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BRIEF REPORT

Evaluation of different methods to detect oxacillin resistance in *Staphylococcus aureus* and their clinical laboratory utility

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Methicillin-resistant *Staphylococcus aureus* strains (MRSA) are major human pathogens responsible for nosocomial and serious community-acquired infections worldwide. Methicillin resistance in *S. aureus* is primarily mediated by a modified penicillin-binding protein (PBP2a) encoded by the *mec*A gene, which is not present in susceptible strains [1–3]. Several additional gene products, such as those encoded by *fem* (factor essential for methicillin resistance) genes and auxiliary genes, can also contribute to MRSA resistance [1, 2, 4]. Rapid and accurate identification of MRSA is necessary for therapeutic and epidemiological purposes. Currently, several methods are available for detecting oxacillin resistance including phenotypic methods, such as disk diffusion, E-test and broth dilution tests, and genotypic methods that detect the *mec*A gene [1, 5, 6]. PCR amplification of the *mec*A gene has been used successfully and is presently considered the gold standard for detecting methicillin resistance in *S. aureus* [1]. Detection of oxacillin resistance using phenotypic methods is problematic because of variation in the degree of *mec*A gene expression in heterogeneous populations of MRSA; thus, routine oxacillin disk diffusion tests often fail to detect heterogeneous MRSA populations. The Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) has consequently recommended use of the cefoxitin disk diffusion test rather than the oxacillin disk diffusion test [7]. In the study presented here, the performance and utility of several different methods for detecting MRSA was evaluated.

PCR for detecting the *mec*A gene was used as the gold standard assay against which we compared oxacillin and cefoxitin disk diffusion tests and the GenoType MRSA molecular genetic assay (Hain Lifescience, Nehren, Germany) for detecting MRSA. The objective was to determine whether these disk diffusion tests and GenoType MRSA could detect all MRSA as accurately as *mec*A PCR and to evaluate their suitability as routine methods for detecting oxacillin resistance in *S. aureus* in clinical microbiology laboratories. GenoType MRSA is a new DNA-based strip assay kit designed to detect oxacillin resistance in *S. aureus*. Its performance is based on amplification of a 71 bp fragment of the *mec*A gene and an 85 bp sequence highly specific for *S. aureus* and subsequent hybridization of the denatured amplicons to their complementary sequences coated on DNA strips.

A total of 224 isolates of *S. aureus* collected from patients of Süleyman Demirel University Hospital were included in this study. The isolates were collected from different clinical specimens and at different times. All strains were identified using biochemical procedures. Reference strains included ATCC33591 (MRSA) and ATCC29213 (MSSA). Disk diffusion tests were performed for each of the 224 isolates using the following method recommended by the National Committee for Clinical Laboratory Standards [7]: 1 µg oxacillin disks (Oxoid, Basingstoke, UK) were incubated at 35°C and at 30°C on Mueller-Hinton agar (Difco, Detroit, MI, USA) with and without 2% NaCl, and 30 µg cefoxitin disks (Oxoid) were incubated at 35°C on Mueller-Hinton agar. After incubation, inhibition zone sizes for oxacillin and cefoxitin were noted and compared. For cefoxitin, isolates showing a zone of inhibition of ≤19 mm were considered

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Table 1 Comparison of susceptibility testing results achieved with *mec*A PCR, oxacillin disk diffusion, cefoxitin disk diffusion and GenoType MRSA for 224 *S. aureus* isolates

mecA PCR	No. of isolates			Disk diffusion			
		GenoType MRSA		Oxacillin		Cefoxitin	
		Positive	Negative	S	R	S	R
Positive	124	124	0	5	119	1	123
Negative	100	0	100	100	0	100	0

S oxacillin susceptible, *R* oxacillin resistant

oxacillin resistant while those showing zones of inhibition of ≥20 mm were considered oxacillin susceptible. For oxacillin, isolates showing an inhibition zone <13 mm were considered resistant.

For DNA extraction, a single bacterial colony was obtained from a fresh culture and suspended in 100 µl of lysis solution (%1 TritonX-100, 10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA). The suspension was boiled at 100°C for 10 min. After centrifugation, 2 µl of the supernatant containing the bacterial DNA was used as a template for PCR amplification. The *mec*A gene was amplified with primers *mec*A1 (5′- AAA ATC GAT GGT AAA GGT TGG C-3′) and *mec*A2 (5′- AGT TCT GCA GTA CCG GAT TTG C-3′), yielding a PCR product of 533 bp. Each 50 µl reaction mixture contained 2 µl DNA template, 0.5 µl *Taq* polymerase (Fermentas, Vilnius, Lithuania), 200 µM dNTPs (each), 1.5 mM $MgCl_2$, and 250 nM of each of the two primers *mec*A1 and *mec*A2. DNA amplification was carried out as follows: initial denaturation at 94°C for 5 min, followed by 30 min of amplification (denaturation at 94°C for 45 s, annealing at 50°C for 45 s, extension at 72°C for 1 min), and ending with a final extension at 72°C for 5 min. PCR products were analyzed on an agarose gel.

For the GenoType MRSA assay, five colonies grown after overnight incubation were selected and suspended in 150 µl of distilled water. Bacterial DNA was released by incubating the solution at 95°C for 15 min followed by sonication for 15 min. For PCR, 5 µl of the supernatant was used. The multiplex PCR was carried out using a hot-start polymerase and biotin-labelled dNTPs. Amplification was performed as follows: 1 cycle for 5 min at 95°C, and 22 cycles, each consisting of 95°C for 20 s and 60°C for 30 s. The hybridization step in this assay includes chemical denaturation of the amplicons, hybridization of the single-stranded, biotin-labeled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin-alkaline-phosphatase (AP) complex and AP-mediated staining reaction. Results were evaluated visually using a template provided with the kit. The whole test procedure takes about 3 h.

A total of 224 clinical strains of *S. aureus* were evaluated with oxacillin and cefoxitin disk diffusion tests and the new DNA-based strip assay GenoType MRSA. The overall results obtained with the different techniques are shown in Table 1. The *mec*A PCR allowed us to categorize 124 of the 224 isolates as *mec*A positive and 100 as *mec*A negative. All of the positive isolates produced the predicted 533 bp product on PCR (data not shown). The isolates identified as *mec*A negative by PCR were also shown to be oxacillin susceptible by the oxacillin and cefoxitin disk diffusion tests.

Five of 124 *mec*A-positive isolates showed discrepant results with the oxacillin disk diffusion test; the test falsely identified these five *mec*A-positive isolates as oxacillin susceptible (sensitivity 95.9%). Extension of the incubation time to 48 h, addition of 2% NaCl and incubation at 30°C did not change the sensitivity of the oxacillin disk diffusion test. The cefoxitin disk diffusion test detected oxacillin resistance correctly in all but one isolate (sensitivity 99.1%) compared with presence of the *mec*A gene. This isolate was reported as susceptible by the cefoxitin disk method but was different from the five resistant isolates reported as susceptible by the oxacillin method. The size ranges of the inhibition zones around the cefoxitin disk for *mec*A-positive and *mec*A-negative isolates were as follows: 124 *mec*A-positive isolates produced halos sized between 0 and 18 mm whereas one isolate produced a halo of 20 mm; 100 *mec*A-negative isolates produced halos between 24 and 32 mm. There were no false-resistant results with either the oxacillin or the cefoxitin disk diffusion test (specificity 100%).

In this study, we also evaluated the performance of the new GenoType MRSA assay. All 124 isolates typed as oxacillin resistant by PCR were also identified as oxacillin resistant by the GenoType MRSA test. No discrepant results were identified between these two methods.

In summary, previous studies have shown higher sensitivity and specificity values for the cefoxitin disk test in comparison with the oxacillin disk test for detecting heterogeneous MRSA populations [8, 9]. The enhanced accuracy of the cefoxitin disk test can be explained by the fact that cefoxitin is a stronger inducer of the *mec*A gene than oxacillin, and our study confirmed this test's superiority. However, as shown in this and other studies, no phenotypic method is completely reliable for the detection

of MRSA [10]. In fact, genotypic methods are not 100% accurate either since non-*mec*A-mediated resistance can occur and strains can be *mec*A-positive but the gene may be defective for expressing methicillin resistance (*fem*A mutant). In this study, however, we found that the GenoType MRSA test was highly specific and sensitive for the detection of MRSA. In comparison with regular PCR, it doesn't require agarose gel electrophoresis, for which additional time and experienced staff are required, and it gives a reliable result within 3 h.

The results of this study indicate the GenoType MRSA assay was the most sensitive of the methods tested for detecting oxacillin resistance; however, this test is rather expensive to incorporate into the daily routine (~€70/per strip) and is not available in most clinical microbiology laboratories. Thus, in the absence of molecular techniques, the cefoxitin disk test is a highly accurate alternative to the oxacillin disk test in clinical laboratories.

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