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Rapid Determination of Methicillin Resistance among *Staphylococcus aureus* Clinical Isolates by Colorimetric Methods

Ahmet Yilmaz Coban

Ondokuz Mayis University, Medical School, Department of Medical Microbiology, Samsun, Turkey

In the present study, the effectiveness of a rapid and colorimetric nitrate reductase analysis (NRA) method and resazurin microplate assay (REMA) for rapid determination of methicillin resistance among *Staphylococcus aureus* was investigated. A total of 275 clinical isolates of *S. aureus* were included in the present study. Among these isolates, 151 had the *mecA* gene and were resistant to methicillin. The remaining 124 isolates were methicillin susceptible and did not have the *mecA* gene. Cefoxitin MICs of all isolates were detected by NRA, REMA, and reference broth microdilution methods. Category and essential agreement were determined as 100% and 99.6%, respectively, comparing both NRA and REMA with the reference method. No minor, major, or very major discrepancy was observed for either of the methods. The time necessary to have the MIC results was 5 h for NRA, whereas it was 6 h for REMA. Early detection of MRSA is an important public health concern, and the results of this study showed that both of the colorimetric methods are easy to perform and save time in the determination of MRSA. These methods have a potential use for early detection of MRSA for laboratories unable to use molecular techniques.

Staphylococcus aureus is one of the most commonly encountered bacteria in clinical practice. Despite effective antimicrobial agents and hygiene measures, it has remained an important community- or hospital-acquired infectious agent (12). Methicillin resistance among staphylococci has been increasing all over the world. However, methicillin-resistant *S. aureus* (MRSA) usually causes health care-associated infections; it has also emerged recently as a cause of community-acquired infections (3, 9).

Nosocomial infections caused by MRSA are associated with increased antibiotic use and longer hospital and intensive care unit stays (3). Septicemia with *S. aureus* results in longer hospital stays and higher costs. Furthermore, the mortality rate is higher in methicillin-susceptible *S. aureus* (MSSA) septicemia than MRSA septicemia. The early selection of an appropriate antibiotic for the treatment of MSSA and MRSA is critical for outcomes (8). The early selection of an appropriate regime for the treatment avoids the unnecessary use of vancomycin. In addition, early detection reduces mortality, the length of hospitalization, and costs associated with bloodstream infections caused by *S. aureus* (8). Therapy is also complicated because of limited antibacterial options for MRSA infections (2). However, glycopeptides are the preferred antibacterial agents for the treatment of MRSA, but glycopeptide-resistant isolates have been reported recently. The novel antibiotics linezolid and daptomycin seem to be new options for treatment, but their high cost is a problem (16). Therefore, rapid laboratory identification of MRSA is important for the treatment of patients (9). A few methods developed for rapid determination of MRSA are available. Chromogenic agar medium used for determination of nose carriage, a latex agglutination test for penicillin-binding protein 2a (PBP 2a), and determination of the presence of the *mecA* gene by PCR are the most widely used methods (14). Molecular methods are expensive and require technical equipment, and so they can be used only in developed centers.

Resazurin is used as an oxidation reduction indicator in bacterial cell viability assays. It is also used for determination of contamination and antimicrobial activity in addition to its use in cell viability assays (13). Nitrate reductase analysis (NRA) is a method used for biochemical identification of some bacteria (*Mycobacterium tuberculosis* and *S. aureus*). It is based on reduction of nitrate to nitrite by viable bacteria and a color change produced by the addition of a reagent (10).

In the study, the effectiveness of a rapid and colorimetric nitrate reductase analysis method and a resazurin microplate assay (REMA) for rapid determination of methicillin resistance among *S. aureus* isolates according to cefoxitin MIC determination was investigated.

MATERIALS AND METHODS

Bacterial isolates. A total of 275 clinical isolates of *S. aureus* were tested in the study. Of these, 151 isolates had the *mecA* gene and were resistant to methicillin. The remaining 124 isolates were methicillin susceptible and did not have the *mecA* gene. Control strains used in the study were *S. aureus* ATCC 29213 (methicillin susceptible) and ATCC 43300 (methicillin resistant).

Chemical substances. Cefoxitin, resazurin, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and potassium nitrate were purchased from Sigma. Resazurin was prepared at a concentration of 0.02% in distilled water and sterilized by membrane filtration. It was stored at +4°C until used (not longer than 1 week). For the reagent used in the nitrate reductase test, 0.1% *N*-(1-naphthyl)ethylenediamine and 0.2% sulfanilamide in distilled water were prepared and stored at +4°C until used. The reagent was prepared freshly for use as 2 units of 0.1% *N*-(1-naphthyl)ethylenediamine, 2 units of 0.2% sulfanilamide, and 1 unit of concentrated hydrochloric acid.

Preparation of bacterial inoculum. Overnight bacterial cultures on blood agar were used in the test. The solution of each bacterium was adjusted to that of a McFarland 0.5 turbidity standard.

Determination of MIC of cefoxitin. MIC values of cefoxitin were determined by the broth microdilution method according to CLSI recom-

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TABLE 1 Distribution of MICs determined by both colorimetric methods and a microdilution reference method among *S. aureus* clinical isolates

Method[a]	No. of MRSA isolates for which the MIC is:[b]				No. of MSSA isolates for which the MIC is:[b]			Total no. isolates
	>32 μg/ml	32 μg/ml	16 μg/ml	8 μg/ml	4 μg/ml	2 μg/ml	1 μg/ml	
RM	142	2	6	1	50	73	1	275
NRA	138	3	6	4	24	96	4	275
REMA	141	3	6	1	111	12	1	275

[a] RM, reference method, microdilution MIC.
[b] Samples consisted of 151 MRSA isolates and 124 MSSA isolates.

mendations. Cefoxitin was tested in concentrations of 32 to 0.5 μg/ml. Breakpoints of MICs for susceptibility and resistance were defined as ≤4 and ≥8 μg/ml, respectively (4).

Performance of NRA. Nitrate reductase analysis (NRA) was performed with some modification of the broth microdilution method recommended by CLSI. The test was performed in a 96-well microtiter plate with a U-shaped bottom containing cation-adjusted Mueller-Hinton broth with 1,000 μg/ml potassium nitrate. Cefoxitin was prepared in concentrations of 32 to 0.5 μg/ml by serial dilution. Five microliters of bacterial suspension adjusted to a McFarland 0.5 turbidity standard was inoculated into each antibiotic-containing and control (antibiotic-free) well. Plates were incubated at 35°C after inoculation. Fifty microliters of freshly prepared reagent [2 units of 0.1% *N*-(1-naphthyl)ethylenediamine, 2 units of 0.2% sulfanilamide, and 1 unit of concentrated hydrochloric acid] was added into control wells at the fifth hour of incubation (6). It was considered that there was sufficient bacterial growth if a violet-purple color change was seen, and then the same amount of reagent was added to antibiotic-containing wells. The MIC is reported as the last well in which a color change was not seen.

REMA. The resazurin microplate assay (REMA) was performed according to the broth microdilution method defined by CLSI. It was performed in a 96-well microtiter plate with a U-shaped bottom containing cation-adjusted Mueller-Hinton broth. Cefoxitin was prepared in concentrations of 32 to 0.5 μg/ml by serial dilution. Five microliters of bacterial suspension adjusted to a McFarland 0.5 turbidity standard was inoculated into each antibiotic-containing and control (antibiotic-free) well. Following inoculation, plates were incubated at 35°C. Fifteen microliters of 0.02% resazurin (titration studies were performed for resazurin, and the best concentration of resazurin was 15 μl) was added into all wells at the fifth hour of incubation. Plates were incubated for an additional 1 h. When a color change from blue to red was seen in the antibiotic-free control wells, the MIC values were determined. The MIC is reported as the last well in which a color change was not seen.

Analysis of findings. Findings were analyzed according to FDA criteria (7). Minor, major, and very major discrepancy rates and category and essential agreement rates were determined in the analysis.

RESULTS AND DISCUSSION

The results of the MIC testing are presented in Table 1. Cefoxitin MIC values of all 151 *mecA*-positive isolates were determined as ≥8 μg/ml by the reference method and both the NRA and REMA methods. MIC values were ≥32 μg/ml for 142 isolates, 32 μg/ml for 2 isolates, 16 μg/ml for 6 isolates, and 8 μg/ml for 1 isolate by the reference microdilution method. MIC values were ≥32 μg/ml for 138 isolates, 32 μg/ml for 3 isolates, 16 μg/ml for 6 isolates, and 8 μg/ml for 4 isolates by the NRA method. MIC values were ≥32 μg/ml for 141 isolates, 32 μg/ml for 3 isolates, 16 μg/ml for 6 isolates, and 8 μg/ml for 1 isolate by REMA.

MIC values of cefoxitin for 124 *mecA* gene-negative methicillin-susceptible isolates were in the range of 1 to 4 μg/ml by the reference method and both NRA and REMA. MICs were 4 μg/ml for 50 isolates, 2 μg/ml for 73 isolates, and 1 μg/ml for 1 isolate by the reference method. The MIC results by the NRA method were 4 μg/ml for 24 isolates, 2 μg/ml for 96 isolates, and 1 μg/ml for 4 isolates. Using the REMA method the MIC values were 4 μg/ml for 111 isolates, 2 μg/ml for 12 isolates, and 1 μg/ml for 1 isolate. The times necessary for the MIC results were 5, 6, and 16 h by NRA, REMA, and the reference method, respectively.

Analysis of data obtained from our study was done according to FDA criteria, and results are presented in Table 2. Category and essential agreement were found as 100% and 99.6%, respectively, in a comparison of the NRA and REMA methods with the reference method. There were no minor, major, or very major discrepancies for either of the methods.

MRSA causes community-acquired or health care-associated infections, with increasing economic impact and increased mortality and morbidity rates (3). Therefore, it is very important to rapidly distinguish between MSSA and MRSA infections to establish the appropriate antimicrobial therapy. Early determination of MRSA in severe infections like sepsis, endocarditis, meningitis, osteomyelitis, and catheter-related infection allows the early initiation of vancomycin treatment (15).

Resistance patterns for methicillin and other antimicrobial agents have recently been determined by automated systems. Nevertheless, it is not possible to use automated systems in health centers with limited opportunities due to small numbers of patients. Tests used for rapid determination of MRSA are divided into two categories, molecular and conventional methods. Molecular methods are used in commercially available kits, including the BD GeneOhm MRSA assay (BD Diagnostics, Hunt Valley, MD), GeneXpert MRSA assay (Cepheid, Sunnyvale, CA), Hyplex Staphylo Resist test system (Biologische Analysensystem GmbH, Lich, Germany), and BD GeneOhm StaphSR assay (BD Diagnostics, Hunt Valley, MD). The PBP 2a latex agglutination test and

TABLE 2 Comparison of both colorimetric methods with the microdilution reference method

Method	Category agreement (%)	Essential agreement (%)	Minor discrepancy rate (%)	Major discrepancy rate (%)	Very major discrepancy rate (%)
NRA	100	99.6	0	0	0
REMA	100	99.6	0	0	0

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the use of chromogenic media are other methods for the determination of MRSA. Although molecular methods are reliable, they are not commonly used because of their high cost. Therefore, methicillin resistance can be determined by rapid and easily performed colorimetric methods. Coban et al. (6) used both REMA and NRA methods for testing oxacillin- and vancomycin-resistant *S. aureus* isolates, and consistent results were obtained in comparison with results using the liquid microdilution method. NRA has also been used for early determination of *M. tuberculosis* antibiotic susceptibility and growth in Löwenstein-Jensen, Middlebrook 7H10 medium, and blood agar medium, and compatible results were obtained (1, 5, 11).

The methicillin resistance of the isolates was tested by cefoxitin MIC by the liquid microdilution method. Confirmation of methicillin resistance of isolates was done by the PCR method in the study. Results obtained by both REMA and NRA were found to be concordant with the results of the molecular and liquid microdilution methods. In the NRA test, color change is observed immediately after the addition of reagent, allowing results to be obtained 1 h earlier than with the REMA method. Immediately after the addition of reagent, a color change occurs, and so the results can be obtained instantly with the NRA method. Color changes are stable for at least 2 h after the addition of the reagents. The REMA method requires a 1-h incubation after resazurin addition, and the colors are stable for at least 2 h after the incubation period. There are some commercial kits for the rapid detection of coagulase production in *S. aureus* (e.g., BBL's Coagulase Plasma, Rabbit, and Oxoid's Staphylase test). In a clinical microbiology laboratory, following identification of *S. aureus* by use of the above-mentioned commercial kits, determination of methicillin resistance by NRA and REMA in 5 to 6 h allows the clinician to establish the appropriate antibiotic therapy early.

In conclusion, both colorimetric methods are easy to perform and save time in the determination of MRSA, which is one of the most important infectious agents. It is considered that these methods give an option to clinical microbiology laboratories with limited facilities to detect MRSA earlier.

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