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Evaluation of a cefoxitin disk diffusion test for the detection of *mecA*-positive methicillin-resistant *Staphylococcus saprophyticus*

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Abstract

In order to validate the current Clinical and Laboratory Standards Institute (CLSI) criteria for the detection of *mecA*-mediated resistance in *Staphylococcus saprophyticus*, 101 clinical isolates, including 8 *mecA*-positive isolates, were investigated. All the isolates were in the range of the resistant category for coagulase-negative staphylococci with the 1 μg oxacillin disk diffusion method and agar dilution method, despite 93 isolates (92%) being *mecA*-negative. On the other hand, the 30 μg cefoxitin disk diffusion method showed clearly distinguishable zone diameters between the *mecA*-positive and -negative isolates. However, four of the *mecA*-negative isolates that would be considered resistant were false positive, and the current interpretive criteria of the CLSI may thus require reconsideration. This study suggests that the cefoxitin disk diffusion method could be more suitable than the oxacillin disk diffusion method for detecting *mecA*-mediated resistance in *S. saprophyticus*.

Keywords: *Staphylococcus saprophyticus*; Coagulase-negative staphylococci; *mecA*; Cefoxitin

1. Introduction

Staphylococcus saprophyticus is a member of the coagulase-negative staphylococci (CoNS), which frequently cause uncomplicated urinary tract infections (UTIs) in young and middle-aged female outpatients [1–5]. CoNS are part of the normal human flora, but serious problems with opportunistic infection by CoNS have frequently been reported along with the emergence of methicillin-resistant *Staphylococcus epidermidis*, which carries the *mecA* gene encoding penicillin-binding protein 2′ (PBP2′; also called PBP2a) [6–12]. Unlike most other CoNS, *S. saprophyticus* strains are rarely found to be resistant to antibiotics active against Gram-positive organisms [2,4]. The current interpretive criteria for methicillin-resistant CoNS (MRCoNS), except *Staphylococcus lugdunensis*, of the Clinical and Laboratory Standards Institute (CLSI) [13] are as follows: 1 μg oxacillin disk zone diameter of *mecA*-positive isolates ≤17 mm; oxacillin minimum inhibitory concentration (MIC) of *mecA*-positive isolates ≥0.5 μg/mL. These criteria are well adapted to detecting MRCoNS including *S. epidermidis*, *Staphylococcus hominis* and *Staphylococcus haemolyticus*, as described in other reports [8,9]. In contrast, the criteria for methicillin-resistant *S. lugdunensis*, another member of the CoNS, have recently been revised to the same criteria as those for *Staphylococcus aureus* [13]. Other groups have reported that all of their *mecA*-negative *S. saprophyticus* isolates would be considered oxacillin resistant (MIC ≥ 0.5 μg/mL) and the CLSI also states that the interpretive criteria may overestimate resistance for other CoNS, e.g. *S. saprophyticus* [8,9,13,14]. Given that these findings are totally consistent with our observations in this study, some modification of the breakpoint might be required for detection of resistance. We evaluated whether the current standards for MRCoNS are suitable for detection and whether an alternative method using the 30 μg cefoxitin disk diffusion method recommended by the CLSI in 2004 [15–17]

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could be effective for detecting *mecA*-mediated resistance in *S. saprophyticus*.

2. Material and methods

2.1. Bacterial isolates

A total of 101 *S. saprophyticus* strains were clinically isolated from urine specimens (77 isolates) and vaginas (24 isolates) of individual patients at 65 Japanese hospitals from April–December 2003. The strains were identified as CoNS by multiple assays with Gram staining, catalase production, coagulase production (Eiken Chemical Co. Ltd., Tokyo, Japan), DNase production (Eiken Chemical Co. Ltd.), growth on egg yolk mannitol salt agar (Becton Dickinson, Franklin Lakes, NJ) and the novobiocin susceptibility test (Showa Yakuhin Kako Co. Ltd., Tokyo, Japan). The final identification was conducted using ID 32 STAPH by the mini API system (bioMérieux, Marcy l'Etoile, France). *Staphylococcus saprophyticus* ATCC 15305, *S. aureus* ATCC 25923 and *S. aureus* N315 were used as quality control strains for the antimicrobial susceptibility tests.

2.2. Antibiotic susceptibility tests

All isolates were tested using disk diffusion methods with a 1 μg oxacillin disk, a 30 μg cefoxitin disk and a 30 μg ceftizoxime disk (Becton Dickinson) on Mueller–Hinton agar (Becton Dickinson) with 24 h incubation at 35 °C, according to the methods of the CLSI [13] and the National Committee for Clinical Laboratory Standards (NCCLS) [18]. The oxacillin (Sigma, St Louis, MO) MIC was determined by the agar dilution method, according to the CLSI [13] and NCCLS [19]. Oxacillin concentrations were 0.25–256 μg/mL.

2.3. Detection of mecA-positive S. saprophyticus

The *mecA*-positive isolates were detected by dot-blot hybridisation. All 101 isolates of *S. saprophyticus* were cultivated with 200 μL of brain–heart infusion broth in a 96-well tissue culture plate at 37 °C overnight. Fifty microlitres of the culture was mixed with 20 μL of lysis buffer (10 mM Tris/HCl (pH 8.0), 1 mM ethylene diamine tetraacetic acid (EDTA), 2 μg/mL lysostaphin (Wako Pure Chemical Industries Ltd., Osaka, Japan)) and incubated at 37 °C for 1 h to achieve complete lysis of cells. The cell lysate was mixed with 30 μL of DNA denaturing buffer (1 N sodium hydroxide, 0.5 M EDTA) and incubated at 65 °C for 1 h. Five microlitres of each denatured chromosomal DNA aliquot were spotted onto GeneScreen Plus hybridisation membrane (DuPont NEN, Boston, MA) and the membrane was neutralised by immersion in 0.5 M Tris/HCl (pH 7.5). The membrane was subjected to hybridisation at 55 °C for 18 h using *S. aureus* N315 *mecA*-specific gene probe by the AlkPhos Direct Labelling and Detection System (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. The positive hybridised spot was detected by chemiluminescence with CDP-Star as well as with *mecA* polymerase chain reaction (PCR) product as a positive control. The *mecA* PCR product was amplified with oligo primers (5′-GGTAACATTGATCGCAACG-3′; 5′-GAGGTGCGTTAATATTGCC-3′) and with *S. aureus* N315 chromosomal DNA as template.

3. Results

Among 101 *S. saprophyticus* clinical isolates, 8 *mecA*-positive isolates were detected by PCR amplification and dot-blot hybridisation using gene-specific primers and probes, respectively (data not shown). One *mecA*-positive isolate was from a vaginal specimen and the other seven isolates were from urine specimens. The oxacillin MICs of the *mecA*-positive isolates were 64–256 μg/mL and those of the *mecA*-negative isolates were 0.5–4 μg/mL (MIC_{90}, 1 μg/mL) (Fig. 1A). One of the 93 *mecA*-negative isolates showed a relatively high oxacillin MIC (4 μg/mL). The current CLSI criteria define CoNS having an oxacillin MIC ≥ 0.5 μg/mL as MRCoNS, except for *S. lugdunensis*. Thus, all these isolates would be considered MRCoNS even though 93 of them were *mecA* negative. The results of the oxacillin disk diffusion method also indicated that all isolates had a zone diameter of ≤16 mm and were therefore MRCoNS, suggesting that the oxacillin disk diffusion method and the current breakpoint for MRCoNS could be unreliable in the case of *S. saprophyticus* (Fig. 1B).

Since the cefoxitin disk diffusion test is an alternative method for detecting *mecA*-positive staphylococci, the CLSI recommends the use of the 30 μg cefoxitin disk diffusion test. The cefoxitin disk zone diameters of the *mecA*-positive and *mecA*-negative isolates were ≤16 mm and 22–33 mm, respectively (Fig. 1C). The current breakpoint for resistance to β-lactams of MRCoNS using the cefoxitin disk is ≤24 mm, thus four of the *mecA*-negative isolates were false-positive resistant. Using the current standards against the cefoxitin disk diffusion method, the sensitivity of *mecA* detection was 66.7% (8 *mecA*-positive isolates of 12 isolates categorised as resistant) and the specificity was 100% (89 *mecA*-negative isolates of 89 isolates categorised as susceptible). In contrast to cefoxitin, one *mecA*-negative isolate as well as the *mecA*-positive isolates were found to show no inhibition zone using the 30 μg ceftizoxime disk diffusion method, suggesting that the method showed relatively low sensitivity for detecting *mecA*-mediated resistance (Fig. 1D).

A scattergram comparing the results of oxacillin MICs (Fig. 1A) and the zone diameters of the 30 μg cefoxitin disk (Fig. 1C) is shown, with *mecA*-negative and -positive *S. aureus* as control strains (Fig. 2). A good correlation coefficient ($r = 0.8929$) was obtained with the results of these two detection methods, suggesting that cefoxitin