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Original Article

The Prevalence of Resistance to Methicillin in *Staphylococcus aureus* Strains Isolated from Patients by PCR Method for Detection of mecA and nuc Genes

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is the main cause of hospital infection emerged over the last decades. Rapid detection of MRSA is important for patient care and proper usage of infection control. Detection of *mecA* genes (encoding resistance to methicillin and other similar antibiotics) and *nuc* genes (encoding staphylococcal thermostable nuclease) by PCR method is now considered for rapid identification of MRSA strain. The aim of this study was to determine the prevalence of MRSA isolated from patients in Tehran, Iran by PCR method for detection of *mecA* and *nuc* genes.

Methods: Phenotypic method such as microscopic and colony morphology and catalase and coagulase tests were used for identification of *S. aureus* isolates. DNA was extracted from all isolates and the presence of *nuc* and *mecA* gene was detected by PCR method. For determination of MRSA by phenotypic methods, oxacillin disk diffusion test were used. Data were analyzed by SPSS software.

Results: Out of 126 clinical sample identified by phenotypic method, 101 isolates had *nuc* gene. In disk diffusion tests by oxacillin disk, 78.2% of isolates were considered to be MRSA, but in PCR method for *mecA* gene, 69% isolates were positive.

Conclusions: The results showed a high prevalence of methicillin-resistance among S. *aureus* isolates. Identifying MRSA strains, isolating MRSA-positive patients and carrier's treatment in a hospital to prevent MRSA infection is important in limiting the spread of MRSA. The PCR method for detection of *nuc* and *mecA* genes has potential for rapid and accurate diagnosis of MRSA strains.

Keywords: Methicillin-resistant *Staphylococcus aureus,* MRSA*, mecA, nuc*

Introduction

Staphylococcus aureus is one of the most important human pathogens, causing a wide range of nosocomial and community acquired infections, from mild skin and soft tissue infections to wound infections and bacteriemia (1). Currently several methods are used to distinguish *S. aureus* from other bacteria, which include culture methods, followed Gram's staining, grow in mannitol salt agar and fermentation of mannitol and catalase and coagulase tests; however these routine methods are time consuming and cumbersome and the accuracy of most of them has constantly been questioned the course of over time (2). PCR-based method is used as the single most reliable and

sensitive test for accurate and rapid identification of *S. aureus* (3). *S. aureus* produces an extracellular thermostable nuclease, encoded by *nuc* gene, which is one of the most distinguishing and successful characteristics that might be used for distinguishing *S. aureus* from other staphylococcus spp. This suggests that *nuc* gene is a specific marker gene and PCR is a useful method for identifying this gene in *S. aureus* (4).

Methicillin-resistant *S. aureus* (MRSA) is a variant of *S. aureus* bacteria that has evolved resistance to methicillin and other antibiotics including all other beta lactams, aminoglycosides and macrolides (5). Chromosome and plasmids mediated resistance to antimicrobial agents in *S. aureus* strains (6). Methicillin resistance in MRSA is mediated by the presence of 78-kDa penicillin binding protein PBP2' (or PBP2a) which has a very low affinity for beta-lactam antibiotics. PBP2a is encoded by the *mecA* gene (7). *mecA* gene is located on a mobile genetic element (from 21-to 67-kb), that is called staphylococcal cassette chromosome *mec* elements (SCCmec) (8). The first strain of MRSA was reported in the UK and Europe in 1961, just less than 1 year after the introduction of methicillin for clinical use. Since that time, MRSA has become a major public health problem worldwide and the prevalence of MRSA has been dramatically rising in recent years (9). Thus, rapid and exact methods for identification of MRSA in clinical specimens are essential for accurate diagnosis and antimicrobial therapy (10).

Disk diffusion method and agar dilution method are the most commonly used methods in routine clinical diagnostic laboratories to determine methicillin resistance; but these methods have low specificity in detection of methicillin resistance and by the mere results of disk diffusion methods or agar dilution methods, the true prevalence of MRSA cannot be determined (11). Using polymerase chain reaction (PCR) method for detection of *mecA* gene is considered as the gold standard method for the detection and identification of prevalence of MRSA and has been described in recently published reports (12).

The aim of this study was to determine the prevalence of resistance to methicillin in *S. aureus* isolated from patients in Tehran, Iran, by combination of phenotypic and genotypic (PCR method for detection of *mecA* and *nuc* genes) methods.

Materials and Methods

Identification of S. aureus isolates

A total of 126 *Staphylococcus aureus* isolates were collected from January 2008 to June 2008 from various clinical specimens of patients admitted to four university hospitals (Imam Khomeini, Mostafa Khomeini, Shariati, Motahari) in Tehran, Iran. These isolates were transferred to Microbiology Department of Medical School of Shahed University.

Multiple isolates from the same patients, even if the site of infection was different, were excluded. After prepare the new cultures of isolates, Gram's staining were done on isolates smear to ensure the absence of contamination and study of bacteria morphology. All isolates were reconfirmed and identified as *S. aureus* based on colony and microscopic morphology (grape-like Gram-positive cocci) and positivity in catalase and coagulase tests. Altogether, 126 isolates were collected and stored at -70 °C in freezing medium (nutrient broth containing 15% glycerol). Growth on mannitol salt agar medium and fermentation of mannitol were also checked for all isolates.

Detection of methicillin resistance by phenotypic method

Disk diffusion test was performed by oxacillin (1μg) disk (MAST Diagnostics, UK) on Mueller Hinton agar (Merck, Germany) plate inoculated by each isolate according to the Clinical Laboratory Standards Institute (CLSI) guidelines (13). Control strain was *S. aureus* ATCC25923. Intermediate-resistant strains were considered resistant.

DNA extraction

For rapid DNA extraction, five colonies from overnight growth on brain heart infusion (BHI) agar plates were picked up and suspended in 500 μl of sterile distilled water. The suspension was then heated at 100 °C for 15 min. After

centrifugation for 5 min at 14,000 rpm to sediment the debris, the clear supernatant was used as template DNA in PCR method (14).

Primers for PCR

All primers used in this study were synthesized and purchased from Cinnagen Company (Tehran, Iran), according to sequences obtained from GenBank and compared with the sequence of *nuc* and *mecA* genes published by Zhang et al. (4). The primer sequences were as follows: Nuc1 (5'-GCG ATT GAT GGT GAT ACG GTT-3') and Nuc2 (5'-AGC CAA GCC TTG ACG AAC TAA AGC-3'), for *nuc* gene, and MecA1 (5'-CCA ATT CCA CAT TGT TTC GGT CAT A- 3') and MecA2 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A -3') '), for *mecA* gene.

Molecular detection of nuc gene by PCR method

PCR method were performed in a final volume of 20µl, containing 2 µl of template DNA, 2 µl of PCR buffer (10x), 1 µl of $MgCl_2$ (50mM), 4 µl of dNTPs (1mM), 1 µl of each Nuc1 and Nuc2 primers (10 Pmol), 0.25 µl of Taq DNA polymerase (5u/µl) and 8.75 µl of double distilled water. All materials were purchased from Cinnagen Company (Tehran, Iran). PCR amplifications were performed with Techne thermocycler (Touchgene Gradient, UK), using the following cycle conditions: an initial denaturation step at 94°C for 5 min; 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR products were electrophoresed in 2% agarose gels with 1X Tris-acetate-EDTA (TAE) buffer at 100V for 100 min and then gels were stained with ethidium bromide (Cinnagen Co., Tehran, Iran) to see the amplified DNA fragments (279 bp) under UV light box by comparison with a molecular size marker (100 bp ladders, eurobio, UK). Positive and negative controls were *S. aureus* ATCC29213 and molecular grade water, respectively (4).

Detection of mecA gene by PCR method

The PCR reactions were carried out in a 20 µl reaction volume, containing 2 µl of template DNA, 2 µl of PCR buffer (10x), 1 µl of $MgCl_2$ (50mM), 4 µl of dNTPs (1mM), 4 µl of each MecA1 and MecA2 primers (10 Pmol), 0.25 µl of Taq DNA polymerase (5u/µl), and 2.75 µl of double distilled water. All materials were purchased from Cinnagen Company (Tehran, Iran). Thermocycling conditions in a Techne thermocycler (Touchgene Gradient,UK), were as follows: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 30 sec, with a final extension at 72 °C for 5 min. PCR products were run on 2% agarose gel and analyzed as described above for amplified DNA (310 bp). Methicillin resistant *S. aureus* clinical isolate containing *mecA* gene and molecular grade water were used as positive and negative controls, respectively (4). All the personal information of the patients was remained private during all steps of the research.

Results

A total of 126 isolates were identified as *S. aureus* by routine phenotypic methods including Gram's staining, colony morphology and catalase and coagulase test. The results of culture on mannitol salt agar have shown that, 95 (75.9%) of isolates showed positive results with grown on mannitol salt agar and fermentation of mannitol. Thus culture on mannitol salt agar to identify *S. aureus*, is not able to make a definite identification alone.

Figure 1 show image of agarose gel electrophoresis after amplification of *nuc* gene (279 bp) for some isolates by PCR method. By applying PCR method, among the 126 clinical samples that were identified as *S. aureus* with phenotypic methods, 101 (80.2%) isolates were found to be *nuc* positive (Fig. 1). The presence of some discrepancies between the results of phenotypic and genotypic methods for detection of *S. aureus* strain, make it clear that, the method for identification of *nuc* genes is not sufficient alone. So, phenotypic and genotypic methods together were used for identification of S. *aureus* strain. Table 1 to 3 shows the frequency of *S. aureus* isolates, accor-

ding to hospitals, admitted wards and type of clinical specimens.

Table 1: Frequency of *S. aureus* isolates according to hospitals

Hospital	Number of *S. aureus* isolates	Frequency distribution of strains (%)
Imam Khomeini	72	57
Mostafa Khomeini	28	22
Motahari	14	11
Shariati	12	10
Total	126	100

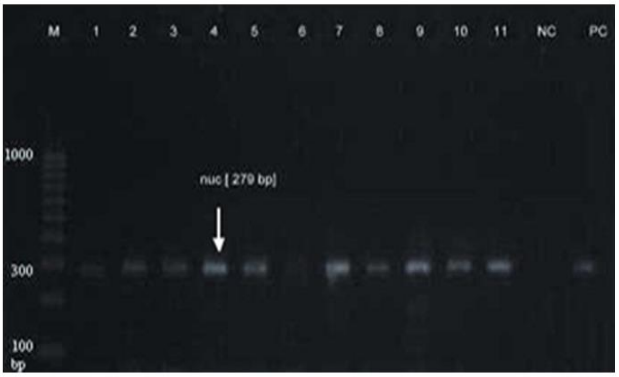

Fig. 1: Image of agarose gel electrophoresis after amplification of *nuc* gene (270 bp) for some isolates by PCR method. Lane M is 100 bp DNA ladder, lanes 1 to 5 and 7 to 11 is *nuc* gene, NC: negative control, PC: positive control (*S. aureus* ATCC29213)

Figure 2 show image of agarose gel electrophoresis after amplification of *mecA* gene (310 bp) for some isolates by PCR method. From 126 *S. aureus* isolates, 87 (69%) isolates harbor the *mecA* gene and identified as methicillin-resistant *S. aureus* (MRSA) and the remaining 39 (31%) isolates were methicillin-susceptible (MSSA).

By disk diffusion test, among 126 isolates of *S. aureus*, 98 (78.2%) of isolates were determined MRSA and 21.8% MSSA. The results of comparison between the phenotypic and genotypic methods are displayed in Table 2. Frequency distribution (percentage) of MRSA and MSSA isolates according to different hospital, admitted wards and clinical specimens were compared and significant differences were observed (Fig. 3 to 5).

Table 2: Frequency of *S. aureus* isolates according to admitted wards/ Departments

Wards/ Departments	Number of *S. aureus* isolates	Frequency distribution of strains (%)
Operation	11	9
Intensive care unit	33	26
Emergency	4	3
Respiratory	28	22
Burn	14	11
Internal	36	29
Total	126	100

Fig. 2: Image of agarose gel electrophoresis after amplification of *mecA* gene (310 bp) for some isolates by PCR method. Lane M is molecular size marker (100 bp DNA ladder), lanes 3 to 6, 8 and 11 is *mecA* gene, PC: Positive control (Methicillin resistant *S. aureus* clinical isolate containing *mecA* gene), NC: Negative control

Table 3: Frequency of *S. aureus* isolates according to type of clinical specimens

Origin	Number of *S. aureus* isolates	Frequency distribution of strains (%)
Respiratory	73	57.6
Wound	31	25
Blood	11	8.7
Urine	1	1.1
Others	10	7.6
Total	126	100

In this study, there was no significant difference between MRSA and MSSA isolates regarding the prevalence of presence of *nuc* gene; the *nuc* genes were observed in 69 (79.3%) of the MRSA isolates (out of 87 isolates) and in 32 (82.1%) of the MSSA isolates (out of 39 isolates).

Table 4: Number of MRSA and MSSA strains detected by oxacillin disk diffusion test and PCR method for comparison of used tests for 126 strains

Result in		Disk diffusion test	
		MRSA	MSSA
PCR method	MRSA	107	10
	MSSA	19	116

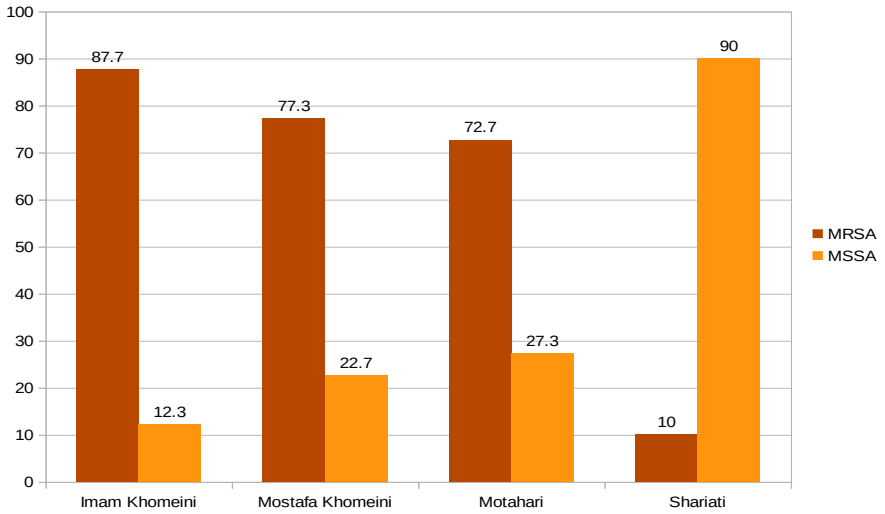

Fig 3: Frequency distribution of MRSA and MSSA isolates in hospitals. Most of MRSA strains were obtained from Imam Khomeini, Mostafa Khomeini and Motahari Hospitals, while most of MSSA strains were obtained from Shariati Hospital

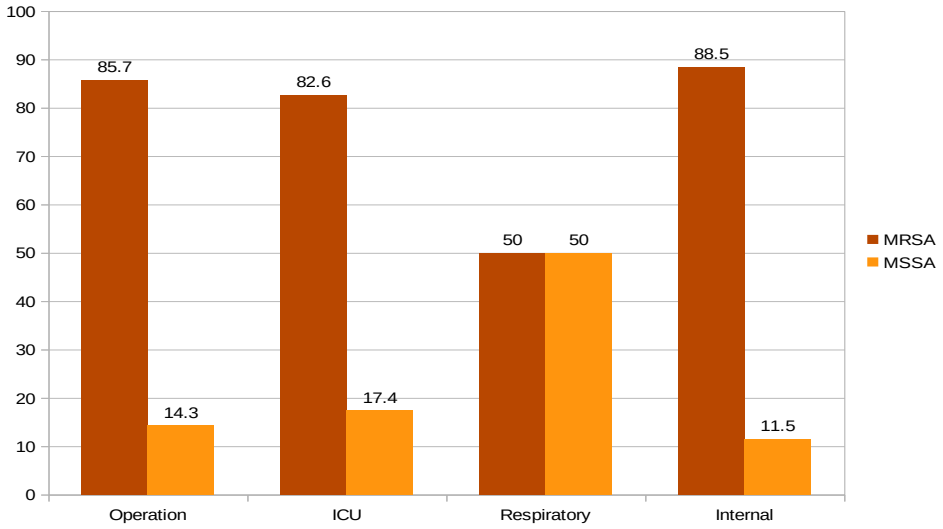

Fig. 4: Frequency distribution of MRSA and MSSA isolates in wards. The majority of the strains obtained from different hospital wards were identified as MRSA strains, except the respiratory ward that the prevalence of MRSA and MSSA were almost same

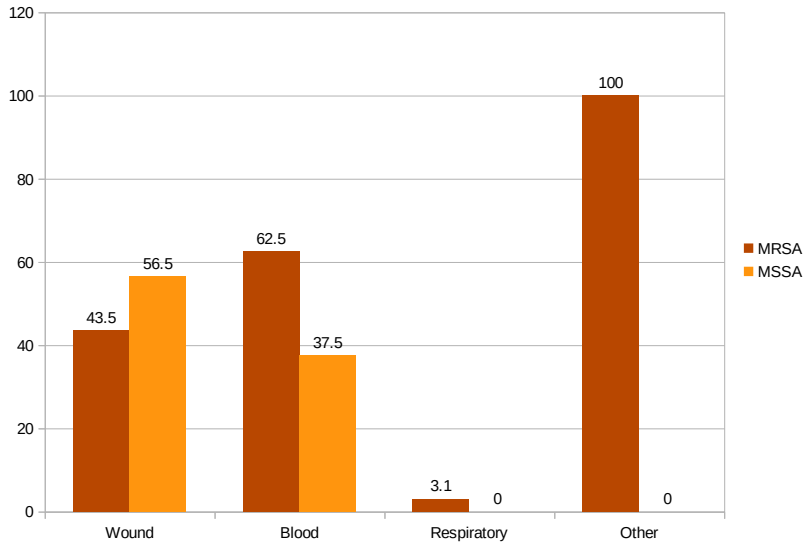

Fig. 5: Frequency distribution of MRSA and MSSA isolates in clinical specimens. The prevalence of MRSA strains in all clinical specimens was significantly higher than MSSA, except for wounds specimen**.**

Discussion

MRSA is type of *S. aureus* that has developed resistance to beta-lactam antibiotics and is responsible for community-associated and nosocomial infections (8,15). Therefore, finding the rapid and accurate techniques for identification of these bacteria, can contribute to the rapid diagnosis and timely treatment of infections. Currently, various laboratory methods for detecting *S. aureus* and MRSA strains have been reported (16). The conventional phenotypic methods have a high ability to detect *S. aureus* and MRSA strains and they are still widely used in clinical laboratories as routine identifications tests. But phenotypic methods have some disadvantages. So many studies are being conducted to improve and increase the sensitivity and specificity of methods for determination of *S. aureus*. On the other hand, there is still no consensus on the best rapid and accurate detection method with high sensitivity; therefore, many articles are published about the comparison of different rapid methods for the detection of these bacteria and advantages of each technique (3).

Our study showed that the mannitol salt agar test was negative for 5% of *S. aureus* isolates; as this shown in other studies (17, 18). *S. aureus* can be easily identified by PCR amplification of *nuc* gene; therefore, *nuc* gene has been used for the detection of *S. aureus* by some researchers (15, 19). The diagnostic values for detection of *nuc* gene by PCR based method were 93.3% sensitivity and 89.6% specificity (1). Brakstad et al. used the *nuc* gene as target DNA to identify *S. aureus* strains and recorded amplification product of *nuc* gene at 279 bp region for all 90 of 90 reference clinical isolates of *S. aureus* (20). Therefore, we used primers of their study in our project. A high percentage (80.2%) of *S. aureus* isolates were positive for the presence of *nuc* gene, that can confirm the ability of PCR method as fast and reliable method for detection of the *nuc* gene to identify *S. aureus* strains. However, some phenotypically *S. aureus* isolates were shown negative result probably due to non-optimal experimental conditions for PCR method, the differences in the nucleotide sequence among the *nuc* genes caused by some mutation or the absence of *nuc* gene in some *S. aureus* strains. So it just seems that, a negative PCR method result for *nuc*

gene cannot prove the absence of *S. aureus* among clinical isolates.

In this study, we used phenotypic (oxacillin disk diffusion test) and genotypic (PCR method for *mecA* gene) methods for detection of MRSA. The results of our study showed that 78.2% and 69% of *S. aureus* isolates were recognized as MRSA by disc diffusion test and PCR method, respectively. Whenever 107 (84.9%) of isolates had shown similar results in phenotypic and genotypic assays, 10 (8.3%) of isolates were *mecA*-positive in PCR but methicillin sensitive in disk diffusion test. This could be attributed to not consistently expression of *mecA* gene. Besides, 19 (15.1%) of the phenotypically methicillin-resistant strains were negative for *mecA* gene. This resistance can be due to the presence of other resistance mechanisms, such as large amounts of produced beta-lactamase or the lack of optimal PCR conditions or change in *mecA* gene due to the mutations. Phenotypically methicillin resistant strains without *mecA* gene and methicillin sensitive strains harboring *mecA* gene are also shown in other studies (21, 22).

Considering that detection of the *mecA* gene by PCR method is gold standard method for identifying methicillin resistance in *S. aureus* isolates, the prevalence of MRSA in this study have determined 69%. This prevalence of MRSA is high and comparable to results of other studies in Tehran, Iran; 53% by Rahbar et al. (23) and 88% by Rahimi et al. (24). This prevalence is also similar to other international studies performed in other areas, such as United States, France, Canada, Australia, European countries (25- 28).

This study also demonstrates that the distribution of MRSA isolates vary in different hospital wards. This definitely shows that some patients are more likely to catch infection (26). In the present study, the maximum and minimum isolation of MRSA was from ICU and emergency ward respectively; as CDC (Centers for Disease Control and Prevention) reported that in the United States, more than 50% of *S. aureus* isolates in ICU and 40% of *S. aureus* infections in other hospital units are methicillin resistant (29). In this study, the prevalence of MRSA was significantly different among various clinical specimens and MRSA isolates showed higher prevalence in most clinical specimens (except wounded samples). The variation in prevalence of MRSA among *S. aureus* isolated from various specimens might be due to the variation in antibiotics usage, infection control practices in different hospitals and prolonged antibiotic treatment of severely sick patients, who generally stay longer in hospital (28). Finally, this study has demonstrated a high prevalence of MRSA, regarding this problem in community, the identification of MRSA in isolates by PCR method, offers a very specific, sensitive and relatively rapid alternative to conventional assays. Moreover, due to the absence of *mecA* gene in some isolates of MRSA, the use of both phenotypic and genotypic tests combined may provide the best information for obtaining general result for detection of MRSA strains.

Conclusion

Considering a significant increase in the prevalence of methicillin-resistance in *S. aureus* strains caused by the indiscriminate and excessive use of antibiotics during the last decade, our study emphasize on identifying MRSA isolates, isolating MRSA-positive patients and carrier's treatment in a hospital to establish effective measures to prevent MRSA infection. This study shows that PCR method is a useful method for detection of *nuc* and *mecA* genes which leads to rapid detection and identification of MRSA cultured from patient's specimens (in less than 6 h) and may provide substantial benefits for infection control by allowing prompt and cost-effective implementation of contact precautions. Here, it is suggested that in order to obtain more reliable results, further studies about the distribution of isolates according to different variables are required.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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