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Comparison of mecA gene-based PCR with CLSI cefoxitin and oxacillin disc diffusion methods for detecting methicillin resistance in Staphylococcus aureus clinical isolates

Soodabeh Rostami[1], Mojtaba Moosavian[2]*, Saeed Shoja[1], Maryam Torabipour1 and Zahra Farshadzadeh[3]

[1]Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Iran.
[2]Deparment of Microbiology, School of Medicine, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences. Iran.
[3]Department of Microbiology, Tehran University of Medical Sciences. Iran.

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The emergence of heterogeneous populations of methicillin-resistant *Staphylococcus aureus* (MRSA) causes major problems in routine screening for MRSA. Cefoxitin is a potent inducer of the *mec*A regulatory system and can be use for detection of heterogeneous populations of MRSA. Detection of the *mec*A gene by PCR was considered to be the "gold standard". In this study we determined the sensitivity and specificity of the oxacillin disk diffusion test, cefoxitin disk diffusion test and oxacillin agar screening for detection of MRSA. A total of 124 non-duplicate isolates of *S. aureus* were included in the study. Methicillin resistance was measured using oxacillin (1µg) and cefoxitin(30µg) disc diffusion method and oxacillin agar screening test (6 mg/ml oxacillin) according to CLSI guideline and PCR for the *mec*A gene. Compared with the molecular detection of methicillin resistance the overall sensitivities and specificities of the phenotypic tests for cefoxitin disc diffusion were 100%, for oxacillin disc diffusion were 91.7 and 92.8% and for oxacillin agar screening were 95 and 95.5%, respectively. We concluded that in the absence of availability of molecular biology techniques, the cefoxitin disc was the best detector of methicillin resistance in *S. aureus* related to the other phenotypic tests.

Key words: Methicillin-resistant *Staphylococcus aureus, mec*A, Cefoxitin, polymerized chain reaction.

INTRODUCTION

Staphylococcus aureus is one of the most common organisms that recovered from clinical bacterial isolates in our area, like other parts of the world (Khorvash et al., 2008a,b; Mohammadtaheri et al., 2010). The reports from all over the world in past two decades showed the gradual increase in methicillin-resistant *S. aureus* (MRSA) infections in hospital settings (Boucher and Corey, 2008; Nimmo et al., 2011; Dar et al., 2006). Resistance of MRSA to the beta-lactame antibiotics is caused by different mechanisms that one of them is the production of an unusual penicillin-binding protein (PBP), designated PBP2' (or PBP2a), which presents low affinity for beta-

*Corresponding author. E-mail: moosavian_m@yahoo.com. Tel :(0098-611)3330074. Fax: (0098-611)3332036.

lactame antimicrobials and that is encoded by *mec*A gene (Katayama et al., 2000). This mechanism has been reported to be the most commonly encountered mechanism (Baddour et al., 2007).

Detection of the *mec*A gene by polymerase chain reaction is the gold standard for identifying of MRSA (Fernandes et al., 2005) but this test is not available in many clinical laboratories and is relatively expensive. Different methods have been developed for the detection of MRSA and widely used in bacteriologycal laboratories such as minimal inhibitory concentrations (MIC) determination [by agar dilution (AD), broth dilution and E-test], the oxacillin screening agar (OSA) method and disc diffusion (DD) testing. The major problem in routine screening for MRSA is the heterogeneous population of MRSA. In these population, a set of cells show low-level resistance to oxacillin with MIC ranging between 1 to 100 mg/l, while on homogeneous MRSA, the MIC for all of the bacteria is >100 mg/l (Cauwelier et al., 2004). Cefoxitin, a cephamycin, is a stronger inducer of the *mec*A regulatory system than the oxacillin. Therefore cefoxitin is considered to better than oxacillin for the detection of heterogeneous MRSA and is recommended by Clinical and Laboratory Standards Institute (CLSI) (Fernandes et al., 2005; Frigatto et al., 2005; Swenson et al., 2005).

In the present study, we evaluated the methicillin resistance of *S. aureus* clinical isolates using oxacillin and cefoxitin disk diffusion method and oxacillin agar screening in comparison with *mec*A-based PCR.

MATERIALS AND METHODS

Clinical isolates

A total of 124 isolates of *S. aureus*, collected from different clinical specimens in teaching hospital microbiology laboratories of Ahvaz Jundishapur University of Medical Sciences (South west of Iran), were entered in the study from November 2009 to October 2010. Confirmation of the strains was done using standard tests like catalase, slide and tube coagulase, growth on Mannitol salt agar (Merck, Germany), produced of DNase and resistance to Bacitracin (0.04 U) (Mast Group Ltd, UK) (Winn et al., 2006). Only one isolate from each patient was included in the study.

Antibiotic susceptibility tests

Antibiotic susceptibility testing for all of *S. aureus* was performed according to the Clinical and Laboratoty Standard Institute guidelines (CLSI, 2007). For phenotypic detection of MRSA, susceptibility to oxacillin (1µg) and cefoxitin (30µg) (Mast Group Ltd, UK) was determined by Kirby-Bauer disk diffusion method. A bacterial suspension equivalent of 0.5 McFarland was inoculated onto a Muller-Hinton's (MH) agar (Merck, Germany). Plates were incubated at 35°C for 24 h. Results were interpreted according to CLSI guidelines (CLSI, 2007). Reference strains included *S. aureus* ATCC 29213 and 25923.

Oxacillin agar screening test

This test was performed according to the CLSI guidelines (CLSI, 2007). A bacterial suspension equivalent of 0.5 McFarland was spotted onto MH agar (Merck, Germany) containing 4% (w/v) NaCl (Merck, Germany) and 6 mg/ml oxacillin (Sigma, USA), and the plate was incubated at 35°C for 24 h. Oxacillin resistance was confirmed by bacterial growth after 24 h of incubation at 35°C. If individual colonies or a light haze of growth appeared, the strain was considered positive (Baddour et al., 2007).

Detection of the mecA gene

All isolates of *S. aureus* were tested by PCR for the presence of *mec*A gene. Staphylococcal DNA was extracted using boiling method. Briefly, frozen bacteria were sub cultured onto Mueller–Hinton agar (Merck, Germany) before DNA extraction. For rapid DNA extraction, one to five bacterial colonies were suspended in 100µL of Tris-EDTA buffer and heated at 100°C for 10 min. After centrifugation at 9 000 Xg for 30 s, 2 µL of supernatant were used as template in a 50 µL PCR reaction (Nunes et al., 1999). Primers (*mec*A-F: 5'-GTAGAAATGACTGAACGTCCGATGA and *mec*A-R: 5'- CCAATTCCACATTGTTTCGGTCTAA) and protocol used were based on the methodology previously mentioned (Tiwari and Sen, 2006). The master mixture consisted of 5µL of the 10X reaction buffer; 3 µL of 25 mM MgCl2; 1 µL of 2.5 mM dNTPs; 1 µL *mec*A-F primer 20pmol/ µL; 1 µL *mec*A-R primer 20pmol/ µL; 0.2µL Taq polymerase 5U/µL; 10µL DNA; and 28.8 µL distilled water. DNA amplified in a Mastercycler Eppendorf (Eppendorf, Germany) under the following conditions: initial denaturation for 4 min at 94°C followed by 35 cycles at 94°C for 1 min, at 62°C for 1 min, then at 72°C for 45 s. Final extension was for 5 min at 72°C and PCR products was kept in 4°C. Amplicons were electrophoresed on a 1.5 % agarose gel with 0.5µg/ml ethidium bromide in 1X Tris Borate EDTA buffer (Primers and all of chemical materials were prepared from CinnaGen, Iran). Gels were visualized and photographed under ultraviolet illumination (Aktas et al., 2007). The positive tests have shown PCR product of 310 bp. *S. aureus* ATCC 29213, reference strain was used as *mec*A negative control strain, and *S. aureus* ATCC 33591 as *mec*A positive control strain.

Statistical analysis

The results were analyzed using the Epi Info 7 (CDC, USA) for Widows software. Cross Tabulation was done and sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of phenotypic tests were calculated against *mec*A gene PCR as the gold standard.

RESULTS

Out of the 124 examined strains, 44.4% (n=55) and 46% (n=57) were methicillin resistant by oxacillin disk diffusion method and oxacillin agar screening respectively. Sixty isolates (48.4%) were resistant to methicillin based on cefoxitin disk diffusion test and all of them were positive for *mec*A gene by PCR method. Sixty isolates (48.4%) were positive for *mec*A gene and so were known as MRSA strains. The results of the some tested isolates

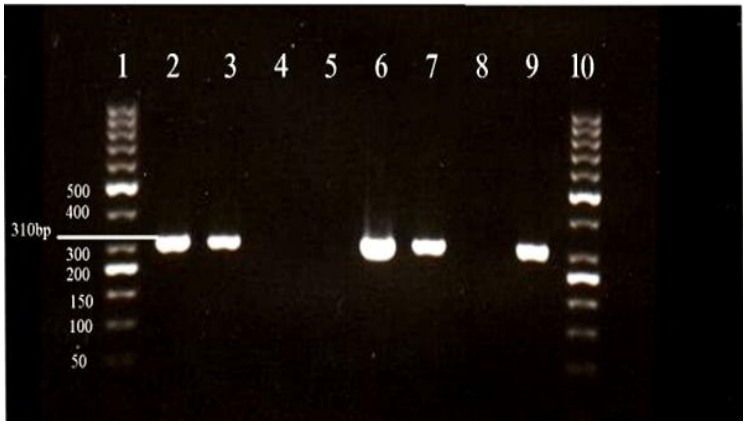

Figure 1. *mec*A PCR results for some of the tested isolates. There are positive bands at 310 bp. Lane1 and 10: 50-bp molecular-weight markers, lanes 2, 3, 6 and 7: Positive clinical isolates for *mec*A gene, lanes 4 and 5 : Negative clinical isolates for *mec*A gene, lane 8: MSSA ATCC 29213 (negative control) and lane 9: MRSA ATCC 33591 (positive control).

Table 1. Evaluation of phenotypic tests for detection of Methicillin resistance in comparison with PCR in 124 *S. aureus* isolates.

PCR *mec*A detection	Number of isolates	OX-agar screen		OX disk diffusion		FOX disk diffusion	
		Growth	No Growth	Resistant	sensitive	Resistant	sensitive
Positive	60	57	3	55	5	60	0
Negative	64	0	64	0	64	0	64
Total	124	124		124		124	

OX, Oxacillin; FOX, Cefoxitin.

Table 2. Sensitivity and specificity of phenotypic and genotypic methods used for the detection of MRSA strains.

Method	Sensitivity (%)	Specificity (%)	PPV(%)	NPV (%)
Oxacillin disk diffusion	100	92.8	91.7	100
Oxacillin agar screening	100	95.5	95	100
Cefoxitin disk diffusion	100	100	100	100
PCR for *mec*A gene	100	100	100	100

PPV, positive predictive value; NPV, negative predictive value.

with the positive and negative controls have been shown In (Figure 1). The results obtained by the phenotypic tests and PCR have been listed in (Table 1). Sensitivity, specificity, PPV and NPV of the three phenotypic tests in comparison with *mec*A-based PCR method are given in (Table 2).

DISCUSSION

Nowadays, different methods are used for detection of MRSA in throughout the world. The identification of *mecA* gene by PCR is considered as the gold standard, but it is not routinely available in most clinical laboratories and it wastes of the time and cost. In this study, the *mecA* gene was observed in 48.4% (60/124) of the *S. aureus* isolates by PCR method. The cefoxitin disk diffusion results were similar to *mec*A gene PCR (60/124) and sensitivity and specificity of this test were 100%, however, oxacillin disk diffusion and oxacillin agar screening showed less specificity. John et al. (2009) evaluated 799 staphylococcus spp and 139 *S. aureus* isolates by phenotypic method. They showed that cefoxitin disk diffusion method was 100% reliable for *S. aureus.* Similarly Cauwelier et al. (2004) found that cefoxitin disks were better than oxacillin

disks in detecting methicillin resistante *S. aureus*. Anand et al. (2009) among 50 *S. aureus* strains found 28 MRSA by routine oxacillin disk diffusion test. They also showed that 30 isolates were MRSA in oxacillin agar screening and 32 isolates were resistant with cefoxitin disk diffusion test and in these 32 isolates *mec*A gene was detected by PCR method. Their study showed that sensitivity and specificity for cefoxitin disk diffusion test was 100% but other tests have less sensitivity and specificity.

Some of the reports have shown different sensitivity and specificity for these three phenotypic tests for detection of MRSA. Baddour et al. (2007) reported that the sensitivity and specificity of the cefoxitin and oxacillin disk diffusion test were 84.6, 84.6, 87.5 and 79.2%; respectively. Also they found that the oxacillin agar screening was 92.3% sensitive and 45.8% specific. In another study by Jain et al., (2008) the sensitivity and specificity of the cefoxitin and oxacillin disk diffusion test were 94.44, 100, 95.83 and 58.33%; respectively. Matos et al., (2010) showed the cefoxitin and oxacillin disk diffusion test and oxacillin agar screening was 100% specific but only the cefoxitin and oxacillin disk diffusion test had 100% sensitivity. Also they found the oxacillin agar screening had the lowest sensitivity (82.2%). In general, in the most conducted studies, cefoxitin disk diffusion test has shown the highest specificity compared to oxacillin disk diffusion and agar screening.

In a laboratory where molecular methods are not feasible as a routine, cefoxitin disk diffusion test is a good surrogate marker for detecting methicillin resistance. Our results suggested that cefoxitin disk can be used for detection of MRSA especially heterogeneous strains. Also, using the oxacillin agar screening and *mec*A gene PCR can be useful for verifying of the results.

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