Evaluation of the Accuracy of Various Phenotypic Tests to Detect Oxacillin Resistance in Coagulase-Negative Staphylococci

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In this study, we determined the accuracy of phenotypic tests (cefoxitin agar dilution, 30μg-cefoxitin and 1μg-oxacillin disks) to detect the oxacillin resistance/mecA gene among coagulase-negative staphylococci (CoNS) isolates. The presence of the mecA gene was detected by PCR technique (gold standard). A total of 176 CoNS isolates from blood of hospitalized patients were evaluated. Of these, 138 (78.4%) harbored the mecA gene. Using 30μg-cefoxitin and 1μg-oxacillin disks we obtained 100 and 98.3% accuracy, respectively. In addition, when cefoxitin was used as marker in an agar dilution method, the higher accuracy (99.4%) was established with 8μg cefoxitin per mL breakpoint. Thus, despite of the agar dilution method using cefoxitin as a marker not being standard for this detection, our results suggested that it is an excellent alternative to detect the oxacillin resistance/mecA gene among CoNS isolates.
Key-Words: Oxacillin, cefoxitin, CoNS.

Coagulase-negative staphylococci (CoNS), as a group, are a frequently isolated microorganism in nosocomial infections. In Brazil, about 10%-20% of bloodstream infections are caused by CoNS and, of the isolates, about 70%-90% present resistance to oxacillin [1].

Oxacillin resistance in CoNS is due to the acquisition of the mecA gene that encodes an additional protein, called PBP 2', with low affinity to the β-lactamic antimicrobials [2,3]. The accurate determination of oxacillin resistance is necessary for the establishment of therapeutic and surveillance programs [4]. The use of 30μg-cefoxitin disks has been established by the Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS). However, no dilution method using cefoxitin as a marker for oxacillin resistance characterization has been adopted. Thus, the aim of this study was to evaluate the accuracy of phenotypic methods (cefoxitin agar dilution, 30μg-cefoxitin and 1μg-oxacillin disks by disk-diffusion) in detecting oxacillin resistance among CoNS isolates and to compare the results with the presence of the mecA gene detected by PCR (gold standard).

Materials and Methods
Bacterial Strains and Identification
From August to December 2004, 176 consecutive CoNS isolates were obtained from blood cultures of patients admitted to three hospitals: Hospital de Clínicas, Irmandade Santa Casa de Misericórdia and Hospital Conceição, in Porto Alegre, RS, Brazil. Only one isolate of each patient was included in the study. The isolates were identified to the species level as previously described [5]. All samples were frozen and stored at -20°C in skim milk with 10% glycerol.

Antimicrobial Susceptibilities Testing
The susceptibilities to cefoxitin and oxacillin were determined by the disk diffusion method on Mueller-Hinton agar (Oxoid, Basingstoke, UK) plates using bacterial suspensions with turbidity adjusted for 0.5 McFarland standard (Densimat, Rome, Italy), after incubation at 35 ºC for 24h. The results were interpreted according to the CLSI (2006) guidelines.

The minimum inhibitory concentrations (MICs) for cefoxitin were determined by the agar dilution method, according to the CLSI guidelines. Briefly, for each strain, colonies isolated from an overnight growth were transferred to sterile saline; the suspensions were adjusted to a 0.5 McFarland standard and inoculated on Mueller–Hinton agar plates containing 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 or 256μg of cefoxitin per mL using a Steers replicator.

Plates containing Mueller-Hinton agar without antimicrobial were used as controls for bacterial growth.

Polymerase Chain Reaction for mecA Gene
A polymerase chain reaction (PCR) procedure was used to verify the presence of the mecA gene. Primers (mecA$_1$: 5'-TGG CTA TCG TGT CAC AAT CG and mecA$_2$: 5'-CTG GAA CTT GTT GAG CAG AG) and protocol used were based on the methodology previously mentioned [8].

ROC Curve
The receiver operator characteristic (ROC) curve was used to determine the best cefoxitin breakpoint to predict oxacillin resistance.

Control Strains
The quality control of the identification tests was done using *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), and *Staphylococcus hominis* (ATCC 27844). The oxacillin-susceptible *S. aureus* (ATCC 29213) and oxacillin-resistant *S. aureus* (ATCC 33591) isolates were used as controls for antimicrobial susceptibility testing and PCR procedure.

Results

A total of 176 CoNS isolates were analyzed. The most frequent species were *Staphylococcus epidermidis* (120 isolates; 68.2%), followed by *Staphylococcus haemolyticus* (23 isolates; 13.1%), *Staphylococcus capitis* (12 isolates; 6.8%), *Staphylococcus hominis* (6 isolates; 3.4%), *Staphylococcus lugdunensis* (5 isolates; 2.8%) and other CoNS species (10 isolates; 5.7%).

One hundred and thirty eight (78.4%) CoNS isolates were considered oxacillin-resistant based on the presence of the *mec*A gene detected by PCR. The presence of the *mec*A gene, using disk-diffusion method, was predicted with 100% and 98.3% accuracy for cefoxitin and oxacillin disks, respectively (Table 1). Table 1 summarizes the data showing the sensitivity, specificity, negative and positive predictive values and accuracy for the phenotypic tests applied. Using 1μg-oxacillin disk, three isolates (*S. epidermidis*, *S. haemolyticus* and *S. saprophyticus*) that did not harbor the *mec*A gene were oxacillin resistant. According to the agar dilution method, only one isolate (*S. epidermidis*) that did not harbor the *mec*A gene was classified as resistant. The others isolates were classified as susceptible to oxacillin when the follow breakpoints were adopted by us: ≥8μg cefoxitin per mL and ≤4μg cefoxitin per mL corresponding to resistant (*mec*A gene present) and susceptible (*mec*A gene absence) to oxacillin, respectively. Table 2 presents the discrimination among CoNS isolates in comparison to the presence/absence of the *mec*A gene and its respective cefoxitin MICs.

Figure 1 indicates receiver operating characteristic (*ROC*) curves for cefoxitin breakpoints when an agar dilution method is used. At this point, our results showed that 8μg cefoxitin per mL is the best breakpoint to discriminate between CoNS isolates that harbored and did not harbor the *mec*A gene.

Discussion

The accurate characterization of oxacillin resistance is essential, mainly for correct antimicrobial therapy. Phenotypic tests are important for laboratorial diagnosis. Although molecular methods are considered to be a "gold standard" for the diagnosis of oxacillin resistance, these methods are expensive for many of our laboratories and the use of rapid and accurate phenotypic tests has become an alternative [9].

Since 2004, the CLSI has recommended the use of 30μg-cefoxitin disk for detection of oxacillin resistance among staphylococci species. Presented in the document M100-S15 are the breakpoints for cefoxitin susceptibility (susceptible ≤8μg cefoxitin per mL, resistant ≥32μg cefoxitin per mL) although they haven't explicitly specified the *mec*A gene's presence as the determinant factor for methicillin-resistance. Even thought researches have shown that an agar screening test containing 6μg oxacillin per mL is not efficient towards CoNS [10], comparative studies to asses the agar screening test and disk-diffusion have indicated that agar screening is more sensitive in the detection of methicillin-resistant isolates [11,12]. We have demonstrated that the application of the agar screen test using 4μg cefoxitin per mL as breakpoint showed 99.4% accuracy for oxacillin resistance

characterization among CoNS [13]. Despite the same accuracy being demonstrated when 4μg oxacillin per mL was used, cefoxitin agar screen is a feasible method in clinical laboratories and, additionally, it is not necessary to add salt to the culture medium, minimizing errors due to methodological variants.

In this study, cefoxitin breakpoints were evaluated by an agar dilution test, also 30μg-cefoxitin and 1ìg-oxacillin disks, for characterization of oxacillin resistance among CoNS species due to the presence of the *mec*A gene.

Several studies have showed that the greatest accuracy for oxacillin resistance characterization may be achieved by use of cefoxitin, basically, by the disk-diffusion method [14,15,16]. In our study, 98 and 100% accuracies were obtained with the use of 1μg-oxacillin and 30μg-cefoxtin disks, respectively. In this respect, our results showed that the 30μg-cefoxitin disk presented equal sensitivity but higher specificity than the 1μg-oxacillin disk, confirming the better performance of cefoxitin as a marker of this resistance (*mec*A-mediated resistance).

Fernandes et al. [17] suggest that a breakpoint of ≤4μg cefoxitin per mL is accurate for the determination of oxacillin susceptible *S. aureus* (not harboring *mec*A gene). The 4μg cefoxitin per mL breakpoint, in our study, showed less accuracy than an 8μg cefoxitin per mL breakpoint (97.1% *vs.* 99.4%) (Figure 1).

The study described values of ≥8μg cefoxitin per mL and ≤4μg cefoxitin per mL as the best breakpoints for characterization of oxacillin -resistant and -susceptible isolates, respectively. Using 8μg cefoxitin per mL, only one *S. epidermidis* isolate (with MIC of 16μg cefoxitin per mL) was mischaracterized as resistant (Table 2). This breakpoint was shown to be highly reliable in the discrimination of isolates harboring the *mec*A gene. On the other hand, discrepancies can be observed between isolates harboring and those not harboring the *mec*A gene because this event (resistance without the presence of the *mec*A gene) may be associated with the hyperproduction of β-lactamase in these isolates or mutations in PBP's other than PBP 2'. Swenson et al. showed that a cefoxitin test can be used alone to predict *mec*A-mediated resistance, when these tests were applied for the characterization of this resistance among *S. aureus* isolates [18,19]. However, despite the excellent concordance between cefoxitin tests and the *mec*A-gene, non-mecA-mediated resistance may be underestimated [18].

In conclusion, the 30μg-cefoxitin disk was shown to be more specific than the 1μg-oxacillin disk in the prediction of oxacillin resistance due to the *mec*A gene among CoNS. Also, our results suggested that 8μg cefoxitin per mL is the more accurate breakpoint (99.4%) when an agar dilution method is applied, despite oxacillin resistance (*mec*A gene) detection not being actually done by it.

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Table 1. Sensitivity, specificity, negative and positive predictive values and accuracy for different phenotypic tests applied to predict the presence of the *mec*A gene considering the PCR procedure as gold standard.

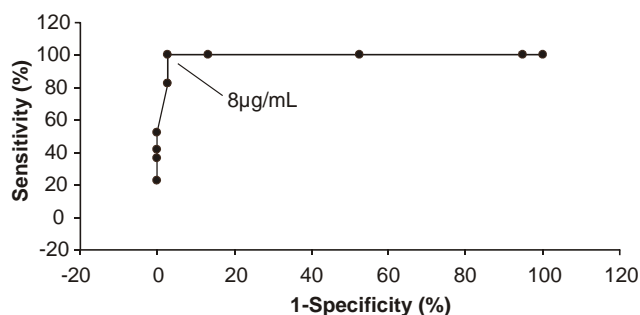| Phenotypic tests	Sens[c] (%)	Spec[c] (%)	NPV[c] (%)	PPV[c] (%)	Accur[c] (%)
1μg-oxacillin disk[a]	100	92.1	100	97.9	98.3
30μg-cefoxitin disk[a]	100	100	100	100	100
8μg cefoxitin per mL[b]	100	97.4	100	99.3	99.4
4μg cefoxitin per mL[b]	100	86.8	100	96.5	97.1

[a]Using a disk-diffusion method; [b]Using an agar dilution method; [c]Sens, sensitivity; Spec, specificity; NPV, negative predictive value; PPV, positive predictive value and Accur, accuracy.

Table 2. Presence of the *mec*A gene and cefoxitin minimum inhibitory concentration among CoNS isolates analyzed.

Nº of isolates	PCR[b]	Number of isolates with cefoxitin MIC[a] (μg cefoxitin per mL)										
		0.25	0.5	1	2	4	8	16	32	64	128	256
38	*mec*A -	2	16	15	4		1					
138	*mec*A +						24	42	14	8	19	31

[a]Cefoxitin MIC determined by an agar dilution method; [b]*mec*A gene was detected by PCR; *mec*A+=gene detected; *mec*A-=gene not detected; Total number of isolates (n)=176; the shaded numbers of isolates in the table were correctly categorized as resistant (*mec*A+) and susceptible (*mec*A-), respectively, when ≥8μg cefoxitin per mL and ≤4μg cefoxitin per mL breakpoints were used.

Figure 1. *ROC* curve established for the cefoxitin breakpoints by an agar dilution method. The best performance including sensitivity and specificity were achieved in 8μg cefoxitin per mL.

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