

Evaluation of oxacillin and cefoxitin disks for detection of resistance in coagulase negative staphylococci

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Coagulase-negative Staphylococcus spp. was considered nonpathogenic until the emergence of multiresistance and the demonstration of their participation as infectious agents. In Brazil, oxacillin resistance may be present in over 80% of isolates, and the Clinical and Laboratory Standards Institute standardized a disk-diffusion method to predict this resistance in Staphylococcus. The aim of this study was to evaluate the variability among commercial disks of oxacillin (1 µg) and cefoxitin (30 µg) widely used in clinical laboratories of microbiology, compared with mecA gene and minimum inhibitory concentration (MIC) of oxacillin. The use of oxacillin and cefoxitin disks simultaneously allowed the detection of important differences, particularly, in less frequent species such as S. cohnii, S. haemolyticus, S. saprophyticus, and S. sciuri. Disks of cefoxitin of the brand 2 displayed good correlation with the mecA gene (98.7%) and oxacillin MIC (97.8%), while major discrepancies were observed using disks of brand 1. One of the critical points in the diffusion disk test is the quality of the disks: the use of better quality disks associated with molecular methods lead to better results to define the best antibiotic therapy.

Key words: coagulase negative staphylococci - mecA gene - methicillin resistance - cefoxitin - susceptibility tests

Coagulase-negative *Staphylococcus* spp. (CoNS) were considered nonpathogenic until their implication as nosocomial agents and the emergence of multiresistance (Beekmann et al. 2003). Healthcare-associated infections caused by these microorganisms are responsible for high mortality rates worldwide. The emergence of CoNS populations with heterogeneous resistance to oxacillin causes a great difficulty to detect them in clinical routine laboratories (Cauwelier et al. 2004). In Brazil, oxacillin resistance may be present in over 80% of isolates in some health institutions (Sader et al. 2001). On this account, vancomycin has been widely used and it is a major cause for the emergence of glycopeptide-resistant isolates (Oliveira et al. 2001, Nunes et al. 2006).

Recently, Clinical and Laboratory Standards Institute (CLSI) standardized a disk-diffusion method to predict resistance mediated by the *mecA* gene in *Staphylococcus* spp., using a cefoxitin disk (30 µg). In fact, studies indicate that this is the best phenotypic test to predict resistance to beta-lactam agents among CoNS (Felten et al. 2002, Pottumarthy et al. 2005). Regardless of the CLSI guidelines, the detection of this resistance through phenotypic methods remains a challenge for clinical laboratories of microbiology (Sejas et al. 2003). Several dis-

crepancies were observed among the laboratories that participated in the Antimicrobial Surveillance Program (SENTRY), suggesting non-observance of the interpretation criteria currently recommended (Mendes et al. 2003). The quality of the antimicrobial disks may affect the results, with failure to detect hetero-resistance (Sejas et al. 2003). This is especially important in the case of oxacillin and cefoxitin, which predict susceptibility to a large group of antimicrobial agents.

The aim of this study was to evaluate two different brands of oxacillin (1 µg) and cefoxitin (30 µg) disks that are commonly used in clinical laboratories of microbiology.

MATERIALS AND METHODS

Isolates - CoNS isolated from blood cultures maintained in "skim-milk" (Difco, Detroit) at -20°C were studied. For the tests, the isolates were cultivated onto agar plates supplemented with sheep blood at 5% (Trypticasein agar - Oxoid, UK), incubated for 24 h at 35°C.

Identification of CoNS - Characterization was made by a set of phenotypic tests (Bannerman 2003): coagulase, catalase, alkaline phosphatase, ornithine decarboxylase, urease, PYR, and utilization of carbohydrates (trehalose, mannitol, mannose, sucrose, maltose, lactose, cellobiose). Anaerobic growth in thioglycolate and susceptibility to novobiocin (> 16 mm), to polymyxin B (≥ 10 mm), and to bacitracin (> 9 mm) by disk diffusion tests were also evaluated. Other tests useful to discriminate between *S. epidermidis* and *S. hominis* were susceptibility to desferrioxamine (≥ 20 mm) and fosfomicin (> 30 mm) (Lindsay et al. 1993, Rosco 2000).

Susceptibility tests - Oxacillin (1 µg) and cefoxitin (30 µg) disks from two different brands, widely used in clinical microbiology laboratories were tested. A 0.5

Financial support: Capes, CNPq, UFRGS, UCPel, FFCMPA

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Received 26 February 2007

Accepted 2 July 2007

Mcfarland standard suspension of each isolate was inoculated onto a Mueller Hinton agar plate (Oxoid), and the disks were distributed maintaining a distance of 30 mm edge to edge. The diameters of the inhibition zones were interpreted according to the criteria recommended by the CLSI (2006), considering a breakpoint diameter for susceptibility inhibition of ≥ 18 mm to oxacillin and ≥ 25 mm to cefoxitin. Using a Steer's replicator apparatus (Craft Machine, Chester, PA, US), a suspension of each isolate was inoculated onto Muller-Hinton agar plates supplemented with 2% NaCl in the following conditions: 0.125 to 4 μg of oxacillin (Sigma Chemical Co., St. Louis, MO). The tests were interpreted after 24 h of incubation at 35°C, and growth of more than one colony was considered a positive result. Both strains *S. aureus* ATCC 29213 (oxacillin susceptible) and *S. aureus* ATCC 33591 (oxacillin resistant) were included as controls.

Detection of the *mecA* gene - A polymerase chain reaction (PCR) based on the methodology previously described (Vannuffel et al. 1998) was used to determine the presence of the *mecA* gene. Briefly, staphylococcal DNA was extracted by boiling (Nunes et al. 1999) followed by PCR using with specific primers: *mecA*¹: 5 TGG CTA TCG TGT CAC AAT CG, *mecA*²: 5 CTG GAA CTT GAG CAG AG. A positive result was indicated by observation of the presence of a 310-bp DNA fragment after gel electrophoresis (Vannuffel et al. 1998). *S. aureus* ATCC 25923 (*mecA* negative) and *S. aureus* ATCC 33591 (*mecA* positive) were the controls used in all sets.

RESULTS

A total of 302 CoNS isolates were identified (Table I). The most frequent CoNS species found was *S. epidermidis*, with 157 isolates, followed by *S. hominis* (56 isolates), *S. haemolyticus* (52 isolates), *S. sciuri* (10 isolates), and *S. warneri* (9 isolates); other species were found at a lower percentage. The percentage of resistance accordingly to oxacillin minimum inhibitory concentration (MIC) was greater than 90% in the three prevalent species.

Resistance according to PCR determination of *mecA* gene was detected in 88.1% of the cases, comparing to 90.4% detected by MIC. The distribution of resistance observed with oxacillin and cefoxitin disks of both brands, as well as MIC and *mecA* results are shown in Table II. Oxacillin MIC and *mecA* gene displayed concordant results in resistant isolates. Resistance to oxacillin was detected in 248 cases (82.1%) by brand 1 and in 271 cases (89.7%) by brand 2 disks. Cefoxitin disks of brand 1 showed concordant results in 154 (50.9%) of the resistant isolates while brand 2 detected 264 (87.4%) of them.

Considering only the results obtained with brand 2, discrepancy between phenotypic and genotypic methods was observed in 14 cases (Table III). The *mecA* gene was observed in only four isolates, all with MIC compatible with resistance, although all of them displayed disagreement with disks (cefoxitin or oxacillin disk).

Among isolates with negative PCR to *mecA* gene, two cases confirmed susceptibility through MIC and cefoxitin disk. The other eight isolates without *mecA* gene

are resistant accordingly to MIC. Of them, the one identified as *S. epidermidis* displayed a considerable high level of resistance (MIC > 4 $\mu\text{g}/\text{ml}$), although considered susceptible to both disks. Susceptibility test with cefoxitin disk agreed in all but one case with negative *mecA* gene. Disagreement with oxacillin disks were observed in this and in other seven cases.

TABLE I
Prevalence and resistance of *Staphylococcus* species

Species	n	Total	
		%	% resistance (<i>mecA</i>)
<i>S. epidermidis</i>	157	52.0	94.2
<i>S. hominis</i>	56	18.5	94.3
<i>S. haemolyticus</i>	52	17.2	94.1
<i>S. sciuri</i>	10	3.3	40.0
<i>S. warneri</i>	9	3.0	75.0
<i>S. saprophyticus</i>	6	2.0	90.0
<i>S. cohnii cohnii</i>	4	1.3	50.0
<i>S. simulans</i>	3	1.0	66.6
<i>S. capitis capitis</i>	2	0.6	50.0
<i>S. xylosum</i>	1	0.3	100
<i>S. capitis ureolyticus</i>	1	0.3	0
<i>S. cohnii urealyticum</i>	1	0.3	100

TABLE II
Resistance of coagulase-negative *Staphylococcus* spp.

Test	n / Total	Resistance	
		%	%
Oxacillin, brand 1	248 / 302	82.1	
Oxacillin, brand 2	271 / 302	89.7	
Cefoxitin, brand 1	154 / 302	50.9	
Cefoxitin, brand 2	264 / 302	87.4	
Oxacillin MIC	273 / 302	90.4	
<i>mecA</i> gene	266 / 302	88.1	

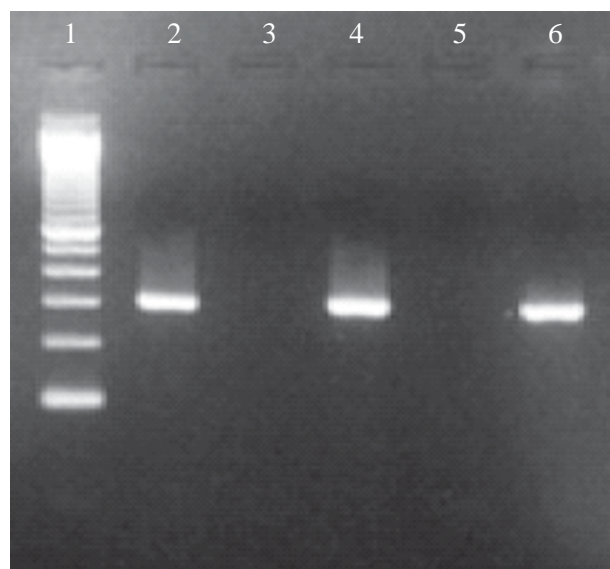
DISCUSSION

Considering that the treatment of staphylococcal infections depends on the methicillin resistance (Chandran & Rennie 2005), and that this is habitually based upon the susceptibility to oxacillin and cefoxitin disks, the inconsistency of results may have important implications on inadequate therapy. It is important to point out that mortality rates in non-treated patients with bacteremia are greater than those of patients receiving adequate antimicrobial therapy (Weinstein et al. 1997) and that false-positive resistance accounts for increased costs, additional clinical work, request of more cultures, and unnecessary use of antibiotics such as vancomycin (Chandran & Rennie 2005).

TABLE III
Discordant results of susceptibility tests (disks brand 2)

<i>Staphylococcus</i> species	<i>mecA</i> gene	Oxacillin MIC ($\mu\text{g/ml}$)	Disk diffusion	
			Oxacillin	Cefoxitin
<i>S. sciuri</i>	Negative	R (0.5)	R	R
<i>S. sciuri</i>	Negative	R (0.5)	R	S
<i>S. sciuri</i>	Negative	R (0.5)	R	S
<i>S. sciuri</i>	Negative	R (0.5)	R	S
<i>S. cohnii cohnii</i>	Negative	R (0.5)	R	S
<i>S. saprophyticus</i>	Negative	R (0.5)	R	S
<i>S. cohnii cohnii</i>	Negative	R (0.5)	S	S
<i>S. epidermidis</i>	Negative	R (> 4)	S	S
<i>S. sciuri</i>	Negative	S (0.25)	R	S
<i>S. sciuri</i>	Negative	S (0.25)	R	S
<i>S. haemolyticus</i>	Positive	R (2)	S	R
<i>S. epidermidis</i>	Positive	R (> 4)	S	R
<i>S. cohnii cohnii</i>	Positive	R (0.5)	R	S
<i>S. hominis hominis</i>	Positive	R (> 4)	R	S

R: resistant; S: susceptible; MIC: minimum inhibitory concentration.



Polymerase chain reaction detection of *mecA* gene of three isolates with discrepancy regarding oxacillin disk. Lanes - 1: molecular size markers (1100 bp); 2: positive control (310-bp band obtained with DNA from *Staphylococcus aureus* reference strain ATCC 33591); 3: negative control (*S. aureus* ATCC 25923); 4: clinical isolate (310-bp band obtained with DNA from *S. haemolyticus*); 5: clinical isolate (absence of band with DNA from *S. sciuri*); 6: clinical isolate (310-bp band obtained with DNA from *S. epidermidis*).

The rates of resistance detected with oxacillin MIC determination were greater than those obtained with any other methods, but the majority of discrepant isolates displayed lower MIC (0.5 $\mu\text{g/ml}$). In fact borderline CoNS isolates are more difficult to detect, and changes in the interpretative zone diameters of cefoxitin have been suggested (Frigatto et al. 2005).

The brands used for the disk susceptibility tests are among those most common used in laboratories of routine microbiology as well as in some research (Sejas et al. 2003, Palazzo & Darini 2006). Brand 1 is cheaper than brand 2 which displayed a better performance. It is important to point out that disks of cefoxitin brand 1 failed to detect resistance in many isolates, and that they are recommended by CLSI to detect resistance in CoNS.

Depending on the brand, cefoxitin disks showed distinct results: with brand 1 disks, only 60.9% of the resistant isolates detected by *mecA* gene were linked. On the other hand, brand 2 displayed higher concordant results (98.7%), either among susceptible or resistant isolates. Oxacillin disks results were less divergent. The major difference of results between the two brands makes complex universal evaluations. We observed that brand 2 showed good correlation between MIC of oxacillin and disk diffusion (98.5% for oxacillin and 97.8% for cefoxitin).

Cefoxitin disks of brand 2 displayed concordant results with *mecA* gene in 298/302 cases, failing to detect resistance in three isolates and susceptibility in one. Oxacillin disk results were concordant in 291 isolates, and also failed to detect resistance in three cases, but the failure to detect susceptibility was greater (eight cases). This data indicates the value of high-quality disks, as already pointed, and corroborates recommendations (Swenson & Tenover 2005, CLSI 2006), to increase the sensitivity and specificity of the cefoxitin disk to detected oxacillin hetero-resistance.

High discrepancy of results between MIC of oxacillin and *mecA* gene was observed. Considering *mecA* gene as standard, eight isolates were false-positive, seven of them displayed a MIC of 0.5 $\mu\text{g/ml}$, contrasting with a higher MIC of oxacillin (> 4 $\mu\text{g/ml}$) observed in the majority of concordant resistance cases. This may be due to the fact that the detection of resistance in iso-

lates that displayed higher MIC is easier than among those with MIC closer to the susceptibility values. It must be pointed that we also observed discrepancy in the results of one isolate with MIC of 2 µg/ml.

Given that *mecA* gene is present in the majority of the methicillin-resistant *Staphylococcus* (MRS), it can be considered the best method, even though discrepancies have been reported in CoNS isolates (Swenson & Tenover 2005, Palazzo & Darini 2006). Isolates with negative results for *mecA* gene and MIC for oxacillin > 0.5 µg/ml displayed controversial results in disk diffusion tests. For uncommon species such as *S. cohnii* and *S. warneri*, this is not surprisingly (Hussain et al. 2000, Palazzo & Darini 2006). Resistance to oxacillin, without *mecA* gene, may be due to the overproduction or overexpression of penicillinase, or by alteration of other penicillin-binding proteins (Caierão et al. 2004).

The disk diffusion test proposed by Kirby-Bauer is one of the most used methods in Brazilian clinical laboratories for determination of bacterial susceptibility. It has already been pointed the importance of the quality of the disks (Sejas et al. 2003). Our study indicated that antimicrobial susceptibility test according to CLSI and the use of qualified disks (brand 2) led to reliable results, although in a few cases the disks were powerless to provide an adequate response, and corroborates the idea that the use of more methods or tests improves the detection of resistance (Frigatto et al. 2005).

Hussain et al. (2000) investigated the correlation of oxacillin MIC with presence of *mecA* by PCR and pointed that the breakpoints for oxacillin correctly categorize the most frequent CoNS but can not succeed for less common species. Our study also observed discrepancy among the less frequent CoNS. According to Skov et al. (2005), false resistance among CoNS is expected to be present in 1 to 9% of the cases. Although no technique alone display 100% of sensitivity and specificity to detect oxacillin resistance among CoNS, the combination of disk diffusion and oxacillin MIC can reduce failure to detection of such resistance.

Although cefoxitin is recommended by CLSI to detect oxacillin resistance in staphylococci, Frigatto et al. (2005) observed discrepant results between oxacillin and cefoxitin disks among *S. epidermidis*, and emphasized the importance of using molecular techniques to confirm disk diffusion results. Palazzo and Darini (2006) reported that the use of both cefoxitin disks and MIC of oxacillin may reduce errors in the detection of resistance to methicillin. While in our study we observed better concordance of *mecA* gene with cefoxitin disks rather than with oxacillin disks, we also observed some cases in which only oxacillin disk detect resistance. For this reason we consider important to perform tests with both disks for the moment.

Laboratories of microbiology need rapid, sensitive, and specific techniques to detect bacterial resistance. Molecular methods were recently introduced to improve results, but for most of the clinical laboratories these techniques are still too expensive. Disk susceptibility

tests are widely available and the use of excellent quality disks can ensure results to properly guide antimicrobial therapy.

ACKNOWLEDGMENTS

To the technical team of the Laboratory of Gram-positive Cocci of the Fundação Faculdade Federal de Ciências Médicas de Porto Alegre.

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