

Cefoxitin disc diffusion test for detection of methicillin-resistant staphylococci

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Staphylococci are the main causative agents of nosocomial diseases. Over the last few years, the increase in the number of methicillin-resistant (MR) staphylococci has become a major clinical problem. Accuracy and promptness in the detection of methicillin resistance are of key importance in ensuring the correct antibiotic treatment in infected patients and control of MR staphylococci in the hospital environment. This study evaluated the accuracy of a cefoxitin disc diffusion (DD) test for the detection of methicillin resistance in staphylococci. A total of 144 clinical isolates [97 *Staphylococcus aureus* and 47 coagulase-negative staphylococci (CoNS)] were tested using a *mecA* gene PCR, a DD test (oxacillin, 1 µg disc; cefoxitin, 30 µg disc), determination of oxacillin MIC by agar dilution (AD), and an oxacillin screen agar test at oxacillin concentrations of 4 and 6 µg ml⁻¹. Of the 97 *S. aureus* and 47 CoNS isolates, 73 (75.26%) and 30 (63.83%), respectively, were *mecA*-positive. The sensitivity and specificity of the cefoxitin DD test were 94.44 and 95.83%, respectively, for *S. aureus* and 80 and 100%, respectively, for CoNS. The oxacillin DD method was 100% sensitive and 58.33% specific for *S. aureus*, and 86.67% sensitive and 70.59% specific for CoNS. The AD test was highly sensitive (98.63%) and specific (98.53%) for *S. aureus* and CoNS (83.33% sensitive and 94.12% specific). The cefoxitin DD test for methicillin-resistance detection was more specific but less sensitive than the oxacillin DD test. Use of DD tests for both cefoxitin and oxacillin can help in more accurate prediction of methicillin resistance. Centres that are not equipped to carry out PCR can use AD methods for confirmation of methicillin resistance, especially in oxacillin-resistant and cefoxitin-sensitive cases.

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INTRODUCTION

In recent years, an increase in the number of methicillin-resistant (MR) *Staphylococcus aureus* and MR coagulase-negative staphylococci (CoNS) strains has become a serious clinical and epidemiological problem, as resistance to this antibiotic implies resistance to all β-lactam antibiotics. Accuracy and promptness in the detection of methicillin resistance are of key importance in ensuring correct antibiotic treatment in infected patients and control of MR staphylococci in the hospital environment (Velasco *et al.*, 2005). MR staphylococci have also emerged as a frequent cause of community-acquired infections (Naimi *et al.*, 2003).

There are several phenotypic methods, such as MIC determination [by agar dilution (AD), broth dilution and E-test], the oxacillin screen agar (OSA) method and disc diffusion (DD) testing, for detection of MR staphylococci. Phenotypic expression of resistance can vary depending on the growth conditions (e.g. temperature, osmolarity and

culture medium supplements such as NaCl or sucrose) (Ferreira *et al.*, 2003; Sakoulas *et al.*, 2001), making susceptibility testing by standard microbiological methods potentially problematic.

The *mecA* gene is highly conserved in staphylococcal strains and thus is a useful marker of methicillin/oxacillin resistance (Ferreira *et al.*, 2003). Its detection is considered the gold standard for detection of MR isolates. However, many laboratories throughout the world do not have the capacity to use molecular techniques to detect MR staphylococci in routine clinical practice.

Susceptibility to oxacillin by disc diffusion has been used for the detection of MR staphylococcal strains in routine testing; however, some recent studies have reported low sensitivity and low specificity of oxacillin compared with cefoxitin for the detection of MR isolates (Palazzo & Darini, 2006; Swenson & Tenover, 2005). The aim of this study was to evaluate the accuracy of a cefoxitin DD test and other phenotypic methods for the detection of oxacillin resistance in staphylococci compared with detecting the presence of the *mecA* gene for routine detection of MR staphylococci.

Abbreviations: AD, agar dilution; CoNS, coagulase-negative staphylococci; DD, disc diffusion; MR, methicillin-resistant; OSA, oxacillin screen agar; PBP2a, penicillin-binding protein 2a.

METHODS

Clinical isolates. We tested 144 clinical isolates of staphylococci: 97 *S. aureus* and 47 CoNS. These isolates were recovered from blood (11 *S. aureus* and 14 CoNS), peripheral intravenous devices (26 *S. aureus* and eight CoNS), skin swabs collected from the site of peripheral intravenous device insertion (20 *S. aureus* and nine CoNS) and anterior nasal fossa (40 *S. aureus* and 16 CoNS) referred to the Department of Microbiology from the Department of Pediatrics, Chhatrapati Sahuji Maharaj Medical University, India. Isolates were collected over a period of 1 year from January to December 2005 and identified to species level using standard methods (Kloos & Bannerman, 1999; Monsen *et al.*, 1998).

Antimicrobial susceptibility testing. Susceptibility to oxacillin and cefoxitin was determined by the DD method on Mueller–Hinton agar plates (Hi-Media Laboratories) using a bacterial suspension with the turbidity adjusted to a 0.5 McFarland standard. Plates were incubated at 35 °C for 24 h. Results were interpreted according to CLSI (2005) guidelines. The interpretive criteria for cefoxitin were: *S. aureus*, sensitive $\geq 20 \mu\text{g ml}^{-1}$, resistant $\leq 19 \mu\text{g ml}^{-1}$; CoNS, sensitive $\geq 25 \mu\text{g ml}^{-1}$, resistant $\leq 24 \mu\text{g ml}^{-1}$. The interpretive criteria for oxacillin were: *S. aureus*, sensitive $\geq 13 \mu\text{g ml}^{-1}$, resistant $\leq 12 \mu\text{g ml}^{-1}$; CoNS, sensitive $\geq 18 \mu\text{g ml}^{-1}$, resistant $\leq 17 \mu\text{g ml}^{-1}$.

OSA test. All plates were prepared with Mueller–Hinton agar supplemented with 4% (w/v) NaCl containing oxacillin. Two types of plate were prepared, one containing oxacillin at a concentration of $4 \mu\text{g ml}^{-1}$ (OSA $4 \mu\text{g ml}^{-1}$) and another containing oxacillin at a concentration of $6 \mu\text{g ml}^{-1}$ (OSA $6 \mu\text{g ml}^{-1}$). All plates were spot inoculated with a cotton swab dipped into a 0.5 McFarland standard suspension of each isolate, according to the procedures outlined by the CLSI (2005). Oxacillin resistance was confirmed by bacterial growth after 24 h incubation at 35 °C.

Agar dilution test. The MIC for oxacillin was determined by an AD method, following CLSI guidelines. Briefly, for each isolate, a minimum of four to five colonies isolated from an overnight growth

were transferred to sterile saline. The suspension was adjusted to a 0.5 McFarland standard (10^8 c.f.u. ml^{-1}) and spot inoculated on Mueller–Hinton agar plates supplemented with 2% NaCl and containing 256–0.125 μg oxacillin ml^{-1} in serial doubling dilutions. The oxacillin Mueller–Hinton plates were incubated at 35 °C for 24 h. The oxacillin susceptibility breakpoint currently recommended by the CLSI for the AD testing method is $\leq 2 \mu\text{g ml}^{-1}$ for *S. aureus* and $\leq 0.5 \mu\text{g ml}^{-1}$ for CoNS.

Detection of the *mecA* gene. Four to five colonies of an overnight bacterial subculture were suspended in 100 μl lysis buffer [10 mM Tris/HCl (pH 8.0), 2 mM EDTA, 0.4% NaCl, 0.1% Triton X-100], vortexed for 5 min and boiled for 15 min followed by centrifugation at 3000 r.p.m. for 10 min at room temperature. The supernatant was used for DNA amplification. The forward (5'-CATTGAGTTC-TGCACTACC-3') and reverse (5'-GCAATACAATCGCACATACATTAATAG-3') primers (Ryffel *et al.*, 1992) were synthesized by Bangalore Genie, India. The reaction mixture (20 μl total) containing 2 μl extracted DNA, 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl_2 , 200 μM each dNTP, 1.0 U *Taq* DNA polymerase and 100 pM each primer was used to PCR amplify the *mecA* gene in an automated thermal cycler (Progene/Techne). The amplified product of 967 bp was detected by ethidium bromide staining following 1.5% agarose gel electrophoresis (Jaffe *et al.*, 2000).

RESULTS AND DISCUSSION

Detection of the *mecA* gene was positive in 73/97 *S. aureus* (75.26%) and 30/47 CoNS (63.83%) isolates. Table 1 shows the results for the phenotypic methods used for detection of meticillin resistance in the present study. The cefoxitin DD test could detect meticillin resistance only in 68/73 *mecA*-positive *S. aureus* and 24/25 *mecA*-positive CoNS. *mecA*-negative *S. aureus* (23/24) and CoNS (17/17) were accurately detected by the cefoxitin DD test. The use

Table 1. Detection of oxacillin resistance in *S. aureus* and CoNS

The results are shown as the number of resistant isolates. The interpretative criteria used in the susceptibility tests are defined in Methods

Strain (no. of isolates)	OSA $4 \mu\text{g ml}^{-1}$	OSA $6 \mu\text{g ml}^{-1}$	AD	Oxacillin DD	Cefoxitin DD
<i>mecA</i> -positive <i>S. aureus</i> (73)	73	72	72	73	68
<i>mecA</i> -negative <i>S. aureus</i> (24)	10	9	1	10	1
<i>mecA</i> -positive CoNS (30)	25	22	25	26	24
<i>mecA</i> -negative CoNS (17)	2	1	1	5	0

Table 2. Statistical analysis of phenotypic methods used for the detection of MR in staphylococci

PPV, positive predictive value; NPV, negative predictive value.

Method	Sensitivity (%)		Specificity (%)		PPV (%)		NPV (%)	
	<i>S. aureus</i>	CoNS	<i>S. aureus</i>	CoNS	<i>S. aureus</i>	CoNS	<i>S. aureus</i>	CoNS
OSA $4 \mu\text{g ml}^{-1}$	100.00	83.33	58.33	88.24	87.95	92.59	100.00	75.00
OSA $6 \mu\text{g ml}^{-1}$	98.63	73.33	62.50	94.12	88.89	95.65	93.75	66.67
AD	98.63	83.33	98.53	94.12	98.63	96.15	95.83	76.19
Oxacillin DD	100.00	86.67	58.33	70.59	87.95	83.87	100.00	75.00
Cefoxitin DD	94.44	80.00	95.83	100.00	98.55	100.00	82.14	73.91

of oxacillin in the DD test and in the OSA 4 µg ml⁻¹ and OSA 6 µg ml⁻¹ tests was more sensitive but less specific than the cefoxitin DD test (Tables 1 and 2). The results of the oxacillin AD test were comparable to those of the *mecA* PCR (Tables 1 and 2). The sensitivity and specificity of each method are shown in Table 2. The cefoxitin DD test was less sensitive than the oxacillin DD test but was more specific for both *S. aureus* and CoNS.

Determination of the oxacillin MIC is a method recommended by the CLSI for the detection of methicillin resistance. As shown in Table 3, we found one *mecA*-positive *S. aureus* strain with an MIC less than the cut-off value of 2 µg ml⁻¹ and one *mecA*-negative *S. aureus* strain with an MIC greater

than the cut-off value. A large number of *mecA*-positive CoNS isolates (5/30) demonstrated an MIC less than or equal to the cut-off point of 0.5 µg ml⁻¹, and one *mecA*-negative CoNS isolate had an MIC greater than the cut-off point.

The discrepant results for *S. aureus* and CoNS are shown in Tables 4 and 5, respectively. The most important discrepancy was encountered in 8/24 *mecA*-negative (methicillin-sensitive) *S. aureus* isolates, which demonstrated oxacillin resistance by the majority of phenotypic methods except for the cefoxitin DD test and the AD method.

In the present study, it was found that the cefoxitin DD test was more specific and less sensitive than the OSA method

Table 3. Oxacillin MIC for *mecA*-positive and *mecA*-negative *S. aureus* and CoNS

Numbers in bold indicate discrepant results. The interpretative criteria used are defined in Methods.

Strain (no. of isolates)	MIC (µg ml ⁻¹)											
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
<i>mecA</i> -positive <i>S. aureus</i> (73)			1			4	2	8	2	4	8	44
<i>mecA</i> -negative <i>S. aureus</i> (24)	2	7	7	5	2		1					
<i>mecA</i> -positive CoNS (30)		2	3			3	2	2	2	1	5	10
<i>mecA</i> -negative CoNS (17)	2	5	9				1					

Table 4. Discrepant results for *S. aureus* isolates

R, Resistant; S, sensitive.

Strain	No. of isolates	OSA 4 µg ml ⁻¹	OSA 6 µg ml ⁻¹	AD	Oxacillin DD	Cefoxitin DD
<i>mecA</i> -positive	1	R	S	R	R	R
	1	R	R	S	R	S
	4	R	R	R	R	S
<i>mecA</i> -negative	1	R	R	R	R	R
	8	R	R	S	R	S
	1	S	S	S	R	S
	1	R	S	S	S	S

Table 5. Discrepant results for CoNS

R, Resistant; S, susceptible.

Strain	No. of isolates	OSA 4 µg ml ⁻¹	OSA 6 µg ml ⁻¹	AD	Oxacillin DD	Cefoxitin DD
<i>mecA</i> -positive	2	S	S	S	S	S
	2	S	S	S	R	S
	1	R	R	R	R	S
	1	S	S	S	S	R
	1	R	R	R	S	S
	3	R	S	R	R	R
<i>mecA</i> -negative	1	R	S	S	R	S
	1	R	R	R	R	S
	3	S	S	S	R	S

and the oxacillin DD test for both CoNS and *S. aureus*, and that it was comparable with MIC determination (AD test) and the *mecA* PCR. Overall, the accuracy of the cefoxitin DD test was better than that of the oxacillin DD test for the detection of MR staphylococci. It also does not require special testing conditions such as a lower incubation temperature (35 °C) and NaCl supplementation in the testing media, as required by the oxacillin DD test. According to previously reported studies, the sensitivity and specificity of the cefoxitin DD test for *S. aureus* has been reported to be 95–100 % and 98–100 %, respectively (Flayhart *et al.*, 2005; Skov *et al.*, 2003; Swenson & Tenover, 2005; Velasco *et al.*, 2005). The reported sensitivity and specificity for CoNS is 92.5–99 % and 98.6–96 %, respectively (Palazzo & Darini, 2006; Swenson & Tenover, 2005). Cefoxitin is considered to be a better predictor than oxacillin for the detection of heteroresistance because it is a stronger inducer of penicillin-binding protein 2a (PBP2a) (Cauwelier *et al.*, 2004; Felten *et al.*, 2002).

The reported sensitivity and specificity of the oxacillin DD test for *S. aureus* is 90.4–98 % and 83–99 %, respectively (Boutiba-Ben Boubaker *et al.*, 2004a; Kircher *et al.*, 2004). Use of the oxacillin DD test for the detection of MR staphylococci was less specific compared with the cefoxitin DD test, as also reported by Palazzo & Darini (2006). It has been reported that oxacillin resistance among staphylococci is caused by expression of PBP2a encoded by the *mecA* gene, which has a low binding affinity to all β -lactam antibiotics available in clinical practice (Boutiba-Ben Boubaker *et al.*, 2004b; Cauwelier *et al.*, 2004; Skov *et al.*, 2003). Detection of oxacillin resistance is complicated because different populations of staphylococci express different levels of resistance.

The reported sensitivity and specificity of the AD method are 94.4–100 % and 85–100 %, respectively, for *S. aureus* (Flayhart *et al.*, 2005; Sakoulas *et al.*, 2001; Swenson & Tenover, 2005). For CoNS, the reported sensitivity and specificity are 81–100 % and 73.5–97.4 %, respectively (DeGiusti *et al.*, 1999; Ferreira *et al.*, 2003; Palazzo & Darini, 2006; Swenson & Tenover, 2005). The discrepancies obtained in our study were also reported by Geha *et al.* (1994), who maintained that such discrepancies occur due to hyperproduction of β -lactamase. Gerberding *et al.* (1991) reported a specific group of MR isolates, called BORSA (borderline oxacillin-resistant *S. aureus*). These strains were characterized by a methicillin MIC at or just above the susceptibility breakpoint. Borderline strains may be divided into two categories on the basis of whether *mecA* is present or not. Borderline strains that contain *mecA* are extremely heterogeneous MR strains that produce PBP2a. These strains have resistant subpopulations of cells, although these may be quite small, that can grow at high drug concentrations (Gerberding *et al.*, 1991). Borderline strains that do not contain *mecA* can be differentiated phenotypically from extremely heterogeneous *mecA*-positive strains. Borderline resistance in *mecA*-negative strains has been hypothesized to result from modification of the

normal PBP gene or overproduction of staphylococcal β -lactamase (McDougal & Thornsberry, 1986; Tomasz *et al.*, 1989).

Previous studies have reported the sensitivity and specificity of the OSA test to be 94.3–100 % and 83–100 %, respectively (Kircher *et al.*, 2004; Sakoulas *et al.*, 2001; Velasco *et al.*, 2005). According to Ferreira *et al.* (2003), the sensitivity and specificity of the OSA test were 99.7 and 100 %, respectively, at a concentration of 4 $\mu\text{g ml}^{-1}$, and 75.7 and 100 %, respectively, at a concentration of 6 $\mu\text{g ml}^{-1}$.

We conclude that the oxacillin DD test is more sensitive but less specific than the cefoxitin DD test. For accurate screening of MR staphylococci, both cefoxitin and oxacillin discs should be included in the DD test. For confirmation of methicillin resistance, especially in oxacillin-resistant and cefoxitin-sensitive strains, the oxacillin AD test is a reliable method and is comparable to *mecA* PCR.

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