Identification and Detection of Methicillin Resistance in Non-*Epidermidis* Coagulase-Negative Staphylococci

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The NCCLS (2004) presented a new methodology to detect, by disk-diffusion agar, oxacillin-resistance using a cefoxitin disk. We identified coagulase-negative staphylococci (SCoN) to the species level and compared the use of cefoxitin disks (30 μg) with oxacillin disks (1 μg), agar dilution (minimum inhibitory concentration of oxacillin) and *mecA* gene detection in isolates of coagulase-negative bacteria other than *Staphylococcus epidermidis* (SCoNne). A total of 238 SCoNne was evaluated; oxacillin-resistance (the *mecA* gene) was detected in 71% of the isolates. All methods gave 100% sensitivity, based on presence of the *mecA* gene. The specificity of the cefoxitin disk was 100%, while the oxacillin disk gave a specificity of 91% and agar dilution oxacillin gave a specificity of 88%. We conclude that the cefoxitin disk is an efficient test, and it is an easy method for use in clinical laboratories to detect oxacillin-resistance in staphylococci.

Key-Words: Coagulase-negative *Staphylococcus*, cefoxitin, oxacillin, *mecA* gene, susceptibility diagnostic.

Coagulase-negative staphylococci (SCoN) are common pathogens of the blood stream, being frequently related to nosocomial infections, especially in neonates and immunocompromised patients; transmission usually involves medical devices, such as catheters and prostheses [1-3]. Correct identification of SCoN species has become important in clinical laboratories, since several species have been recognized as potential pathogens, especially in a nosocomial setting [4]. Although *Staphylococcus epidermidis* causes most SCoN infections, many other species have been identified in association with human infections, for example, *Staphylococcus lugdunensis*, associated with native valve endocarditis and *Staphylococcus haemolyticus*, which can be multiresistant, including reduced susceptibility to vancomycin [5-7].

Methicillin-resistant staphylococci are considered important agents of nosocomial infections and have frequently been isolated in hospitals throughout the world, including Brazilian hospitals [8]. Sader et al. reported that 80% of SCoN recovered from blood in Latin America were oxacillin resistant [9]. Susceptibility testing by phenotypic methods can be problematic for the detection of methicillin resistance in SCoN because of heterogeneous expression in many strains, affected by growth conditions and by the nature of the beta-lactam agents that are used [10]. For this reason, *mecA* gene detection by PCR is considered the gold standard for methicillin resistance detection in *Staphylococcus* spp. [11].

To improve accuracy in the detection of resistance, NCCLS 2004 recommended that clinical laboratories should use cefoxitin disk (30 μg) tests for detection of oxacillin resistance in *Staphylococcus* spp. [12]. Several studies have been performed to compare results obtained with cefoxitin and oxacillin disks and how they correlate with the presence of the *mec*A gene in *Staphylococcus* spp. [13-15]. We identified all SCoN to the species level and compared the use of cefoxitin disks (30 μg) with oxacillin disks (1 μg), agar dilution (MIC of oxacillin), and *mecA* gene detection, in isolates of coagulase-negative other than *Staphylococcus epidermidis* (SCoNne).

Material and Methods

Bacterial Isolates

A total of 238 samples of SCoNne were analyzed, from a collection of samples of SCoN of the Gram-positive Cocci Laboratory of the UFCSPA, stored in skim milk (Difco, Detroit) at –20°C. The samples were obtained from blood cultures collected consecutively, between 2002 and 2004, in the Complexo Hospitalar Santa Casa, Porto Alegre, RS, Brasil.

Identification of Isolates

The isolates were cultured in Tryptic Soy agar (Oxoid, Basingstoke, UK), supplemented with 5% sheep blood, for 24h at 35°C; colony morphology, hemolytic activity and purity were evaluated. Subsequently, phenotypic tests were evaluated by the conventional method proposed by Kloss & Bannerman 1994, and modified by Bannerman 2003, which consists of a set of biochemical tests that determine the utilization of coagulase, catalase, alkaline phospatase, ornithine decarboxylase, urease, PYR (pyrrolidinyl-β-naphthylamide hydrolysis), and acid production from carbohydrates (trehalose, mannitol, mannose, sucrose, maltose, lactose, and cellobiose) [16,17]. Anaerobic growth in thioglycolate and susceptibility to novobiocin, polymyxin B, bacitracin, desferrioxamine and fosfomycin using disk diffusion tests were evaluated [18]. Quality control was performed with *S. epidermidis* ATCC 12228. The samples that gave variable results in the phenotypic test

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identifications, or to confirm less frequent species, were run through an automated method of identification (Microscan Walkway; Dade Behringer, Deerfield, IL, USA). The automated results that gave a low percentage certainty of species identification were submitted to determination of the *sodA* gene by PCR amplification and sequencing with specific primers: d^1: 5'CCITAYICITAYGAYYGCIYTIGARCC-3' and d^2: 5'ARRTARTAIGCRTGYTCCCAIACRTC-3' [19].

Disk Diffusion Test (DD)

The suspensions were adjusted to a 0.5 McFarland standard for each sample to perform the disk diffusion method (Kirby-Bauer) on Mueller-Hinton agar plates (Difco, Laboratories, Detroit, Mich), using cefoxitin (30 µg) and oxacillin (1 µg) disks (Oxoid, Basingstoke, UK), according to the criteria recommended by CLSI 2005 [20]. The plates were incubated at 35° C and screened after 24h.

Agar Dilution Test

Determination of the minimum inhibitory concentration (MIC) for oxacillin was performed by bacterial suspension (0.5 McFarland), diluted 1:10 in saline solution and inoculated on Mueller-Hinton agar plates supplemented with 2% NaCl by using Steers replicator. Concentrations of 0.125µg/mL-4 µg/mL of oxacillin (Sigma Chemical Co, St. Louis, USA) were used for determination of the MIC of oxacillin. The plates were incubated at 35° C and screened after 24h.

Detection of *mecA* Gene

Staphylococcal DNA was extracted in guanidine isothiocyanate solution (Invitrogen, Carlsbad, USA) and the *mecA* gene was detected by PCR with specific primers: $mecA^1$: 5' TGGCTATCGTGTCACAATCG, $mecA^2$: 5' CTGGAACTTGTT GAGCAGAG [21]. Subsequently, 2 µL of bacterial DNA was added to the PCR tube, followed by 23 µL mixture containing 0.2 mM of each of the deoxynucleoside triphosphates (dNTPs), 1 X PCR buffer [20 mM Tris-HCl, 50 mM KCl (pH 8,4)], 1.0 U of *Taq* polymerase (Invitrogen, Carlsbad, USA), 1.5 mM $MgCl_2$; and 0.2 µM/µL of each primer. PCR was performed under the following conditions: initial denaturation for 2 min at 95°C, followed by 35 cycles of amplification: denaturation at 95°C for 20s, annealing at 53°C for 30s, extension at 72°C for 40s and a final extension step at 72° for 10 min, using a PTC 200 thermocycler (MJ Research, GMI, Minnesota, USA). A positive result was indicated by the presence of a 310-bp amplified DNA fragment when compared with a 100-bp DNA ladder (Invitrogen, Carlsbad, USA). The DNA fragment was revealed by electrophoresis on 1.5% agarose gel, staining with ethidium bromide, and visualization under ultraviolet light. Quality control was performed with *S. aureus* ATCC 25923 (*mecA*-negative) and *S. aureus* ATCC 33591 (*mecA* positive).

Slide Latex Agglutination Test

Detection of PBP2a was performed with the latex agglutination test Slidex MRSA Detection (bioMérieux, l'Etoile, France), following the manufacturer's instructions. This test was performed only for samples that showed discrepant results between cefoxitin and oxacillin in our tests.

Results

A total of 238 isolates were identified as SCoNne. The most frequent organism was *S. haemolyticus* (42%-100/238), followed by *S. hominis-hominis* (29.4%-70/238) and *S. warneri* (7.5%-18/238). The prevalence of SCoNne species and of the *mecA* gene are shown in Table 1. Various phenotypic methods were employed to identify SCoN, including morphology of the colonies, antimicrobial resistance, hemolytic activity, enzymatic activity in different substrates and acid production from carbohydrates. The tests were followed for 24, 48, and 72h and 7 days at 35ºC, identifying 238 strains of SCoNne. The most prevalent species, *S. haemolyticus*, was easily identified by a simple scheme, using hemolytic activity, a PYR test and urea and mannose tests.

By conventional and commercial methods of species identification, in addition to giving low accuracy (50%-70%), are more troublesome and require more incubation time [19]. Among the isolates, 75% (178/238) were identified only by phenotypic methods and 25% (60/238) by a commercial method. Molecular methods based on the analysis of products from PCR have been developed for SCoN identification, giving improved consistency and speed [19,22]. Species that are more difficult to identify by phenotypic or commercial methods, such as *S. caprae* and *S. equorum*, were identified by PCR and *sodA* gene sequencing. Although only 3% (8/38) of isolates were identified by the *sodA* gene, the concordance between this method and the commercial method was 62.5% (5/8).

The *mecA* gene was detected in 71% (169/238) of the SCoNne isolates. Concordance between cefoxitin disk, oxacillin disk, agar dilution and *mecA* gene detection was found in 96% (229/238) of the strains. However, discrepancies between the phenotypic methods and *mecA* gene detection were observed in 4% (9/238, Table 2). Disagreement between the oxacillin disk test and *mecA* gene detection was observed in 2.5% (6/238) of the isolates. The cefoxitin disk gave 100% sensitivity and specificity. The oxacillin disk also gave a sensitivity of 100% and a specificity of 91%. The sensitivity of agar dilution was 100%, with a specificity of 88%. When the results obtained in the disk diffusion test and agar dilution were compared, we found that they gave the same sensitivity. However, the specificity, VPP and VPN results were better with the cefoxitin disk (Table 3).

The cefoxitin disk test was proposed in 2001 as an option for the detection of oxacillin resistance mediated by the *mecA* gene, considering that many laboratories do not have a latex agglutination test or a PCR technique for routine use [23]. Earlier studies indicated that the cefoxitin disk is a helpful tool for the detection of oxacillin resistance, with 99%-100% sensitivity and 96% specificity to predict the *mecA* gene in *S.* aureus and SCoN [14,24,25].

Table 1. Occurence of the *mecA* gene among 238 SCoNne isolates.

Species	Occurrence		*mecA* Pos Isisolates		*mecA* Neg isolates	
	N	%	N	%	N	%
S. haemolyticus	100	42.0	91	91.0	9	9.0
S. hominis-hominis	70	29.4	53	76.0	17	24.0
S. warneri	18	7.6	7	39.0	11	61.0
S. capitis-capitis	9	3.7	1	11.0	8	89.0
S. sciuri	9	3.7	1	11 .0	8	89.0
S. saprophyticus	6	2.5	4	67.0	2	33.0
S. hominis novobiosepticus	6	2.5	5	83.0	1	17.0
S. capitis urealyticus	5	2.1	2	40.0	3	60.0
S. cohnii cohnii	4	1.6	0	0. 0	4	100.0
S. xylosus	3	1.3	3	100.0	0	0.0
S. cohnii urealyticus	3	1.3	1	33.0	2	67.0
S. lugdunensis	1	0.4	0	0.0	1	100.0
S. simulans	1	0.4	1	100.0	0	0.0
S. auricularis	1	0.4	0	0.0	1	100.0
S. caprae	1	0.4	0	0.0	1	100.0
S. equorum	1	0.4	0	0.0	1	100.0
Total	238	100	169	71.0	68	29.0

Table 2. Discrepant results for nine SCoNne isolates that are mecA and latex (PBP2a) negative, by phenotypic methods (DD and MIC).

Species	Isolate number	DD cefoxitin	DD oxacillin	Agar dilution (MIC µg/mL)
S. sciuri	224	S	R	0.5 (R)
S. sciuri	247	S	R	0.5 (R)
S. sciuri	201	S	R	0.5 (R)
S. sciuri	216	S	R	0.25 (S)
S. saprophyticus	327	S	S	0.5 (R)
S. saprophyticus	633	S	S	0.5 (R)
S. cohnii cohnii	226	S	S	0.5 (R)
S. cohnii cohnii	244	S	R	0.5 (R)
S. cohnii-cohnii	605	S	R	0.5 (R)

R=resistant; S=susceptible.

Table 3. Sensitivity, specificity and positive and negative predictive values for phenotypic methods in comparison with the PCR results for detection of oxacillin resistance among SCoNne isolates.

	DD cefoxitin		DD oxacillin		MIC oxacillin	
	N	%	N	%	N	%
Sensitivity	169/169	100	169/169	100	169/169	100
Specificity	69/69	100	63/69	91	61/69	88
VPP	169/169	100	169/175	97	169/177	95
VPN	69/69	100	69/69	100	69/69	100
Accuracy	238/238	100	232/238	97	230/238	97

Discussion

We evaluated a higher number of isolates of SCoNne than other studies performed in Brazil, and we found high rates of resistance to oxacillin by PCR (71%) in these isolates, though earlier studies also included isolates of *S. epidermidis* [26-28]. In our study, among the SCoNne isolates, 100 were identified as *S. haemolyticus*, with 91% presenting a *mecA*-gene-positive PCR result. Other studies have shown that *S. haemolyticus* and *S. epidermidis* are the species most frequently associated with antimicrobial agent resistance [7,8]. A study performed by Palazzo & Darini in 2006 described a sensitivity of 92.5% to both cefoxitin and oxacillin disk tests,

a specificity of 98.6% to cefoxitin disks and 96% to oxacillin disks. In this study, six isolates showed a false negative result in the cefoxitin disk test (five isolates of *S. epidermidis* and one isolate of *S. caprae*) [29]. Perazzi et al. in 2006 found a lower sensitivity for the cefoxitin disk (84%) than that obtained with the oxacillin disk (87%), in a study performed with isolates of SCoN other than *S. saprophyticus*. The specificity of the cefoxitin disk was 100% [30].

Among the species for which there were discrepancies between the tests, susceptibility to cefoxitin disk with resistance with oxacillin disk was observed in 44% (4/9) isolates of *S. sciuri*, all of them giving negative results in a PCR of the *mecA* gene. Oxacillin resistance was observed in three of these isolates and oxacillin susceptibility in one of them with an agar dilution test. Resistance to oxacillin was observed in 33% (2/6) of *S. saprophyticus* isolates by the agar dilution test. The PCR results for the *mecA* gene were negative in these isolates, which showed susceptibility to cefoxitin and oxacillin by disk diffusion. Seventy-five percent (3/4) of the isolates identified as *S. cohnii-cohnii*, which gave a negative PCR for the *mecA* gene, had discrepancies with the oxacillin disk and/or agar dilution results. Two of these isolates were resistant to oxacillin by disk and agar dilution, and one of them was resistant only by agar dilution. All of them were susceptible when tested with the cefoxitin disk. Results obtained for the other species did not differ among the methods (Table 2). Pottumarthy et al. in 2005 found 3% of very major errors with the cefoxitin disk test and 4% with the oxacillin disk test [31]. We only found major errors (false-resistance) with the oxacillin disk in 2.5% of the isolates, including *S. sciuri*, *S. saprophyticus* and *S. cohnii-cohnii*. Similar discrepancies between phenotypic and genotypic results have been described for *S. saprophyticus*, *S. cohnii*, *S. warneri*, *S. capitis*, *S. lugdunensis* and *S. xylosus* [32].

Detection of oxacillin resistance in SCoN is a challenge for clinical laboratories because many false negative results can be attributed to heterogeneous resistance to oxacillin expressed by this type of organism [26,29,33]. The cefoxitin disk test is considered a better predictor for resistance than oxacillin, especially when there is heteroresistance to oxacillin. This can be explained by the fact that cefoxitin has a strong ability to induce PBP2a and also has a high affinity for PBP4, a protein involved in resistance in *Staphylococcus* spp. These organisms, although less frequent in the laboratory routine, could be more affected by the lower specificity of the oxacillin disk diffusion test and/or the oxacillin agar dilution test [32,34]. According to NCCLS 2001, the disk diffusion test with oxacillin is not recommended for *S. saprophyticus*, since most isolates that are *mecA* gene negative can express resistance based on phenotypic methods [35]. The *S. saprophyticus* isolates evaluated in our study gave similar results when cefoxitin/oxacillin disk diffusion and *mecA* gene detection were compared. However, agar dilution results showed poor concordance with the *mecA* gene test, since two isolates were resistant to oxacillin based on agar dilution (MIC of 0.5 μg/mL).

Studies performed to detect resistance in *Staphylococcus* spp. have demonstrated discrepancies between the phenotypic methods and the gold-standard, especially in SCoN. False positive results (resistant isolates that do not harbor the *mecA* gene) can be associated with hyper-production of β-lactamases, resulting in hydrolysis of the beta-lactam agent and changes in PBPs other than PBP2a [33]. Total concordance between the latex agglutination test for PBP2a and *mecA* gene detection by PCR has been observed in isolates that showed false resistance with oxacillin disk diffusion or dilution. The PBP2a test was performed with and without induction, and identical results were observed with the different methods [32,36]. When we analyzed the false positive results obtained with agar dilution (Table 2), we found that eight isolates gave MICs of 0.5 μg/mL (low level of resistance or borderline resistance). These strains presented oxacillin resistance between 0.5 and 2.0 μg/mL, near the breakpoint for resistance.

In conclusion, though the phenotypic methods (manual or automated) are troublesome and time consuming, they are the most widely used in clinical laboratories, because they are easy to use. Molecular methods of identification of SCoN are still restricted to research laboratories and should be employed for the less prevalent species.

The cefoxitin disk test (30μg) gave the same sensitivity and better specificity than the oxacillin disk (1μg) for the detection of *mecA*-gene-mediated oxacillin resistance in SCoNne isolates. To improve the performance of the cefoxitin disk, concomitant use of cefoxitin (30μg) and oxacillin (1μg) in the clinical laboratory routine would reduce mistakes made in the detection of oxacillin resistance, restricting PCR or agglutination tests to PBP2a to resolve discrepancies between disk tests.

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