JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2006, p. 934–937 Vol. 44, No. 3
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.3.934–937.2006

Evaluation of Phenotypic and Molecular Methods for Detection of Oxacillin Resistance in Members of the *Staphylococcus sciuri* Group

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Received 25 October 2005/Returned for modification 15 December 2005/Accepted 1 January 2006

In this paper we report on an experimental evaluation of phenotypic and molecular methods as means for the detection of oxacillin resistance in members of the *Staphylococcus sciuri* group. A total of 109 *S. sciuri* group member isolates (92 *S. sciuri* isolates, 9 *S. lentus* isolates, and 8 *S. vitulinus* isolates) were tested by the disk diffusion method, the agar dilution method, the oxacillin salt-agar screening method, slide latex agglutination for PBP 2a, and PCR assay for *mecA* as the reference method. The *mecA* gene was detected in 29 *S. sciuri* isolates, and the true-positive and true-negative results of the other tests were defined on the basis of the presence or the absence of the *mecA* gene. For the different methods evaluated, the sensitivities and specificities were as follows: for the disk diffusion test with a 1-μg oxacillin disk, 100% and 55.9%, respectively; for the disk diffusion test with a 30-μg cefoxitin disk, 93.5% and 100%, respectively; for the agar dilution method, 100% and 50%, respectively; for the oxacillin salt-agar screen test (with 6 μg of oxacillin per ml and 4% NaCl) 100% and 100%, respectively; and for the slide latex agglutination test for PBP 2a, 100% and 100%, respectively. The disk diffusion test with various β-lactam antibiotics was performed to evaluate their use for the prediction of oxacillin resistance. The results indicate that meropenem, cefazolin, cefamandole, cefuroxime, cefotetan, cefoperazone, cefotaxime, ceftriaxone, moxalactam, cefaclor, and cefprozil may be used as surrogate markers of oxacillin resistance, although further studies of their use for the detection of oxacillin resistance are required.

Staphylococcus sciuri, *Staphylococcus lentus*, and *Staphylococcus vitulinus* are novobiocin-resistant, oxidase-positive, coagulase-negative staphylococci (CoNS) which compose the *Staphylococcus sciuri* group. The rate of isolation of these bacteria from humans ranges from 0.042% from the urinary tract (31) to 0.087% from the female genital tract (32). They constitute between 0.79% and 4.3% of the total number of CoNS isolated from human clinical samples (12, 31). Although they are rarely isolated from humans, 21.4% of the *S. sciuri* group isolates were found to be clinically significant (29). These bacteria have been associated with serious human infections, such as endocarditis (14), peritonitis (37), septic shock (15), urinary tract infections (31), endophthalmitis (1), pelvic inflammatory disease (32), and, most frequently, wound infections (28, 30). The sources of *S. sciuri* group bacteria for humans are animals (13, 16, 33); food of animal origin (10, 24); and the environment, such as soil, sand, and water (16, 23), as well as the hospital environment (7).

Oxacillin-resistant staphylococci are recognized as important nosocomial and, recently, community pathogens. The major mechanism of oxacillin resistance in staphylococci is mediated by the production of PBP 2a, which is encoded by the *mecA* gene (3). *S. sciuri* attracted special attention after it was suggested that the *mecA* gene of oxacillin-resistant staphylococci originated from an evolutionary relative of the *mecA* homologue that has been identified in *S. sciuri* (5, 6, 26). The *mecA* homologue performs a normal physiological function in this bacterium unrelated to oxacillin resistance (5, 6, 26). Strains of *S. sciuri* showing resistance to oxacillin have both the *S. sciuri mecA* homologue and the "true" *mecA* (the *mecA* gene of oxacillin-resistant staphylococci) (5). However, a few natural *S. sciuri* strains exhibiting resistance to oxacillin, which is mediated by a high rate of transcription of the *S. sciuri mecA* homologue and which is caused by a single nucleotide alteration or insertion of IS*256* upstream of the *mecA* homologue, have been reported (6).

Oxacillin-resistant staphylococci are frequently resistant to other antibiotics. The treatment of choice for infections caused by these microorganisms is often vancomycin. On the other side, infections caused by oxacillin-susceptible staphylococci are more effectively treated with β-lactam antibiotics because of their higher intrinsic activities, better concentrations in tissues, low incidence of side effects, more rapid bactericidal action, and low therapeutic cost (38). Moreover, the recent emergence of staphylococci with reduced susceptibility to vancomycin, including *S. sciuri* (4, 36), further emphasized the importance of the exact differentiation between oxacillin-susceptible and oxacillin-resistant staphylococci. However, numerous studies have reported problems with the detection of oxacillin resistance, particularly in strains of CoNS other than *Staphylococcus epidermidis*. In this study we evaluated phenotypic and molecular biology-based methods for the detection of oxacillin resistance in members of the *S. sciuri* group.

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TABLE 1. Correlation between inhibition zone diameter and the presence of the *mecA* gene after 24 h of incubation at 35°C

Antibiotic	Inhibition zone diam (mm) (avg [range])			
	mecA-positive *S. sciuri* (n = 29)	*mecA*-negative *S. sciuri* (n = 63)	*mecA*-negative *S. lentus* (n = 9)	*mecA*-negative *S. vitulinus* (n = 8)
Oxacillin (1 μg)	6–13 (6.41)	6–20 (10.87)	6–19 (12.56)	18–22 (20.50)
Methicillin (5 μg)	6 (6.0)	13–26 (18.02)	13–24 (18.56)	21–24 (22.75)
Meropenem (10 μg)	13–28 (23.10)	31–42 (34.43)	36–40 (39.11)	38–42 (39.75)
Imipenem (10 μg)	27–40 (33.72)	40–50 (43.30)	44–50 (47.56)	44–54 (48.50)
Cefazolin (30 μg)	6–26 (17.24)	27–44 (32.38)	32–36 (34.11)	36–40 (38.75)
Cefamandole (30 μg)	6–26 (19.55)	29–44 (33.33)	35–39 (36.44)	38–42 (39.25)
Cefuroxime (30 μg)	6–14 (7.21)	16–29 (23.48)	21–31 (25.44)	29–37 (32.75)
Cefotetan (30 μg)	6–14 (8.52)	15–24 (18.95)	15–23 (19.22)	23–26 (24.25)
Cefoxitin (30 μg)	6–28 (16.24)	24–38 (29.62)	31–36 (34.78)	34–36 (34.75)
Cefoperazone (75 μg)	6–21 (14.24)	24–33 (27.84)	28–36 (30.78)	31–36 (33.63)
Cefotaxime (30 μg)	6–14 (7.28)	20–30 (23.43)	21–32 (26.33)	28–34 (31.50)
Ceftizoxime (30 μg)	6–11 (6.17)	14–35 (22.81)	11–31 (22.22)	26–35 (31.88)
Ceftriaxone (30 μg)	6–12 (7.07)	15–27 (20.90)	20–35 (25.00)	26–32 (29.38)
Moxalactam (30 μg)	6–19 (11.83)	20–31 (24.92)	23–32 (28.33)	28–35 (32.75)
Cefaclor (30 μg)	6–22 (10.97)	28–39 (31.67)	33–40 (35.56)	34–40 (38.25)
Cefprozil (30 μg)	6–25 (10.69)	27–39 (31.41)	32–38 (34.78)	33–42 (37.75)

MATERIALS AND METHODS

Bacterial strains. A total of 109 *S. sciuri* group isolates, isolated between 1998 and 2004, were investigated in this study. The group of isolates tested comprised 92 *S. sciuri* isolates, 9 *S. lentus* isolates, and 8 *S. vitulinus* isolates. Of these 109 isolates, 26 were isolated from humans (wound, central venous catheter, urine, cavum douglasi, cervix, vagina), 38 were isolated from animals (dog, piglet, chicken), and 45 were isolated from the environment (hospital environment, slaughterhouse, house, house yard, bakery). All isolates included in the survey were unrelated according to the results of pulsed-field gel electrophoresis analysis (7; unpublished data). The isolates were stored at −80°C until they were analyzed. Some of the isolates have been reported previously but were not investigated for their oxacillin resistance by the methods presented in this study (7, 29, 33).

Susceptibility testing. The susceptibilities of the *S. sciuri* group isolates were tested by the disk diffusion method, the agar dilution method, and the agar screening method. Mueller-Hinton agar (Oxoid Limited, Basingstoke, United Kingdom) was used for propagation and testing of the strains. Bacterial suspensions equal to a 0.5 McFarland standard were prepared in 0.9% saline by using a Densimat densitometer (bioMérieux, Marcy l'Etoile, France) and were further diluted as required.

The sensitivity of each test was calculated as the ratio of the number of isolates with true-positive results (*mecA*-positive isolates) and the sum of the number of isolates with true-positive and the number of isolates with false-negative results. Specificity was calculated as the ratio of the number of isolates with true-negative results (*mecA*-negative isolates) and the sum of the number of isolates with false-positive and the number of isolates with true-negative results.

Disk diffusion test. Oxacillin disk diffusion susceptibility testing was performed with 1-μg oxacillin (BBL, Becton Dickinson and Company, Cockeysville, Md.) and 30-μg cefoxitin (Bioanalyse Co., Ltd., Ankara, Turkey) disks. The test was carried out on Mueller-Hinton agar without NaCl supplementation, following the recommendations given by CLSI (formerly the National Committee for Clinical Laboratory Standards) (21). Zone diameters were measured after 24 h of incubation at 35°C and were interpreted according to the CLSI recommendations (22).

Disk diffusion testing with 5-μg methicillin (Oxoid), 10-μg meropenem (Oxoid), 10-μg imipenem (BBL), 30-μg cefazolin (Bioanalyse), 30-μg cefamandole (Oxoid), 30-μg cefuroxime (Bioanalyse), 30-μg cefotetan (Bioanalyse), 75-μg cefoperazone (Bioanalyse), 30-μg cefotaxime (BBL), 30-μg ceftizoxime (Bioanalyse), 30-μg ceftriaxone (Oxoid), 30-μg moxalactam (HiMedia, Mumbal, India), 30-μg cefaclor (Oxoid), and 30-μg cefprozil (Bioanalyse) disks was performed to evaluate the use of these antibiotics for the prediction of oxacillin resistance.

Agar dilution method. The MIC of oxacillin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was determined by the agar dilution method on Mueller-Hinton agar containing 2% NaCl, in accordance with the CLSI recommendations (20).

Agar screening method. Oxacillin-salt agar (Mueller-Hinton agar containing 4% NaCl and 6 μg oxacillin/ml), which is recommended by the CLSI, was used for the agar screening method (20). Inoculation of the oxacillin-salt agar was performed by spreading a 1-μl loopful of a 0.5 McFarland suspension over an area of 10 mm in diameter.

β-Lactamase test. Production of β-lactamase was determined by the nitrocefin method. Colonies from the edge of the inhibition zone of a 10-U penicillin disk (Oxoid) were streaked onto a nitrocefin disk (BBL), and β-lactamase production was characterized by the appearance of a pink color. The amoxicillin-clavulanic acid disk test was performed to determine β-lactamase hyperproduction (11).

Slide latex agglutination for PBP 2a. The presence of PBP 2a was determined for 45 randomly selected isolates by using a rapid latex agglutination test, the MRSA-Screen (Denka Seiken, Tokyo, Japan). Colonies from the edge of the inhibition zone of a 1-μg oxacillin disk (Oxoid) were used for the test.

PCR for *mecA* genes. The primers described by Couto et al. (5) were used to evaluate all isolates tested for the presence of the *S. sciuri mecA* homologue gene. All isolates were evaluated for the presence of the *S. aureus mecA* gene by use of previously described primers and protocols (2, 18).

RESULTS

Among the 109 *S. sciuri* group isolates tested, the *mecA* gene was detected in 29 isolates (26.6%). The *mecA* gene was detected in 6 of 21 (28.6%) human *S. sciuri* isolates, 9 of 34 (26.5%) animal *S. sciuri* isolates (all from healthy domestic dogs), and 14 of 37 (37.8%) environmental *S. sciuri* isolates (all from the hospital environment). The true-positive and true-negative results of the other tests were defined on the basis of the presence or the absence of the *mecA* gene. The presence of the *S. sciuri mecA* homologue gene was detected in all 92 *S. sciuri* isolates. It was not detected in any *S. lentus* or *S. vitulinus* isolate. All oxacillin-resistant *S. sciuri* isolates had both copies of the *mecA* gene, the *S. sciuri mecA* homologue, and the *mecA* gene of oxacillin-resistant staphylococci.

The disk diffusion test with a 1-μg oxacillin disk (zone diameter, ≤17 mm) identified oxacillin resistance not only in all 29 *S. sciuri mecA*-positive isolates but also in 63 *mecA*-negative isolates, 55 *S. sciuri* isolates, and 8 *S. lentus* isolates. The overall sensitivity of the disk diffusion method with a 1-μg oxacillin disk was 100%, while its specificity was only 55.9%. We repeated oxacillin susceptibility testing by the disk diffusion method and also performed the test with oxacillin disks from other manufacturers (Oxoid and Torlak, Belgrade, Serbia), but the specificity remained low (data not shown). Much better

TABLE 2. Correlation between oxacillin MIC and presence of the *mecA* genes

Species	No. of strains for which the oxacillin MIC (μg/ml) is as follows:												
	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
S. sciuri (n = 92)		36	27					2[a]		3[a]	16[a]	7[a]	1[a]
S. lentus (n = 9)		2	7										
S. vitulinus (n = 8)		8											

[a] *mecA*-positive isolates.

results were obtained with the cefoxitin disk. Oxacillin resistance, determined by disk diffusion test with a 30-μg cefoxitin disk (zone diameter, ≤24 mm), was identified in 27 *S. sciuri mecA*-positive isolates but not in any of the *mecA*-negative isolates, resulting in 93.5% sensitivity and 100% specificity. The results of the disk diffusion test obtained with other β-lactam antibiotics are summarized in Table 1.

Oxacillin resistance, as determined by the agar dilution method (oxacillin MIC, ≥0.5 μg/ml), was identified in all 29 *S. sciuri mecA*-positive isolates, as well as in all 80 *mecA*-negative isolates (Table 2). The MIC for *S. aureus* ATCC 29213 was 0.25 μg/ml, and the MIC for *S. aureus* ATCC 25923 was 0.125 μg/ml. The results indicate 100% sensitivity and 50% specificity for this test.

All *mecA*-positive isolates were detected by the agar screening method after an incubation period of 24 h at 35°C. Furthermore, none of the *mecA*-negative isolates grew under these conditions (100% sensitivity and 100% specificity).

The slide latex agglutination for PBP 2a was performed with 11 *mecA*-positive *S. sciuri* isolates, as well as with 23 *S. sciuri*, 8 *S. lentus*, and 3 *S. vitulinus mecA*-negative isolates. The test showed 100% sensitivity and 100% specificity, since all *mecA*-positive isolates gave positive results and all *mecA*-negative isolates gave negative results.

β-Lactamase production was noted in 19 of 29 (65.5%) *mecA*-positive *S. sciuri* isolates, as well as in 39 of 80 (48.7%) *mecA*-negative *S. sciuri* group members isolates, 31 *S. sciuri* isolates, and 8 *S. lentus* isolates. β-Lactamase production was not detected in any *S. vitulinus* isolate tested. β-Lactamase hyperproduction was not observed in any of the isolates tested.

DISCUSSION

The molecular biology-based methods that search for the presence of the *mecA* gene are considered the "gold standard" for the detection of oxacillin resistance in staphylococci, even though these methods do not detect resistance unrelated to *mecA* (3). In the present study, the *mecA* gene was detected in one-third of the *S. sciuri* isolates tested. Since animals and the environment are considered the main reservoirs of *S. sciuri*, the result of particular interest is the presence of relatively high numbers of isolates with the *mecA* gene among animal (26.5%) and environmental (37.8%) *S. sciuri* strains. *S. lentus* and *S. vitulinus* are less frequently isolated from humans than *S. sciuri* (29). In this study the *mecA* gene was not detected in any of the isolates of these two species tested, irrespective of their origin.

Owing to the technical difficulties associated with the molecular biology-based method and its high cost, the disk diffusion method remains the method of choice for routine screening for oxacillin resistance in a number of bacteriological laboratories. In our study the disk diffusion method with a 1-μg oxacillin disk showed 100% sensitivity, but false-positive results were found for 63 isolates, resulting in a low specificity of 55.9%. The discrepancy between the presence of *mecA* and the results of susceptibility testing obtained by the disk diffusion method with a 1-μg oxacillin disk in various staphylococci, including *S. sciuri* strains, was also noted by other authors (9, 17, 19, 34).

Recently, the CLSI recommended the use of cefoxitin for screening for oxacillin resistance. No false-positive results were noted in our study with a 30-μg cefoxitin disk. However, two *mecA*-positive *S. sciuri* isolates gave false-negative results after 24 h of incubation. Other studies confirm the better performance of the 30-μg cefoxitin disk test compared to that of the 1-μg oxacillin disk test for the detection of *mecA*-mediated oxacillin resistance (8, 25, 34). However, even cefoxitin was not able to completely separate *mecA*-positive from *mecA*-negative isolates in our study, as was also noted by other authors (19, 34).

Therefore, we tested other β-lactam antibiotics as potential markers of oxacillin resistance. With the exception of imipenem and ceftizoxime, there was no overlap of the size of the inhibition zone between *mecA*-positive and *mecA*-negative strains for the other β-lactam antibiotics tested. This indicates that these β-lactam antibiotics may potentially be used as markers of oxacillin resistance. The application of β-lactam antibiotics as potential markers of oxacillin resistance was also studied by other authors (8, 25). While the differences in interpretative criteria make comparison of the results difficult, there is general agreement that other β-lactam antibiotics may be used as surrogate markers for the detection of oxacillin resistance (8, 25).

The oxacillin salt-agar screen plate test exhibited 100% sensitivity and 100% specificity. These results are similar to some previous results (9, 17) but not to others (27, 35). It was shown that several factors, such as the source of the Mueller-Hinton agar base, the procedure used to prepare the medium, the inoculum size used, the temperature of incubation, and the length of incubation, may influence the results obtained by the oxacillin salt-agar screen plate test (9, 17, 27, 35).

According to the currently recommended breakpoints for oxacillin, all strains with the *mecA* gene were correctly recognized as oxacillin resistant by the dilution method. However, the dilution method exhibited very low specificity, since all *mecA*-negative isolates were also classified as oxacillin resistant due to the oxacillin MICs of 0.5 and 1 μg/ml obtained. Similar oxacillin MICs for *mecA*-negative *S. sciuri* isolates were also obtained by other authors (5, 9, 26, 34). Discrepancies between the oxacillin MICs obtained and the actual presence of the *mecA* gene were also noted in other studies which tested CoNS isolates other than *S. epidermidis* (9, 17, 34, 38). *S. epidermidis* is the major species of CoNS tested in clinical laboratories, and the oxacillin MIC breakpoint was selected to be the best choice for maximization of the sensitivity for detection of *mecA*-positive *S. epidermidis* isolates (35). Therefore, the CLSI recommends detection of the presence of *mecA* or PBP 2a for CoNS other than *S. epidermidis* for which oxacillin MICs are 0.5 to 2 μg/ml. Various rapid slide latex agglutination tests for PBP 2a have been extensively evaluated. It has been shown that the sensitivity of the assay was improved when the test was per-

formed with isolates in which oxacillin resistance has been induced or when a larger inoculum was used (9, 17, 19). In this study we enhanced PBP 2a expression only before testing. After that the latex agglutination test showed 100% sensitivity and 100% specificity.

In conclusion, the cefoxitin disk diffusion method was found to be a more suitable alternative than the oxacillin disk diffusion method. The other β-lactam antibiotics tested seem to have significant potential as surrogate markers for the detection of oxacillin resistance, although these results need to be confirmed with a large number of different staphylococcal species. The technically simple slide latex agglutination test for PBP 2a was also accurate for the detection of oxacillin resistance in members of the *S. sciuri* group.

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