Polish Journal of Microbiology
2006, Vol. 55, No 4, 339–343

Detection of Methicillin Resistance in Hospital Environmental Strains of Coagulase-negative Staphylococci

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Received 3 July 2006, revised 24 September 2006, accepted 29 September 2006

Abstract

The aim of this study was to evaluate methicillin resistance detection methods currently used when studying coagulase-negative staphylococci (CoNS). The resistance to oxacillin of 142 strains from seven species of CoNS isolated from the Intensive Care Unit environments was tested. The methods used were: disc diffusion test with cefoxitin (FOX_{30}) and oxacillin (OX_1), oxacillin agar screen test with 6 mg/l of oxacillin (MHOXA), latex test for PBP2a (LA) and detection of *mecA via* PCR. One hundred and one isolates were methicillin-resistant in at least one of methods used, but only 74 were *mecA*-positive. Of the 68 *mecA*-negative strains: two were positive by OX_1, the LA and MHOXA methods; three by the LA and MHOXA; and 22 only by OX_1 test. Most of these strains were from the novobiocin-resistant CoNS group. The results obtained for all tested strains using FOX_{30} showed complete concordance with the presence of the *mecA* gene.

Key words: cefoxitin disc test, coagulase-negative staphylococci, methicillin resistance

Introduction

The number of nosocomial infections, particularly bloodstream infections caused by coagulase-negative staphylococci (CoNS) has increased in recent years (Marshall *et al.*, 1998). The majority of clinical isolates are resistant to β-lactam antibiotics mainly thought to be due to methicillin (oxacillin) resistance mechanism (Pfaller *et al.*, 1998). More than 70% of CoNS isolates worldwide are resistant to oxacillin (Diekema *et al.*, 2001). *Staphylococcus epidermidis* is often isolated from clinical specimens but other species including novobiocin-resistant ones can also cause serious infection (Ishihara *et al.*, 2001; Mastroianni *et al.*, 1995; Okudera *et al.*, 1991; Tselenis-Kotsowilis *et al.*, 1982). It is vital that the appropriate antimicrobial therapy is applied for patients with these infections as inaccurate detection of oxacillin resistance can lead to important adverse clinical consequences. Furthermore, due to the emergence of vancomycin-resistant staphylococci, it is important for clinical laboratories to distinguish between oxacillin-susceptible (MSCNS) and oxacillin-resistant coagulase-negative staphylococci (MRCNS) strains in order to limit unnecessary use of vancomycin. Thus, accurate and rapid detection of methicillin resistance in CoNS is essential for the success of this strategy.

Laboratories use various tests to determine methicillin resistance, including: disc diffusion, oxacillin agar screening (MHOXA), broth microdilution, agar dilution and the latex agglutination (LA) test for PBP2a. Detection of *mecA* by PCR is still considered the "gold standard", but this methodology is not feasible in every laboratory. Detection of the gene product – protein PBP2a as a marker for methicillin resistance is recommended as an alternative for *mecA* PCR by the National Committee for Clinical Laboratory Standards (NCCLS, 2002).

In 2003, Ferreira *et al.* (2003) showed the accuracy of the MHOXA method with 4 mg/l of oxacillin and detection of PBP2a by the LA test for detection of oxacillin susceptibilities of CoNS strains, suggesting both methods as good alternatives for *mecA* PCR. Since 1999 use of the agar screen test has not been

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recommended by the NCCLS (1999), while Kohner *et al.* (1999) support the use of the MHOXA method for CoNS strains as well as for *Staphylococcus aureus* strains. In 2004, the NCCLS recommended a new disc diffusion test with 30 μg of cefoxitin for detection of oxacillin resistance as a further attempt to detect the correlation between oxacillin resistance detection and the presence of the *mecA* gene (NCCLS, 2004).

Our study evaluated the oxacillin susceptibilities of 142 well-characterized strains of a wide group of CoNS novobiocin-susceptible and novobiocin-resistant species isolated from hospital environments. Four different phenotypic methods were compared with *mecA* gene assay to examine the resistance and the usefulness of these methods for the detection of methicillin resistance in CoNS with special attention to novobiocin-resistant strains which haven't previously been discussed as they are seldom isolated and studied.

Experimental

Materials and Methods

Bacterial strains and identification. Coagulase-negative staphylococci: 142 well-characterized strains of seven species belonging to novobiocin-resistant group (*Staphylococcus saprophyticus*, *Staphylococcus cohnii* and *Staphylococcus xylosus*) and novobiocin-susceptible group (*Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Staphylococcus warneri*) were tested. Strains were collected in 1997 and in 2003 from the hospital environments of the Intensive Care Unit at the Teaching Paediatric Hospital in Łódź, Poland. Identification was performed using standard criteria with particular reference to Kloos and Bannerman (1999) and Freney *et al.* (1999). The API StaphSystem (bioMerieux, Marcy l'Etoile, France) was used as a parallel method of identification.

Disc diffusion test. All strains were tested with 30 μg cefoxitin disc (FOX_{30}) and with 1 μg oxacillin (OX_1) disc on Mueller-Hinton Agar 2 (bioMerieux). Plates were incubated for 24 h in ambient air at 33–35°C (for cefoxitin, results may be reported after 18 h if resistant) according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (CLSI, 2005).

Oxacillin agar screen test (MHOXA). Strains were plated on Mueller-Hinton agar (Difco Laboratories, Detroit, USA) supplemented with 4% NaCl containing 6 mg/l of oxacillin using a cotton swab dipped into a 0.5 McFarland standard suspension of each test strain according to the procedures outlined in the CLSI guidelines for *S. aureus* (CLSI, 2005). Oxacillin resistance was demonstrated by bacterial growth after 48 h of incubation at 35°C.

Detection of PBP2a (DR900A – Oxoid, Basingstoke Hampshire, England). Strains were grown on Columbia agar with 5% sheep blood plates with a 1 μg/ml oxacillin disc placed on the main inoculum. After overnight incubation, cells that grew around the disc were used to perform the test. The test was carried out according to the manufacturer's instructions using 1.5×10^9 CFU/ml inoculum of bacteria. Oxacillin-susceptible *S. aureus* ATCC 29213 and oxacillin-resistant *S. aureus* ATCC 43300 were used as control organisms.

Detection of *mecA* gene. Staphylococcal DNA was extracted by lysostaphin and heating at 99°C as described by van Griethuysen *et al.* (1999). The *mecA* gene was detected by PCR according to the manufacturer's instructions (DNA-GDANSK II, Gdansk, Poland). A 533-bp fragment of the *mecA* gene was amplified in a thermal controller (Tpersonal; Biometra, Göttingen, Germany) and then revealed by electrophoresis on 1.5% agarose gel at 120 V for 40 min.

Results

One hundred and one (71%) of 142 tested strains belonging to seven species of CoNS were methicillin-resistant according to at least one of methods used in this research (Table I), but only 68 presented the same results in all methods used in this research (Table II). Among the 142 CoNS strains 74 were *mecA*-positive and 68 were *mecA*-negative.

Table I
The number of strains suspected of showing methicillin resistance among tested species on the basis of at least one of methods used

Species		Number of strains	Methicillin-susceptible	Methicillin-resistant
Novobiocin-susceptible	*S. epidermidis*	38	21	17
	S. hominis	18	8	10
	S. haemolyticus	7	1	6
	S. warneri	2	1	1
Novobiocin-resistant	*S. cohnii*	73	9	64
	S. xylosus	2	1	1
	S. saprophyticus	2	0	2
	TOTAL	142	41	101

Table II
Detection of methicillin resistance by the recommended methods compared to *mecA* PCR

Number of strains	Methicillin resistance demonstration				
	PCR *mecA*	LA test PBP2a[a]	FOX_{30}[b]	MHOXA[c]	OX_1[d]
68	+	+	+	+	+
22	–	–	–	–	+
3	–	+	–	+	–
6	+	–	+	+	+
2	–	+	–	+	+

[a] LA test PBP2a, the latex agglutination test for PBP2a
[b] FOX_{30}, disc diffusion test with 30 µg of cefoxitin
[c] MHOXA, oxacillin agar screen test with 6 mg/l of oxacillin
[d] OX_1, disc diffusion test with 1 µg of oxacillin

Table III
Credibility of currently used tests for determining the methicillin resistance in particular species of staphylococci

Species	Methicillin resistance demonstration					
	Number of strains	PCR *mecA*	LA test PBP2a	FOX_{30}	MHOXA	OX_1
S. epidermidis	16	+	+	+	+	+
	1	–	+	–	+	+
S. hominis	7	+	+	+	+	+
	2	–	+	–	+	–
	1	–	+	–	+	+
S. haemolyticus	6	+	+	+	+	+
S. warneri	1	+	+	+	+	+
S. cohnii	38	+	+	+	+	+
	19	–	–	–	–	+
	6	+	–	+	+	+
	1	–	+	–	+	–
S. xylosus	1	–	–	–	–	+
S. saprophyticus	2	–	–	–	–	+

The results obtained for all tested strains using cefoxitin disc diffusion showed complete concordance with the presence of the *mecA* gene. Of the 68 *mecA*-negative strains: two were positive by OX_1, the LA and MHOXA methods; three by the LA and MHOXA; and 22 only by OX_1 test (Table II). Our research did not show the correlation between methicillin resistance estimated by expression of protein PBP2a and presence of *mecA* gene in every strain tested (Table II and III).

Almost 18% of strains presented methicillin resistance although *mecA* gene was not found. Such results occurred in the oxacillin disc test (19 *S. cohnii* strains, two *S. saprophyticus* and one *S. xylosus*), but also in MHOXA method and latex agglutination (two strains *S. hominis* and one strain *S. cohnii*). One *S. epidermidis* and one *S.hominis* strains were positive in both the oxacillin tests and in the LA test. Although *mecA* gene was present, the LA test revealed methicillin susceptibility in six strains of *S. cohnii* (Table III).

Discussion

Numerous studies have been conducted to determine the optimal methods for phenotypic detection of methicillin resistance in CoNS. The disc diffusion method currently recommended by the CLSI for all staphylococci is the 30 µg cefoxitin disc method (CLSI, 2005). In our study, all of the MRCNS detected as

methicillin-resistant by this method possessed the *mecA* gene. Our results were in full concordance with data from Stierna-Johsen *et al.* (2005) who tested 110 CoNS isolates from monomicrobial bacteraemia. In our experiments many strains possessed the *mecA* gene and this was in agreement in all cases with cefoxitin resistance. The high percentage positive results, when using other phenotypic methicillin-resistance tests, suggest that resistance to methicillin may be connected with other mechanisms (genes) in these cases.

Among the species most often isolated from clinical sources, the group of novobiocin-susceptible staphylococci, a discordance where results were positive and *mecA* was absent, applied to only 12% of tested strains. Among novobiocin-resistant staphylococci this discordance applied to more than 34% of strains. In the group of novobiocin-resistant strains *S.cohnii* dominated in hospital environments (Szewczyk *et al*., 2000). Six strains of these species in current study also did not demonstrate methicillin resistance in LA test although they were *mecA* and other phenotypic tests positive. Our results clearly show heterogeneity in the coagulase-negative staphylococci group. It seems however, that phenotypic testing with cefoxitin, which easily detects the methicillin resistance of all CoNS species, may be used to determine this feature. Nevertheless, negative opinions appearing in publications cannot be overlooked. Frigatto *et al.* (2005) observed discrepant results between oxacillin and cefoxitin when testing CoNS by disc diffusion and suggested that the detection of low-level methicillin resistance in CoNS by the cefoxitin disc could be problematic. Methicillin resistance in novobiocin-resistant staphylococci detected by phenotypic methods has already been mentioned in some articles concerning the optimisation of such methods for all CoNS (Ramotar *et al.*, 2001; Tenover *et al.*, 1999). However, these results usually came from a limited range of novobiocin-resistant strains and remained outside the main interest of the papers' authors and were left unclear. Analysing studies of Suzuki *et al.* (1992), York *et al.* (1996) and Hussain *et al.* (2000) we noticed that such results have not lead to discussion regarding a few species of staphylococci, mainly: *S. saprophyticus*, *S. cohnii*, *S. xylosus* and *S. lugdunensis*. These data, however, confirmed our observations. Undertaking studies of CoNS strains specifically isolated from hospital environments allowed us to perform a unique review of a large novobiocin-resistant group relatively seldom isolated from clinical materials, a case in point being *S. cohnii* (Basaglia *et al.*, 2003; Gauduchon *et al.*, 2003). Kloos (1997) suggested that the ability of *S. cohnii* and *S. hominis* to share plasmids and antibiotic resistance genes may indicate that these species can act as a bridge for genetic exchange by horizontal gene transfer between novobiocin-susceptible *Staphylococcus epidermidis* group and novobiocin-resistant *Staphylococcus saprophyticus* group. This suggests a clear potential for interspecies spread of antibiotic resistance in CoNS and forces us to consider results relating to novobiocin-resistant species in the hospital.

While the availability of PCR techniques is limited, the cefoxitin disk diffusion test is preferable to the other phenotypic methods, including LA test for detection of MRCNS and seems to be suitable for routine use. The MHOXA test with 6 mg/l of oxacillin, routinely used for *S. aureus* testing, gives more accurate results than the LA test which, in turn, is better than the oxacillin disc method in classifying *mecA*-negative CoNS as oxacillin susceptible. It may be possible that methicillin resistance of the six strains of *S. cohnii*, which were negative in LA test, was due to the presence of proteins other than PBP2a or different unknown mechanisms.

The recent suggestion of Chandran and Rennie (2005) to discontinue routine antibiotic susceptibility testing (AST) of CoNS isolates from blood cultures because of common methicillin resistance, in light of the data put forward by us, could be seen as very controversial. Another conclusion drawn was that the CoNS group is not homogenous and the mechanisms of resistance to β-lactam antibiotics remains, to a large extent, unexplained.

Therefore, we believe that the detection of susceptibility or resistance to methicillin even when using the cefoxitin disc diffusion test should not be considered the only possible option when leading to choices in antimicrobial therapy.

Acknowledgments. This research was presented as a poster during the IX Symposium Advances in Medicine of Infection, December 2005, Warsaw, Poland. This study was supported by grant from the Medical University of Łódź No. 502-13-074.

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