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Full Length Research Paper

Comparison of an automated system with four phenotypic methods for the detection of methicillin-resistant *Staphylococcus aureus*

Meryem Iraz[1]*, Mehmet Sait Tekerekoglu[2], Baris Otlu[2] and Selma Ay[2]

[1]Department of Medical Microbiology, Faculty of Medicine, Bezmialem Vakif University, Adnan Menderes Bulvari 34093; Fatih/Istanbul, Turkey.
[2]Department of Medical Microbiology, Faculty of Medicine, Inonu University, Malatya, Turkey.

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Correct and rapid detection of methicillin resistance in *Staphylococcus aureus* is very important for treatment of infected patients. Detection of the *mecA* gene or PBP2a by polymerase chain reaction (PCR) is considered the gold standard for determination of methicillin resistance in staphylococci. In most clinical laboratories, phenotypic methods are used for the detection of methicillin resistance in *S. aureus* because PCR is not suitable for routine usage. In this study, we aimed to compare different phenotypic methods: disk diffusion, agar screening, latex agglutination and an automated system employed to establish the presence of methicillin-resistant *S. aureus* (MRSA). Presence of the *mecA* gene via PCR was used as the marker for MRSA positivity. Afterward, 214 samples were analyzed for methicillin resistance via oxacillin or cefoxitin disk diffusions, oxacillin agar screening, MRSA latex agglutination and the automated BD Phoenix system. Sensitivity, specificity, negative and positive predictive values of these phenotypic methods were evaluated. In the detection of MRSA, the cefoxitin disk-diffusion method was found to be more useful than oxacillin disk diffusion. The automated MRSA strain-detection system was found to be more successful than the other phenotypic methods. These results showed that the automated system could be used safely for routine MRSA detection.

Key words: Phoenix, cefoxitin disk diffusion, *mecA*, oxacillin, methicillin-resistant *Staphylococcus aureus*, latex agglutination.

INTRODUCTION

Staphylococci are one of the most important causes of hospital-acquired infections worldwide. About one-quarter of healthy people carry one or more strains asymptomatically at any given time and infections commonly originate from these carriers (von Eiff et al., 2001; Waldvogel, 2000). Although antibiotics and surgical drainage are the basis of treatment for staphylococcal infections, the appearance of resistances to methicillin and other agents leads to compromised therapy (Grundmann et al., 2006; Wenzel et al., 1991). Methicillin resistance is mediated by the *mecA* gene which encodes a penicillin-binding protein 2a (PBP2a) with low affinity for beta-lactam antibiotics and permits organisms to grow and divide in the presence of methicillin and other β-lactam antibiotics (Chambers, 1988, 1997; Mulligan et al., 1993). Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an increasingly important pathogen as it spreads by direct person-to-person contact, especially between hospital staff and patients, leading to hospital-acquired infections (Wang et al., 2001; Ayliffe, 1997; Berger-Bachi, 2008). Additionally, invasive procedures, the use of broad-spectrum antibiotics and therapeutic strategies that

*Corresponding author. E-mail: meriraz@mynet.com. Tel: +90 212 4531700/1775.

damage mucosa and skin in hospitals may lead to major outbreaks of MRSA infections. The selection of appropriate antibiotics to treat MRSA infection depends on several factors such as the severity of the disease and on identifying the strain of MRSA involved (Moellering, 2008; Johnson and Decker, 2008). There are several phenotyping methods to detect methicillin resistance including classical methods determining the minimal inhibitory concentration (MIC) by the E-test or broth dilution, screening techniques with solid culture medium containing oxacillin, oxacillin or cefoxitin disk diffusion, automated-system methods, and genotypic methods that detect the *mecA* gene or its protein product (the PBP2a protein) (Babel and Decker, 2008; Hackbarth and Chambers, 1989; Swenson et al., 2007).

Detection of the *mecA* gene by polymerase chain reaction is considered the gold standard for the determination of methicillin resistance (Predari et al., 1991). Since many laboratories do not have experienced technicians or the relevant, adequate equipment, application of molecular techniques is difficult or not feasible. Consequently, phenotypic screening methods seem to be more practical than molecular methods. However, phenotypic methods may not always be reliable. For instance, routine oxacillin tests often fail to detect very heterogeneous MRSA populations, which consequently are considered as methicillin-susceptible *S. aureus* (MSSA). Therefore, several parameters have been recommended to improve results such as increasing the inoculum, growth at a low temperature, an oxacillin screening test with NaCl or prolonged incubation (Mackenzie et al., 1995). On the other hand, the cefoxitin disk-diffusion method is considered as a better predictor than oxacillin for the detection of heterogenous methicillin resistance and is the gold standard recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011).

The present study was performed in order to elucidate the efficacy of routine phenotypic methods such as oxacillin disk diffusion, cefoxitin disk diffusion, oxacillin agar, latex agglutination, and an automated system-BD Phoenix- in the detection of MRSA isolates and to discuss the suitability of an automated system as a routine method in comparison with the *mecA* detection by PCR in clinical microbiology laboratories.

MATERIALS AND METHODS

Strains

Between January 2005 and September 2007, 214 clinical isolates of *S. aureus* were collected. The 214 isolates were verified as *S. aureus* by using colony morphology, Gram staining, catalase tests, mannitol fermentation and coagulase tests. They were isolated from different patients and anatomical locations. *S. aureus* ATCC 43300 was used as positive control for the *mecA* gene and the MSSA strain ATCC 29213 was used as a negative control for the diagnostic procedures. All isolates were kept frozen at −80°C (Heraeus, Hansu, Germany) in skim-milk medium (Oxoid, Hampshire, England) until analyzed.

Detection of the *mecA* gene by PCR

Isolation, purification of DNA and amplification procedures were performed as follows: two loops of bacterial colonies were collected in 2 ml of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)]. After washing two times, bacterial lysis was carried out by using lysostaphin (100 µg/ml) and lysozyme (20 mg/ml). After phenol-chloroform extraction and ethanol precipitation, the DNA was resuspended in 25 µl of TE buffer. The PCR reaction mixture (50 µl) contained 5 µl of genomic DNA, 50 pmol of each primer (mecA1: 5'-GAT GAA ATG ACT GAA CGT CCG ATA A-3', mecA2: 5'-CCA ATT CCA CAT TGT TTC GGT CTA A-3'), 2.5 units of Taq DNA polymerase (Promega Corporation, USA), 200 µmol deoxynucleoside triphosphate mix, 10 mM Tris-HCl (pH: 8.0), 50 mM KCl and 1.5 mM $MgCl_2$. The reaction mixture was amplified (MJ Research Inc. PTC-200, Peltier Thermal Cycler Massachusetts, USA) under the following conditions: initial denaturation for 5 min at 94°C; and 30 cycles each consisting of 45 s at 94°C, 45 s at 50°C and 60 s at 72°C. Amplification products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and 448 base pairs of the amplification bands were visualized under UV illumination.

Oxacillin and cefoxitin disk diffusion

Antimicrobial susceptibility was tested by the agar disk diffusion method on Mueller–Hilton agar (MHA) plates according to the guidelines of the Clinical Laboratory Standards (CLSI). After the disks were laid on the plate surface, incubation was performed for 24 to 48 h at 35°C. Oxacillin and cefoxitin resistances were determined both with 1 µg oxacillin and 30 µg cefoxitin disks (Oxoid, England) according to the CLSI. Disk inhibition zone diameters were determined and compared according to CLSI information (2011).

Oxacillin agar screening

MHA plates containing 4% NaCl (Sigma O1002) and 6 µg/ml oxacillin were prepared. 1 µl of a 0.5 McFarland suspension was spotted onto the oxacillin screening agar and incubated at 35°C for 48 h. Plates were inspected for growth after 24 and 48 h. The MSSA strain ATCC 29213 and MRSA strain ATCC 43300 were used as controls for this procedure.

Latex agglutination—detection of PBP2a

The MRSA screen test which is based on the agglutination of latex particles sensitized with monoclonal antibodies against PBP2a was used according to the manufacturer's instructions (Oxoid, Hampshire, United Kingdom).

Automated system

The 'Phoenix' automated-microbiology system (BD Diagnostic Systems, Sparks, MD) was used according to the manufacturer's instructions. Briefly, an inoculum was prepared for each strain by suspending colonies from a fresh subculture in Phoenix ID broth

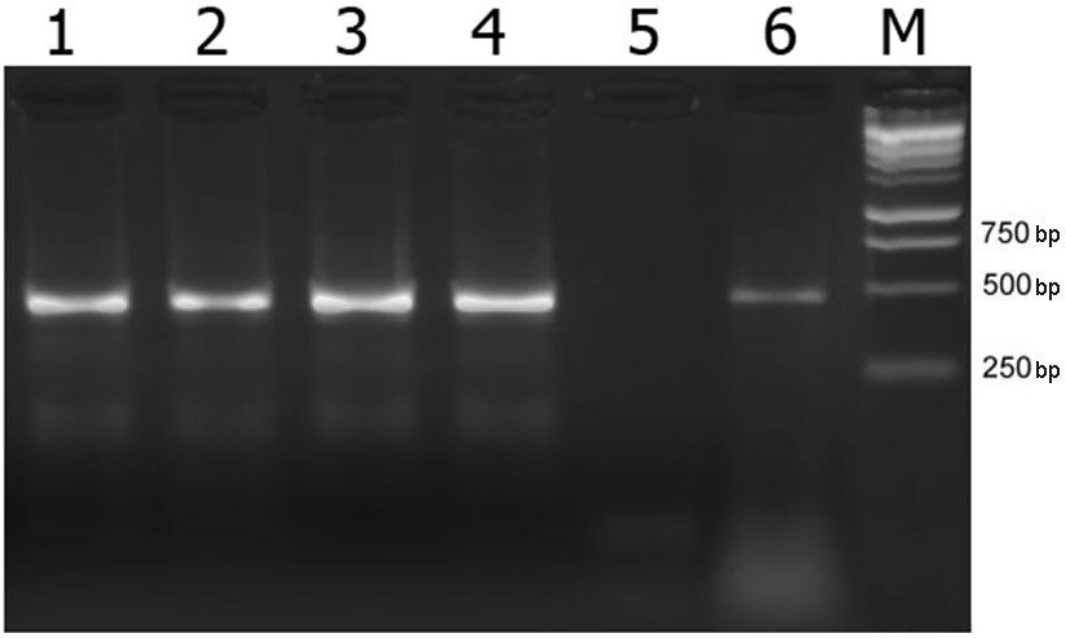

Figure 1. 1 to 4 samples; *mecA* positive (strain numbers 15, 57, 130 and 184), 5; *mecA* negative control, 6; *mecA* positive control (448 bp) and M; 1 kb DNA ladder (Promega).

and adjusting it to a 0.5 McFarland Standard. One drop of AST (antimicrobial-susceptibility testing) indicator was transferred to each Phoenix AST broth tube prior to inoculation. Each test strain was diluted in Phoenix AST broth to an inoculum density equivalent to 5×10^5 cfu/ml and loaded into a Phoenix panel containing oxacillin in doubling dilutions from 0.06 to 4 µg/ml. Phoenix system was processed according to the manufacturers' instructions and oxacillin results interpreted as susceptible if the MIC was ≤ 2 µg/ml and resistant if the MIC was ≥ 4 µg/ml.

Data evaluation

In order to understand the overall performance of phenotypic methods in the identification of MRSA isolates, sensitivity, specificity, positive and negative predictivity values were calculated according to the *mecA* gene positivity of MRSA strains.

RESULTS

In this study, 214 *S. aureus* isolates were obtained from routine individual patient isolates of whom 64 (30%) were from high intensive-care units, 72 (34%) were from surgery units and 78 (36%) were from internal medicine units at Inonu University Hospital, the Turgut Ozal Medical Center, Malatya, Turkey. Of the 214 isolates tested, 86 (40%) were *mecA* positive and were regarded as MRSA and 128 (59.8%) were *mecA* negative. The distribution of *mecA* positive isolates according to units was as follows: 43 (67%) isolates were from intensive-care units; 31 (43%) were from surgery units; and 12 (15%) were isolated from internal medicine units. Of the MRSA positive isolates, 48 (22.4%) were isolated from blood; 16 (7.4%) from scar tissue; 3 (1.4%) from urine; 12 (5.6%) from tracheal aspiration; and 7 (3.2%) were from other types of aspiration samples.

Determination of *mecA* positive isolates using phenotypic methods

Although phenotypic methods can be easily performed to detect *S. aureus* isolates under routine laboratory conditions, some isolates can be falsely identified as MSSA. In order to compare the efficacy of phenotypic methods, we identified *S. aureus* isolates as MRSA and MSSA by using oxacillin disk diffusion, cefoxitin disk diffusion, oxacillin agar screening, PBP2a latex agglutination and an automated system—BD Phoenix—method, following *mecA* detection by PCR (Figure 1). As shown in Table 1, four *mecA*-positive isolates were falsely identified as MSSA and six *mecA*-negative isolates were falsely identified as MRSA by the oxacillin disk diffusion method. Three *mecA*-positive isolates were falsely identified as MSSA and two *mecA*-negative isolates were falsely identified as MRSA by the cefoxitin disk diffusion method. Four *mecA*-positive isolates were falsely identified as MSSA and two *mecA*-negative isolates were falsely identified as MRSA by the oxacillin agar screen, three *mecA*-positive isolates were falsely identified as MSSA and two *mecA*-negative isolates were falsely identified as MRSA by PBP2a latex-agglutination method.

Table 1. Distribution of *S. aureus* isolates by *mecA* gene presence and number of isolates identified as MRSA or MSSA by five distinct phenotypic methods.

Methods	*mecA* (+) (86)		*mecA* (-) (128)	
	MRSA	MSSA	MSSA	MRSA
Oxacillin disk diffusion	82	4	122	6
Cefoxitin disk diffusion	83	3	126	2
Oxacillin agar screen	82	4	126	2
PBP2a latex agglutination	83	3	126	2
Phoenix automated system	85	1	125	3

Table 2. Overall performance of oxacillin disk diffusion, cefoxitin disk diffusion, oxacillin agar screen, PBP2a latex agglutination and BD Phoenix automated system relative to *mecA* gene presence.

Methods	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Oxacilin disk diffusion	95.3	95.3	93.1	96.8
Cefoxitin disk diffusion	96.5	98.4	97.6	97.6
Oxacillin agar screen	95.3	98.4	97.6	96.9
PBP2a latex agglutination	96.5	98.4	97.6	97.6
Phoenix automated system	98.8	97.6	96.5	99.2

On the other hand, only one *mecA*-positive isolate was falsely identified as MSSA and three *mecA*-negative isolates were falsely identified as MRSA were identified by the automated BD Phoenix system.

Sensitivity and specificity of phenotypic methods

The overall performance values for the five distinct methods are presented in Table 2. As shown, the specificity and positive and negative predicative values of the oxacillin disk method were lower than those of the other methods. The sensitivity and negative predictive values of the BD Phoenix automated system were better than those of the other methods. MRSA has been defined as *S. aureus* having the *mecA* gene or showing an MIC of oxacillin higher than 4 mg/l (Lowy, 1998). However, some clinical isolates can be *mecA* positive and oxacillin-susceptible (Hososaka et al., 2007). Therefore, we surveyed the occurrence of *S. aureus* having the *mecA* gene and an oxacillin MIC of less than 2 µg/ml (oxacillin-susceptible MRSA; OS–MRSA). Despite the existence of the *mecA* gene (Figure 1), four (5%) isolates were oxacillin-susceptible *in vitro*, since the oxacillin MIC values of these four samples were < 2 µg/ml. After that, the same four isolates were investigated with the automated Phoenix system, latex agglutination, oxacillin agar screening, and the oxacillin and cefoxitin disk-diffusion methods. Interestingly, the four isolates were identified as susceptible via the agar screening and oxacillin disk-diffusion tests. The latex-agglutination method detected one of the four isolates as MRSA.

The BD Phoenix automated system detected three of the four samples as methicillin-resistant. The cefoxitin disk-diffusion test detected one isolate (isolate 184) as MRSA even though it was detected as susceptible by the other four phenotypic methods.

DISCUSSION

In this study, we evaluated the performance of five distinct methods—the oxacillin and cefoxitin disk methods, oxacillin agar method, latex-agglutination method and the BD Phoenix automated-system method—on MRSA detection in relation to the gold standard method of *mecA* PCR. Currently, MRSA is a serious health problem all over the world because of limited treatment options and financial outcomes of infection-control procedures. Selection of appropriate antibiotics for MRSA infection depends on several factors including the severity of the disease and the types of MRSA strains (Moellering, 2008; Hososaka et al., 2007). Therefore, accurate and early determination of methicillin resistance is a crucial step in the prognosis of *S. aureus* infections. However, many reports have highlighted the difficulties and errors in the identification of MRSA when using phenotypic identification, automated systems or molecular-based test methods in laboratories. False negative results in MRSA detection not only affect choice of treatment but also increase the risk of spreading of MRSA isolates in the community and hospitals (Kaka et al., 2006; Felten et al., 2002; Kohner

et al., 1999; John et al., 2009). Our results demonstrate that sensitivity and negative predictive value of the BD Phoenix automated system was better than the other phenotypic methods (Table 2). The oxacillin disk-diffusion method is widely used for the detection of the *mecA* gene mediated methicillin resistance in *S. aureus* and coagulase negative staphylococci. However, the susceptibility results of this method may not always prove reliable (Mackenzie et al., 1995). Since cephamycins are more potent inducers of the *mecA* gene, recent studies also showed that the cefoxitin disk-diffusion method is more robust than oxacillin disk-diffusion and oxacillin agar-screening methods.

Accordingly, it has been accepted as a reference method for the detection of MRSA by CLSI (Anand et al., 2009; Velasco et al., 2005; Cauwelier et al., 2004; CLSI, 2011). Our data showed that the oxacillin agar-screening test gives the lowest performance values in comparison to other phenotypic methods. We observed high sensitivity and high positive–negative predictive values with the cefoxitin disk-diffusion method in comparison to the oxacillin method (Tables 1 and 2). Methicillin resistance in *S. aureus* is mediated by the production of an altered penicillin-binding protein such as PBP2a encoded by the *mecA* gene which has a low affinity for beta-lactam antibiotics (Chambers, 1997). Detection of the *mecA* gene or PBP2a is the most accurate marker for methicillin resistance in *S. aureus*. The MRSA screening latex-agglutination test designed to detect PBP2a is rapid, easy to perform and has similar accuracy to PCR for *mecA* gene detection with respect to sensitivity (100%) and specificity (99.1%) (Zhu et al., 2006; Nakatomi and Sugiyama, 1998; van Griethuysen et al., 1999; Louie et al., 2000; Yamazumi et al., 2001). However, PBP2a latex agglutination often requires induction, which increases the circle time, especially in coagulase-negative staphylococci (CoNS). Hussain et al. (2000) noticed that latex-agglutination test is better than other conventional tests in classifying *mecA* negative CoNS as oxacillin susceptible. In this study, despite the high specificity (98.4%) in comparison to other conventional methods, it has only modest sensitivity (96.5%) in comparison with the other methods, except for the BD Phoenix automated-system method. Although three *S. aureus* isolates (isolates 57, 130 and 184) possessed the *mecA* gene, they were found to be oxacillin-susceptible by the latex screen test, O-MIC and oxacillin–cefoxitin disk-diffusion tests.

Oxacillin salt agar is a reliable, practical and economical test; therefore, it is usually used to confirm other methods.The sensitivity of this method approaches 100% for the detection of MRSA and 95% for coagulase-negative strains (Hussain et al., 2000). In this study, the oxacillin agar-screening test had one of the lowest sensitivities in comparison to the other methods. On the other hand, it gave an almost similar specificity to the latex-agglutination test. Furthermore, although it is used to confirm other conventional phenotyping methods, it could not detect four *mecA* positive isolates, whereas three of them were detected by the BD Phoenix automated system and only one of them was detected by latex screening.

We believe that temperature, duration of incubation, and components of MHA may cause such dissimilarities between the oxacillin agar-screen test and the other phenotypic methods. Besides, some *S. aureus* strains have an extraordinary type of oxacillin resistance which is not related to the presence of the *mecA* gene. That resistance mechanism is probably due to overproduction of β-lactamase or altered PBP, except for PBP2a and PBP2. These isolates are referred to as borderline-resistant *S. aureus* (BORSA). The oxacillin screen-agar test generally does not detect BORSA in which resistance is heterogenous (Swenson et al., 2007). In MRSA infections, accurate and rapid diagnosis may reduce the mortality rate and hospitalization time. So it may provide cost effectivity. In relation to this, the automated system offers a huge advantage regarding the time involved in identifying MRSA infection in comparison to other phenotypic methods. While the automated system takes an average of 4 (2 to 12) h, other phenotypic methods take an average of 6 (3 to 15) h to identify MRSA isolates (Chambers, 1993; Stamper et al., 2007).

Spanu et al. (2004) demonstrated that an automated system has identified 223 *mecA* gene-positive *S. aureus* isolates in the bloodstream with 100% sensitivity, specificity, positive and negative predictive values. Horstkotte et al. (2004) proved that the Phoenix system had 99.2% sensitivity in detecting methicillin resistance in comparison to *mecA* gene detection by PCR. They concluded that the BD Phoenix system showed high reliability as a phenotypic method for the detection of resistance to oxacillin in *mecA*-positive CoNS (Horstkotte et al., 2004). In this study, BD Phoenix detected methicillin resistance with the highest sensitivity and the highest negative predictive value in comparison to the other phenotypic methods. Additionally, moderate specificity and positive predictive values were observed and three of the four *mecA* positive isolates with O-MIC values < 2 µg/ml were identified as MRSA by the BD Phoenix system.

In conclusion, our study demonstrated that the automated system had the highest sensitivity values in the determination of MRSA strains in comparison to the other phenotypic methods.

Additionally, the Phoenix system may detect most OS-MRSA strains directly. However, our evaluation included only a small number of *S. aureus* isolates (n = 214) and the genetic diversity of the isolates is unknown. These observations need to be confirmed with a large number of isolates.

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