African Journal of Microbiology Research Vol. 3(12) pp. 925-929 December, 2009
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2009 Academic Journals

Full Length Research Paper

Evaluation of phenotypic and genotypic methods for detection of methicillin resistance in *Staphylococcus aureus*

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Accepted 12 November, 2009

Rapid and reliable identification of methicillin-resistant *Staphylococcus aureus* (MRSA) is important for provision of both appropriate therapy and control measures. However, the heterogeneous nature of methicillin resistance in *S. aureus* limits accuracy and reliability of phenotypic methods for detecting resistance. In this study, phenotypic methods for determination of methicillin resistance were compared with polymerase chain reaction (PCR) for detection of *mecA* gene. A total of 160 clinical isolates of *S. aureus* were tested to detect resistance by disc diffusion method, MRSA screen test, and MiniAPI System. The minimum inhibitory concentration values of oxacillin were also determined by broth microdilution method in 115 isolates. A total of 87 (54.4%) isolates were *mecA* positive and 73 (45.6%) isolates were *mecA* negative. The sensitivities of disc diffusion method, broth microdilution method, MRSA screen test, and MiniAPI System were 98.8, 98.2, 97.7 and 98.8%; and specificities were 97.2, 98.2, 94.5 and 89% respectively considering PCR as the reference method. The differences in sensitivities or specificities were not statistically significant (p > 0.05). We conclude that an algorithm should be designated for correct identification of MRSA in routine laboratories because none of the phenotypic tests is completely reliable for the detection of methicillin resistance in *S. aureus.*

Key words: Methicillin resistance, *Staphylococcus aureus*, polymerase chain reaction.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen causing both nosocomial and community-acquired infections worldwide. Since most of these bacteria carry multiple resistance genes against commonly used antibiotics, they show multiple antibiotic resistance patterns and thus cause important treatment problems (Huletsky et al., 2005; Grisold et al., 2002; Waldvogel, 2000; Babel and Decker, 2008). In many cases, glycopeptide antibiotics such as vancomycine and teicoplanine are the only therapeutic alternatives However glycopeptide resistance is expected to become an important problem in future, because of reduced susceptibility of *S. aureus* strains to this group of antibiotics (Brown et al., 2005; Tenover et al., 2004; Appelbaum, 2006).

Rapid and reliable identification of MRSA is very important in order to choose appropriate therapy, to prevent unnecessary use of glycopeptide antibiotics and to take necessary measures for infection control. Also correct identification enables to avoid economic loss caused by unnecessary infection control precautions (Appelbaum, 2006; Prasad et al., 2000; Udo et al., 2000).

Resistance mechanism most commonly observed in MRSA is the synthesis of penicillin binding protein 2a

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Abbreviations: MRSA; Methicillin-resistant *Staphylococcus aureus,* **PCR;** polymerase chain reaction, **PBP2a;** penicillin binding protein 2a, **CLSI;** Clinical Laboratory and Standards Institute, **MIC;** minimum inhibitory concentration.

(PBP2a) which is encoded by the *mecA* gene and shows low affinity for ß-lactam antibiotics. Phenotypic expression of MRSA isolates which are *mecA* positive shows commonly heterogeneous character and is affected by the conditions such as incubation time, temperature, inoculum concentration and NaCl content. Consequently phenotypic methods can fail to detect methicillin resistance (Babel and Decker, 2008; Brown et al., 2005). Studies that assess phenotypic and genotypic methods for the detection of MRSA are extensive in literature, and different recommendations have been presented regarding the most reliable method for routine use (Brown et al., 2005; Prasad et al., 2000; Udo et al., 2000).

In this study, we aimed to evaluate the performance of *in vitro* susceptibility testing methods for detection of MRSA, namely disc diffusion, broth microdilution methods, a semi-automated antimicrobial susceptibility testing system, and a latex agglutination test showing the presence of PBP2a and to compare the results with polymerase chain reaction method (PCR) for detecting *mecA* gene as a reference method.

MATERIALS AND METHODS

Bacterial strains

A total of 160 *S. aureus* strains isolated from various clinical samples sent to the Clinical Microbiology Laboratory of the Ankara Training and Research Hospital were included in this study. The bacteria identified by conventional methods as *S. aureus* were stored in nutrient media at room temperature. The strains were inoculated on to blood agar plates before use in the study. Antibiotic susceptibility tests were performed according to the Clinical Laboratory and Standards Institute (CLSI) guidelines (CLSI M2-A9, 2006; CLSI M7-A7, 2006). *S. aureus* reference strains, ATCC 43300 and ATCC 29213, were used as control microorganisms.

Disc diffusion method

The bacterial inoculum was prepared in Trypticase Soy Broth (Difco, USA) directly from an overnight culture on blood agar plates. The direct colony suspension of organisms adjusted to 0.5 McFarland standard ($1 - 2 \times 10^8$ cfu/ml) was inoculated on to Mueller-Hinton Agar (Biolab, Hungary) with a swab and 1µg oxacillin disc (Oxoid, England) was placed. The plates were incubated at 35℃ for 24 h. Isolates showing inhibition zone sizes ≤10 mm were considered as resistant; 11 - 12 mm were considered as intermediate resistant and ≥13 mm were considered as susceptible (CLSI M2-A9, 2006).

Broth microdilution method

The minimum inhibitory concentration (MIC) of oxacillin was determined using the broth microdilution method in Mueller-Hinton Broth (Merck, Germany) supplemented with 2% NaCl according to the recommendations of CLSI (CLSI M7-A7, 2006). Last inoculum concentration of 5×10^5 CFU/ml was obtained in sterile U based microtiter plates containing the test concentrations of oxacillin (0.125 - 256 µg/ml) and incubated at 35℃ for 24 h. The lowest concentration of antibiotic which inhibited the visible bacterial growth was determined as the oxacillin MIC of the isolate. The strains with oxacillin MICs ≤ 2 µg/ml were considered as susceptible and ≥ 4 µg/ml were considered as resistant to methicillin.

Mini-API semi-automated susceptibility system

(ATB Staph System, bioMerieux, Marcy-l' Etoile / France) The oxacillin susceptibilities of all isolates were evaluated with the Mini-API System using ATB Staph test strips. The test was performed according to the manufacturer's instructions.

MRSA screen test

(Denka Seiken Co. Ltd. Tokyo, Japan) A slide latex agglutination test that was composed of latex particles coated with monoclonal antibodies against PBP2a (PBP2') to detect this protein product was used. Test was performed according to the manufacturer's instructions. For PBP2a extraction, a loopful of *S. aureus* colonies from an overnight culture on blood agar plate was suspended in 4 drops (200 µl) of extraction reagent 1 (0.1 M NaOH) by using a 1.5 ml microcentrifuge tube. The suspension was boiled for 3 min at 100ºC in heat-block (Techne, Dri-block, England). After cooling, 1 drop (50 µl) of extraction reagent 2 (0.5 M KH_2PO_4) was added, mixed and then centrifuged at 1,500 x g for 5 min at room temperature. The supernatant was used as test sample; 50µl of the supernatant was mixed with 25 µl of sensitized latex on the test card and also 50 µl of the supernatant was mixed with 25 µl of control latex as a negative control. Test card was then rotated by hand for 3 min, and presence of agglutination was assessed as positive result.

Detection of *mecA* gene by PCR

A 533-bp fragment of the *mecA* gene was amplified using the primers 5' AAA ATC GAT GGT AAA GGT TGG C-3' and 5' AGT TCT GCA GTA CCG GAT TTG C-3' (Sigma-Genosys, USA). For DNA extraction (Jackson et al., 1993), bacterial suspension adjusted to 0.5 McFarland was prepared in 1ml sterile water from a fresh subculture of *S. aureus*. Microcentrifuge tubes containing this suspension were centrifuged at 13,000 rpm for 1 min and the supernatant was removed. The remaining solution was vortexed after adding 100 µl sterile distilled water and 50µl of this solution was transferred to a clean microcentrifuge tube. Then 50 µl of digestion buffer (50 mM KCl, 10 mM Tris, 1% NP_{40}, 1% Triton x100, 1 mg/ml Proteinaz K) was added onto the suspension. The tubes were incubated in heat-block for 2 h at 65℃ and then for 10 min at 95℃. Following this procedure, the tubes were centrifuged at 13,000 rpm for 2 min. The supernatant was used for amplification. 5 µl of the supernatant was added to 45 µl of the PCR reaction mixture containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin, 10 mM dNTP, primers, *Taq* polymerase and nuclease free water (Sigma, USA). Thermal cycling (Techne, England) conditions were as follows: first denaturation at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 45 s, extension at 72℃ for 1 min and final extension at 72℃ for 5 min. The amplified products were detected by 1% agarose gel electrophoresis after staining with ethidium bromide (1 µg/ml) and examined under UV transilluminator (Vilber Lourmat, France).

Statistical analysis

Chi-square and Fisher's absolute chi-square tests were used to compare the sensitivities and the specificities of the test methods.

Table 1a. Comparison of phenotypic methods for detection of methicillin resistance with a molecular method for detection of *mecA* in *S. aureus* isolates (n).

PCR *mecA*	No. of isolates	Disc diffusion		mini - API		MRSA screen test		No. of isolates	Broth microdilution MIC (µg/ml)	
		Sensitive	Resistant	Sensitive	Resistant	Negative	Positive	58	≤ 2	≥ 4
Positive	87	1	86	1	86	2	85	57	1	57
Negative	73	71	2	65	8	69	4	115	56	1
Total	160	72	88	66	94	71	89	58	57	58

RESULTS

A total of 160 clinical isolates of *S. aureus* were tested for methicillin (oxacillin) resistance by disc diffusion method, MiniAPI ATB Staph System, MRSA-screen latex agglutination test and PCR method. The oxacillin MIC values of 115 isolates were determined by broth microdilution method.

The results of oxacillin resistance testing of the clinical isolates are shown in Table 1 and 18 discordant results are presented in Table 2. A total of 87 (54.4%) isolates were *mecA* positive and 73 (45.6%) isolates were *mecA* negative. Disc diffusion method failed to detect one *mecA* positive isolate that had an MIC value of 8 µg/ml [isolate number (no): 47] and identified two *mecA* negative isolates as oxacillin resistant (no: 46 and 119).

Mini-API semi-automated susceptibility system determined false-positive results for seven isolates that resulted as susceptible by other tests (no: 21, 24, 41, 54, 106, 127, 128) and determined false-negative result for one isolate that resulted resistant by other tests (no: 50).

MRSA screen test that searched for PBP2a resulted as negative in two *mecA* positive isolates (no: 52 and 53). The oxacillin MICs of these isolates were 256 µg/ml and 32 µg/ml; respectively. Besides, the test resulted as positive for four isolates that possessed no *mecA* gene (no: 56, 57, 89, 126) and were determined as methicillin susceptible by other phenotypic methods.

The oxacillin MICs of the isolates were ≥ 4 µg/ml in 57 of 58 *mecA* positive isolates and ≤ 2 µg/ml in 56 of 57 *mecA* negative isolates (Table 1).

Performance characteristics of the methods used are shown in Table 3. All methods were considered to be satisfactory in detecting methicillin resistance and showed similar sensitivities. Although the specificity and positive predictive value of mini-API semi-automated system seemed to be lower than the other methods, differences in sensitivities or specificities were not statistically significant ($p>0.05$).

DISCUSSION

The heterogeneous nature of methicillin resistance in *S. aureus* limits accuracy and reliability of phenotypic methods such as disc diffusion, broth and agar dilution tests (Babel and Decker, 2008; Prasad et al., 2000). Recently PCR-based methods have been used by reference laboratories for detecting the *mecA* gene, but they are not practical for routine use in clinical laboratories (Brown et al., 2005).

In a number of studies, sensitivity and specificity of disc diffusion method have been reported between 61.3 - 100 % and 50 - 99.1 % respectively (Prasad et al., 2000; Udo et al., 2000; Smyth et al., 2001; Krishnan et al., 2002; Cavassini et al., 1999; Swenson et al., 2001; Boutiba-Ben Boubaker et al., 2004). The performance of disc diffusion method differs due to the heterogeneous nature of phenotypic expression of resistance in MRSA isolates. Detection of resistance requires special conditions such as incubation time, temperature, and media. In recent studies, cefoxitin disc diffusion method is considered a better predictor than oxacillin for the detection of heterogeneous methicillin resistance (Brown et al., 2005; Boutiba-Ben Boubaker et al., 2004; Swenson and Tenover, 2005). In this study, the sensitivity (98.8%) and the specificity (97.2%) for disc diffusion method was found to be satisfactory. An isolate that possessed *mecA* gene and MIC value of 8 µg/ml was found methicillin sensitive by this method (isolate no: 48). This result was considered to be due to heterogeneous expression of the isolate. One isolate that possessed no *mecA* gene and PBP2a was found as resistant by phenotypic methods (isolate no: 46, the samples that has discrepant results were tested twice). This result suggests that this isolate may have another mechanism different from PBP2a production for methicillin resistance.

MRSA screen test, a rapid (20 min for a single test) slide latex agglutination test, based on detection of PBP2a is considered very sensitive and specific with *S. aureus* (Udo et al., 2000; Cavassini et al., 1999; Nakatomi and Sugiyama, 1998; Sakoulas et al., 2001; Van Griethuysen et

Table 2. Discrepancies between *mecA* PCR, disc diffusion, mini-API semi-automated system, MRSA screen test and broth microdilution test results.

No. of isolate	*mecA* PCR	Disc diffusion	mini-API semi automated system	MRSA screen test	Broth microdilution MIC (µg/ml)
1	+	R	R	+	2 S
21	-	S	R	-	2 S
24	-	S	R	-	2 S
41	-	S	R	-	2 S
46	-	R	R	-	64 R
47	+	S	R	+	8 R
50	+	R	S	+	4 R
52	+	R	R	-	256 R
53	+	R	R	-	32 R
54	-	S	R	-	0.5 S
56	-	S	S	+	<0.125 S
57	-	S	S	+	<0.125 S
89	-	S	S	+	0.5 S
106	-	S	R	-	0.250 S
119	-	R	S	-	Na
126	-	S	S	+	Na
127	-	S	R	-	Na
128	-	S	R	-	Na

+: positive; -: negative; R: resistant; S: sensitive. Na: MIC values of these isolates are not available.

Table 3. Performance characteristics of phenotypic methods considering *mecA*-PCR as the reference method.

Method	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Disc diffusion	98.8	97.2	97.7	98.6
mini - API	98.8	89.0	91.4	98.4
MRSA screen test	97.7	94.5	95.5	97.1
Broth microdilution	98.2	98.2	98.2	98.2

al., 1999; Mohanasoundaram and Lalitha, 2008). The method requires no special equipment and is recommended for confirmation of resistance or equivocal test results in routine clinical laboratories (Brown et al., 2005; Udo et al., 2000). MRSA screen test resulted as false-positive for four isolates that possessed no *mecA* gene and were phenotypically sensitive to methicillin (specificity, 94.5%). Additionally, it resulted as false-negative for two isolates that possessed *mecA* gene and were phenotypically resistant. It was thought that the latter isolates produced lower amounts of PBP2a than the detection limit of MRSA screen test, but repeated tests with higher inoculum size resulted again as negative.

We obtained the lowest specificity value as 89% by mini-API semi-automated system. Although the difference was not statistically significant, we think that methicillin resistant results obtained with this method should be confirmed with another phenotypic or genotypic method. Similar previous studies reported that automated susceptibility systems might give false-positive results for MRSA detection (Prasad et al., 2000; Udo et al., 2000; Ribeiro et al., 1999). However there are other studies that obtained successful results with these systems. Arbique et al. (2001) reported that BBL Crystal MRSA ID system had 99% sensitivity and 100% specificity, PCR as the reference method. In another study, Sakoulas et al. (2001) have determined the sensitivities of Vitek-1 and Vitek-2 systems as 99 and 99.5% and the specificities as 100 and 97.2% respectively.

We conclude that an algorithm should be designated for correct identification of MRSA in routine laboratories because none of the phenotypic tests is completely reliable for the detection of methicillin resistance in *S. aureus.* The algorithm should include a combination of tests and apply a genotypic method for confirmation of resistance or discrepant results.

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