with respect to at least one susceptibility test. Nine isolates gave false sensitivity results for the LA test, the second least accurate method for the detection of MRSA isolates used in the present study.

In this study, we evaluated the sensitivity of five phenotypic methods in the detection of MRSA isolates carrying SCC*mec* types II, III and IV allotypes and we verified that all the tests had 100% specificity. Moreover, the DD test using cefoxitin or oxacillin detected all MRSA isolates, showing high accuracy in MRSA detection. Although CLSI/NCCLS has just recommended the cefoxitin disk instead of the oxacillin disk for the detection of MRSA, in this study we verified that both disks were able to identify all 118 isolates, including those carrying the SCC*mec* type IV allotype. This is an interesting finding, as the DD test is the simplest and least expensive assay to detect MRSA isolates. Because of the simplicity and affordability of the DD test, all 118 MRSA isolates used in this study can be easily detected in any standard clinical microbiology laboratory.

Our MIC results correctly classified all isolates with SCC*mec* types II and III as methicillin resistant. Despite the fact that two type IV isolates were found to be sensitive to oxacillin suing the AD test, this test had a high overall sensitivity for the detection of MRSA (98.3%), as has been reported by other authors (Swenson et al. 2005, Velasco et al. 2005). However, neither of these studies investigated the different SCC*mec* types.

The OAS with 6 µg/mL of oxacillin has been recommended by CLSI/NCCLS to confirm ambiguous results obtained by the DD test. It has been shown by other studies that there is a high concordance between the results of OAS and the results of genotypic methods for the detection of MRSA (Felten et al. 2002, Velasco et al. 2005). Nevertheless, in our study, OAS showed the lowest sensitivity (82.2%), with one-third of type IV isolates going undetected. Moreover, a few type IV isolates presented non-confluent growth on this medium, that is, they showed heteroresistance to methicillin. This fact could explain the low sensitivity observed for the other tests used in this study. The lower concentration of oxacillin (4 µg/mL) was also used to determine if using a lower concentration would reduce the percentage of MRSA isolates that were undetected. Even at the lower oxacillin concentration, almost 1/4 of type IV isolates were not detected, giving the test a sensitivity of 88.1%.

CHROMagar™ MRSA is a chromogenic medium used to detect MRSA isolates. The advantage of this medium is that it allows the presumptive identification of bacterial species and their methicillin resistance profiles in a single step. Recent studies have found that this test has a high sensitivity, up to 95.4% (Diederen et al. 2005) or 96%

(Tandé et al. 2008). In our study, CHROMagar™ MRSA performed better (sensitivity of 94%) than the OAS test, with a number of isolated remaining undetected being three times smaller.

The slidex MRSA detection test, which involves checking for the presence of the *mec*A gene product (PBP2a), has been used as a rapid method for the detection of methicillin resistance. In our study, this test presented the second lowest sensitivity (86.4%) as a result of classifying 16 type IV isolates as sensitive. Recently, Akcam et al. (2009) used the latex technique to analyze 60 MRSA isolates and they found a similar sensitivity of 88.3%. When we performed the latex test again with the bacterial growth from the edge of the cefoxitin inhibition halo, these results became positive, showing that the isolates were not expressing the gene products that result in resistance to methicillin. In another study from our group (Ferreira et al. 2003), we verified the influence of resistance induction in this test by analyzing coagulase-negative staphylococci isolates; for these experiments, we observed an increase in sensitivity from 97-99%.

In the present study, we verified that the susceptibility results were influenced by the SCC*mec* IV allotypes. The presence of the type IV allotype, which causes a low level of methicillin resistance, resulted in the most discordant results. According to Memmi et al. (2008), an increased sensitivity to β-lactams in type IV isolates is associated with the loss of PBP4, which is responsible for mediating resistance to these drugs. To conclude, our results demonstrate the high accuracy of the cefoxitin and oxacillin DD tests in the detection of MRSA. Some isolates carrying the SCC*mec* IV allotype could not be detected by other phenotypic methods. Therefore, the interpretations of the results of MRSA studies should be carefully evaluated, taking into consideration the genotypic characteristics of the MRSA isolates analyzed.

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