Detecting methicillin resistance in *Staphylococcus aureus*: comparison of different phenotypic methods and the polymerase chain reaction

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ABSTRACT

Cefoxitin is a more potent inducer of the *mecA* regulatory system compared to oxacillin in *Staphylococcus aureus*. It has been recommended for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) when using disk-diffusion testing. The aim of this study is to compare the results of cefoxitin and oxacillin disk-diffusion methods against the polymerase chain reaction (PCR) for the detection of MRSA. A total of 75 strains of *S. aureus* isolated from different clinical specimens (e.g., pus, blood, wound swabs, tracheal aspirates, eye swabs and cerebrospinal fluid [CSF]) were screened for methicillin resistance by PCR and cefoxitin and oxacillin disk-diffusion tests. The antibiotic susceptibility testing was performed by the Baur disk-diffusion method using oxacillin (1 µg) and cefoxitin (30 µg) disks on Mueller Hinton agar (MHA), and zone diameters as recommended by CLSIs were read at 18 h and 24 h. All MRSA isolates detected phenotypically were confirmed by PCR for the amplification of *mecA* and *nucA* genes. Of the 75 isolates screened, 27 were resistant to oxacillin, 30 were resistant to cefoxitin using the disk-diffusion method, while 30 isolates were confirmed as MRSA by PCR. Performing the cefoxitin disk-diffusion method using a 30 µg disk could be a reliable and more accurate method to detect methicillin resistance in *S. aureus* strains in situations where *mecA* PCR cannot be performed.

KEY WORDS: Cefoxitin.
Disk diffusion antimicrobial tests.
mecA gene.
Methicillin-resistant Staphylococcus aureus.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have recently become the focus of intensive media attention. Over the past few years, national surveillance data and public health research have demonstrated that healthcare-associated infections (HAIs) take a major human toll on society. The overall morbidity and mortality associated with HAI are enormous. Some 5–10% of all hospital admissions are complicated by HAI in the USA and Western Europe.[1] In the United States, an estimated 1.7–2 million people per year develop an HAI and nearly 100,000 die.[2] Therefore, HAIs are among the top 10 leading causes of death in the USA.[2]

Methicillin-resistant *S. aureus* is one of the major pathogens associated with serious nosocomial infection because these strains generally show multiple drug resistance which limits treatment possibilities.[3] Historically, infection by *S. aureus* were controlled using methicillin and its analogues, but in 1961 the first strain of MRSA was isolated.[4–6] Since then, MRSA has been found worldwide, especially in hospitals and nursing homes.[4,7,8]

Hospital-acquired infections due to MRSA have been associated with an increase in length of hospital stay, mortality rate and healthcare costs.[9,10] The emergence of MRSA has become a worldwide problem[11] and isolates are now becoming multidrug resistant.[12]

Methicillin-resistant *S. aureus* is now a major health problem and is no longer confined to healthcare settings.[13] Reports of MRSA infections occurring in the community (e.g., schools, day care centres, sports teams) and deaths in healthy children and adults have heightened public awareness. The press has labelled it a 'superbug' which killed more people in the USA in 2005 than did acquired immune deficiency syndrome (AIDS).[14]

Rapid and accurate identification of MRSA is required for therapeutic and epidemiological reasons, in order to commence appropriate antimicrobial therapy and avoid the spread of these strains.[15,16] Genotypic tests involving detection of the *mecA* gene by the polymerase chain reaction (PCR) is the preferred method,[17,18] but this is not practical for routine use in many clinical laboratories.

Detection of methicillin resistance using routine susceptibility methods, include oxacillin disk diffusion (ODD), oxacillin agar screening (OAS) and determination of minimum inhibitory concentration (MIC) of oxacillin by broth dilution or E-test, are known to be problematic due to factors including temperature and concentration of NaCl, temperature, inoculum and test agent.[15] Thus, there remains a need for a reliable test that can be performed easily in routine situations.

Recently, the cefoxitin disk-diffusion method has been proposed as an alternative method for detecting MRSA.[19] Cefoxitin, a semi-synthetic cephamycin, is a potent inducer

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Table 1. Oxacillin (1 µg) inhibition zone diameters.

		Results at zone diameter (mm)												
	No	<6	7	8	9	10	11	12	13	14	15	16	17	18
Positive	27	15	12											
Negative	48									27	8	10	3	

Table 2. Cefoxitin (30 µg) inhibition zone diameters.

		Results at zone diameter (mm)												
	No	<14	15	16	17	18	19	20	21	22	23	24	25	26
Positive	30	14	7	6	3									
Negative	45											23	13	9

of the *mecA* regulatory system.[20] Many strains of *S. aureus* that are hyper-producers of β-lactamases appear resistant to oxacillin (1 µg), while some hetero-resistant strains appear sensitive. These problems could be circumvented by cefoxitin as it detects all *mecA*-positive staphylococci including hyper-producers of β-lactamases and hetero-resistant strains.[21]

According to recent guidelines issued by the Clinical and Laboratory Standards Institute (CLSI), the cefoxitin disk-diffusion method is recommended for the detection of MRSA; therefore, the present study aims to compare the performance of oxacillin disk-diffusion and cefoxitin disk-diffusion methods for the detection of MRSA, and compare them to *mecA* PCR, which is considered the gold standard.

Materials and methods

A total of 75 strains of *S. aureus* were isolated from various clinical specimens, including from blood, pus, wounds, tracheal aspirates, cerebrospinal fluid (CSF) and eyes. All specimens were collected prior to the use antibiotic therapy, and all were identified as *S. aureus* by standard procedures (i.e., Gram staining, catalase, coagulase and DNase tests).[22]

Susceptibility testing

Methicillin resistance was determined using oxacillin (1 µg) and cefoxitin (30 µg) disks (Oxoid, UK), as recommended by CLSI.[23] Overnight *S. aureus* cultures were adjusted to a turbidity consistent with a 0.5 McFarland standard, and bacterial suspension was spread on Mueller-Hinton agar (MHA) plates and oxacillin (1 µg) and cefoxitin (30 µg) disks were applied. All plates were incubated at 35–36˚C for 18–24 h before reading the results.

DNA extraction

Extraction of DNA from the *S. aureus* isolates was performed using a standard method.[24] Briefly, a loop of growth from a fresh culture on sheep blood agar plates was transferred to an Eppendorf tube containing 1 mL sterile distilled water. The suspension was heated for 15 min at 100˚C in a water bath and centrifuged at 10,000 x*g* for 10 min. The procedure was standardised and it was established that 1 µL boiled suspension yielded sufficient template DNA for the PCR reaction. It was then suspended in 100 µL lysis solution (20 mmol/L Tris HCl, 140 mmol/L NaCl, 5 mmol/L EDTA [pH 8.0]). Three units of lysostaphin were added and the suspension was incubated at 37˚C for 3 h. Distilled water (200 µL) was added and incubated at 95˚C for 5 min. Phenol-chloroform extraction and ethanol precipitation steps were then performed for DNA extraction.

The PCR method was performed to amplify a 533 bp portion of the *mecA* gene using the primers mecA1 (5-AAA ATC GAT GGT AAA GGT TGC C-3) and mecA2 (5-AGT TCT GCA GTA CCG GAT TTG C-3) and a 275 bp portion of the *nucA* gene using the primers nucA1 (5-GCG ATT GAT GGT GAT ACG GTT-3) and nucA2 (5-AGC CAA GCC TTG ACG AAC TAA AGC-3). Amplification of the reaction mixture was carried out in a thermal cycler (PTC-100, MJ Research, USA).

The reaction mixture contained 200 mmol/L dNTP, 2.5 mmol/L $MgCl_2$, 10x reaction buffer, 2.5 units *Thermus aquaticus* (*Taq*) DNA polymerase (Sigma, St. Louis MO, USA), 30 ng DNA and 0.2 µmol/L each primer in a total volume of 25 µL in a PCR vial.[25] The PCR reaction consisted of denaturation for 10 min at 94˚C; 30 cycles of 30 sec at 94˚C; 30 sec at 55˚C; 30 sec at 72˚C; and a final 10-min extension step at 72˚C. The amplified products were detected on 0.8% agarose gel containing ethidium bromide and electrophoresed at a constant 150 V for 30 min. The gels were then visualised using an ultraviolet (UV) transilluminator and photographed.

Results

Of the 75 *S. aureus* isolates examined, 22 were from blood, 19 from pus, 18 from wound swabs, 12 from tracheal aspirates, three from CSF and one from an eye swab. Of

Table 3. Evaluation of different methods for detection of MRSA.

	Resistant	Susceptible	Sensitivity (%)	Specificity (%)
Cefoxitin disk (30 µg)	30/75	45	100	100
Oxacillin disk (1µg)	27/75	48	91	99
PCR	30/75	45	100	100

these isolates, MRSA were found in 11 pus samples, nine wound swabs, six blood samples and four tracheal aspirates. The presence of MRSA was confirmed by the oxacillin (1 μg) disk-diffusion method, where a zone of inhibition <10 mm was observed in all resistant isolates and >13 mm was noted in all susceptible strains (Table 1). An inhibition zone <21 mm has been set as resistant and >22 mm as sensitive for cefoxitin disks, and results are shown in Table 2. Sensitivity and specificity results are shown in Table 3. All the *S. aureus* isolates were sensitive to vancomycin. Using the PCR method, only 30 isolates were shown to be positive for the *mecA* gene (Fig. 1), while all 75 strains examined were positive for the *nucA* gene.

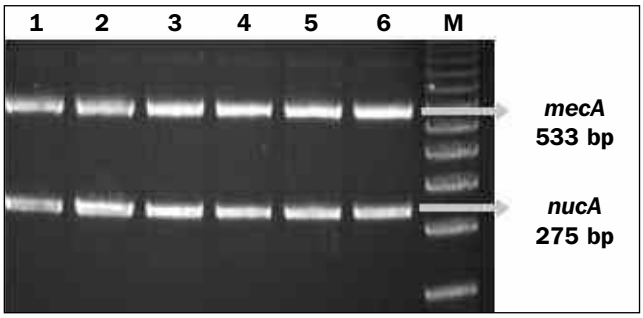

Fig. 1. Agarose gel electrophoresis patterns showing amplification products of *mecA* (533 bp) and *nucA* (275 bp) genes of methicillin-resistant *Staphylococcus aureus* isolates. Lanes 1–6: MRSA; Lane M: DNA molecular size marker (100-bp ladder).

Discussion

The accurate and prompt determination of methicillin resistance is of key importance in the diagnosis of *S. aureus* infections. In this study, an attempt was made to evaluate different methods for the detection of MRSA. Many studies have reported that use of a cefoxitin disk offers high sensitivity and specificity.[26] Oxacillin resistance in *S. aureus* is caused by expression of penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene complex, and detection of the *mecA* gene is considered the gold standard for confirmation of MRSA.[27]

Various conventional and molecular methods are used to detect methicillin resistance in *S. aureus*. The PBP2a latex agglutination test (Oxoid) is a 20 min phenotypic test that detects PBP2a in isolated colonies.[28] This assay is faster, less complicated and has been shown to be more sensitive than other phenotypic methods such as oxacillin screen agar.[29–31] The present study was unable to use this assay due to unavailability of the kit, so it evaluated the presence of the *mecA* gene in 30 *S. aureus* isolates by multiplex PCR and compared the results with cefoxitin and oxacillin disk-diffusion methods.

Using the oxacillin disk (1 μg), 27 (36%) isolates were confirmed as MRSA, whereas 30 (40%) isolates were confirmed as MRSA using the cefoxitin disk (30 μg). Similar results were noted using multiplex PCR, which identified 30 *S. aureus* isolates as MRSA. Levels of sensitivity and specificity identified in the present study are in agreement with those found by others.[32] Cefoxitin was observed to be a better predicator than oxacillin for detecting oxacillin hetero-resistance, due to its better PBP2a detection. In addition, it shows high affinity for staphylococcal PBP4.

Recent studies indicate that the cefoxitin disk-diffusion test is superior to most currently recommended phenotypic methods, and is now an accepted method for the detection of MRSA by many reference groups,[33] and in the absence of available molecular methods is the best predictor for methicillin resistance in *S. aureus* among other available techniques.[34] In terms of sensitivity and specificity, studies have reported conflicting results for the comparison between cefoxitin and oxacillin.[15,19,27] However, errors in determining oxacillin resistance may have serious adverse clinical consequences. False-negative susceptibility results may lead to treatment failure and the spread of MRSA, especially if appropriate infection control measures are not applied. Use of PCR methodology is generally limited to referral laboratories, especially in developing countries, and neither method is used widely for routine methicillin susceptibility tests in diagnostic laboratories.

The present study showed that the cefoxitin disk-diffusion test is a good alternative method for detection of MRSA, as the test is accurate and easy to perform in routine laboratories and shows greater accuracy than the oxacillin disk-diffusion test. Thus, the cefoxitin disk-diffusion test has the potential for wider use in diagnostic microbiology laboratories and can be used as a surrogate marker to PCR.

Conclusions

The present study confirmed that the cefoxitin disk can be used as an accurate method in routine susceptibility testing, compared to other phenotypic methods available for the detection of MRSA. Combining CDD and ODD methods would improve the sensitivity of the cefoxitin and specificity of the oxacillin disk-diffusion methods, and this approach could be used in clinical laboratories for the detection of MRSA. Moreover, cefoxitin disk-diffusion can be used as an alternative to PCR in laboratories were such diagnostic facilities are not available, as this method proved to be a simple, accurate and cost-effective alternative. □

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