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Typing of HVR, Frequency of *blaZ*, and Detection of *mecA* Promoter Mutations in Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT

Aims: Methicillin resistant *Staphylococcus aureus* (MRSA) strains are a major contributor to the development of hospital- and community-acquired infections. The aim of this study was to evaluate the polymorphism of *mecA* gene, frequency of *blaZ* gene, and detection of *mecA* promoter mutations in clinical isolates of methicillin-resistant *S. aureus* strains.

Materials & Methods: Susceptibility of 85 *S. aureus* clinical strains to methicillin was evaluated using disc diffusion method. The polymorphism of *mec*-associated hypervariable region (HVR), presence of *blaZ* genes, and mutation in *mecA* promoter were determined by PCR and sequencing.

Findings: A total of 40 (47.1%) out of 85 *S. aureus* isolates were identified as methicillin resistant by phenotypic assays and PCR-based detection of *mecA* gene in MRSA strains. Seven different groups of repeats were found among these strains. Also, 39 MRSA strains harbored *blaZ* gene, and according to the sequence analysis of *mecA* promoter, R226S mutation was identified in 1 out of 10 isolates tested.

Conclusion: According to the obtained results, there was a high variation in the polymorphic region of *mecA* gene in clinical isolates of *S. aureus*. In addition, it was appeared that beta-lactamase enzyme production and antibiotic hydrolysis played an important role in the occurrence of resistance to beta-lactam antibiotics, and the effect of mutation in genes regulating *mecA* gene expression was negligible.

Keywords: *S. aureus*; Methicillin resistance; Molecular typing

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were first isolated in 1961. Nowadays, studies have revealed a steady increase in the incidence of a wide range of hospital- and community-acquired infections caused by this bacterium [1]. Also, an increase in the frequency of methicillin resistant *S. aureus* strains has been reported in several studies in Iran [2]. The main mechanism of resistance to methicillin in *S. aureus* strains relies on the presence of *mecA* gene and the expression of a new penicillin-binding protein called PBP2a, which has a low-binding affinity with beta-lactams. PBP2a is eventually substituted for PBP as a transpeptidase in the synthesis of the bacterial cell wall peptidoglycan despite the presence of a high concentration of beta-lactam antibiotics [3, 4]. Therefore, methicillin-resistant *S. aureus* strains express cross-resistance to almost all currently available beta-lactam antibiotics [5, 6]. There are several other factors known to influence resistance to this widely used class of antimicrobials, including the production of a beta-lactamase (encoded by *blaZ*) that decreases the activity of beta-lactam antibiotics. Furthermore, MRSA can develop various mutations conferring resistance to these antibiotics [4, 7]. Point mutation in the binding site of the repressor proteins in *mecA* promoter can lead to an increase in *mecA* gene transcription rate, and subsequently, resistance rate [3, 8]. Considering the high mortality rate and treatment costs associated with MRSA infections, the control of these infections must be improved using an effective, easy, and accurate typing method. One of the proposed typing methods involves the typing of methicillin-resistant staphylococcal gene (*mec*) hypervariable region (HVR). The DNA sequence between IS431mec and *mecA* is called HVR which is composed of 40bp direct repeat unit (DRU) elements. Since the number of these repeated units may be different between isolates, the length polymorphisms of HVR-PCR products among different staphylococcal isolates can be used to type and classify MRSA strains [9, 10]. The present study aimed to investigate *mecA* gene HVR polymorphism, frequency of *blaZ*, and mutation in *mecA* promoter in methicillin-resistant *S. aureus* clinical isolates in Rasht, Guilan province, northern Iran.

Materials and Methods

Sample collection: During 2018, 85 *S. aureus* strains were isolated from various sources including urine, skin, blood, and other body fluids in Guilan province, Iran. The isolates were identified using several tests such as Gram staining, catalase, growth onto MSA, haemolysis onto blood agar, and tube coagulase tests. Of each patient, only one isolate was included in this study.

Methicillin-resistant strains: All *S. aureus* isolates were screened for *mecA*-mediated oxacillin resistance by standard disc diffusion (30 µg cefoxitin disc) on Mueller Hinton agar (prepared from High Media-India), according to CLSI guideline [11]. The tests were repeated twice, and standard strain of ATCC 33591 was used as control.

DNA extraction and *mecA* HVR typing: The bacterial genomic DNA was extracted using DNA extraction kit (Roch, High Pure PCR Template Preparation Kit, Germany). The *mecA* HVR was amplified using specific primers, as described previously (Table 1), with a thermal cycling program at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 30 sec. The final elongation step

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was done at 72 °C for 7 min. HVR-PCR products were electrophoresed in 1.5% agarose gel, stained with safe DNA stain, and visualized under UV light. The 100-bp marker (MBI Fermentas) was used as a size standard for detecting the size of HVR polymorphism, and Chromas software was used to analyze HVR-PCR sequencing and to determine the number of their direct repeat units.

Detection of *blaZ:* The *blaZ* gene was amplified using specific forward and reverse primers as described previously (Table 1), with a thermal cycling program at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec. The final elongation step was done at 72 °C for 7 min. PCR products were electrophoresed in 1.5% agarose gel, stained with safe DNA stain, and visualized under UV light. The 100-bp marker (MBI Fermentas) was used as a size standard for detecting the 421bp *blaZ* gene amplicons, which were confirmed by sequencing.

Study of mutations in *mecA* promoter: To amplify *mecA* promoter in *mecA*-positive *S. aureus* strains, specific primers of these genes were used (Table 1). The materials and the thermocycler program were the same as the previous step. However, the annealing temperature for *mecA* promoter primers was set at 60 °C. After assuring the production of desired products, PCR products were sent to Bioneer (South Korea) for nucleotide sequencing. After determining the nucleotide sequences of the *mecA* promoter, the changes in base and amino acid sequences were compared with the standard strain of *S. aureus* NCTC 8325 in the GenBank using online software such as BLAST, Chromas version 1.45, and CLC Main Workbench version 3.5.

Findings

Identification of *S. aureus* and detection of MRSA strains: A total of 85 *S. aureus* strains were isolated from various sources including urine (38), wound and surgical ulcers (30), blood (15), and synovial fluid (2). In phenotypic assays, 40 isolates (55.3%) were recognized as MRSA.

HVR typing: HVR amplicons sequence analysis identified 7 dru types (dt) in 40 MRSA isolates. The dru types contained 7-11 repeats, a majority of which contained 8 repeats (16 isolates). The dt8i was the most common dru type present in 30% of the sequenced isolates. The rest of the recognized dru types and their frequency were as follows; dt10m (25%), dt11v (12.5%), dt8h (10%), dt10a (10%), and dt7h (5%), and ....

Table 1) Nucleotide sequences of primers used in this study

Gene	Primer sequence	Amplicon size (bp)	Ref.
mecA-F	5′- ACTATTCCC TCAGGCGTCC - 3′	variable	12
mecA-R	5′-GGAGTTAATCTACGT CTCATC-3′		
mecA promoter-F	5′- GTTATCGCAACCAGCCCTAC-3′	1016	3
mecA promoter-R	5′- AGGTCGAACACCTGGAACAC-3′		
blaZ-F	5′- ATGCGAATCAGCATCTTTGGT -3′	421	5
blaZ-R	5′- CTACCAGCAGATGCCCTCGGC-3′		

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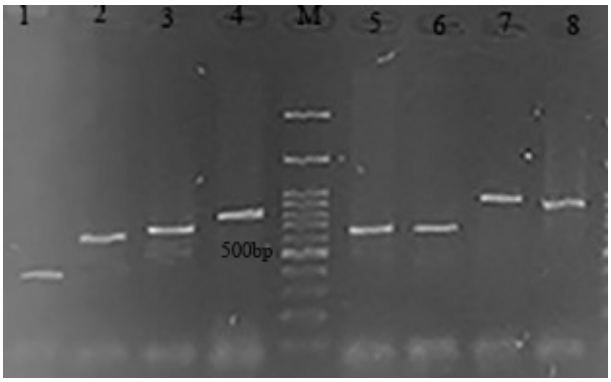

Figure 1) Agarose gel electrophoresis of *mecA* gene PCR product. Lane M: The 100bp DNA marker. Lanes 1 - 8: Polymorphism size in *mecA* gene HVR in tested bacteria.

Detection of *blaZ* gene in MRSA: The presence of *blaZ* gene was evaluated in *S. aureus* isolates, and PCR amplicon with approximate length of 421 bp was identified in 75 isolates, which were considered as the strains with the potential for beta-lactamase enzyme production. Among which, 39 isolates were identified as MRSA. The agarose gel electrophoresis of *blaZ* gene PCR products is shown in Figure 2.

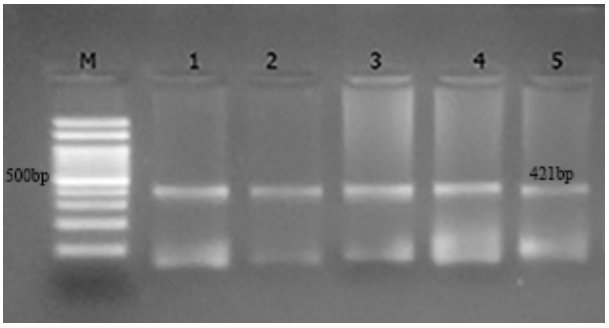

Figure 2) Agarose gel electrophoresis of *blaZ* gene PCR product. Lane M: 100bp DNA marker. Lanes 1-5: 421 bp PCR product of *blaZ* gene confirmed by sequencing.

PCR amplification of *mecA* promoter and sequence analysis: *MecA* promoter was amplified in 10 selective isolates which were resistant to all tested β-lactam antibiotics. Agarose gel electrophoresis of PCR amplicons indicated the production of 1016 bp fragments (Figure 5). Based on the results of sequence analysis of *mecA* promoter in 10 tested isolates, a missense mutation in codon 226 was identified only in one sample, in which the replacement of A with T resulted in the amino acid alteration (Arg> Ser). The sequence of *mecA* promoter in this isolate was deposited in GenBank under the accession no. MK801105.

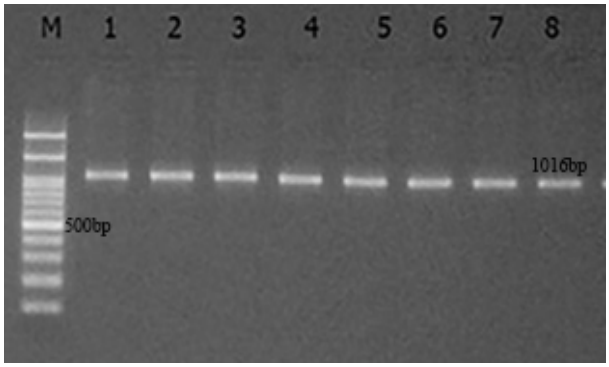

Figure 3) Agarose gel electrophoresis of *mecA* promoter. Lane M: 100bp DNA marker. Lanes 10-1: Lanes 1-8: *mecA promoter* PCR products.

Discussion

S. aureus is considered as a major cause of hospital- and community-acquired infections; the species of which have acquired resistance to a wide range of antibiotics including beta-lactams, aminoglycosides, tetracyclines, fluoroquinolones, and macrolides. Today, a limited number of antibiotics is available for *S. aureus* infections treatment.

In this study, 85 *S. aureus* strains were isolated from clinical samples in Rasht city. Of which, 40 isolates were identified as methicillin resistant by phenotypic assays and PCR-based detection of *mecA* gene. In dru typing, 7 different types containing 7-11 repeats were identified. Previously, Nia *et al*. (2013) reported 11 different PCR based HVR types in *S. aureus* strains isolated from nasal carriers and clinical samples in Tehran, Iran [11]. According to Mirkarimi *et al.*'s (2016) study in Tabriz, Iran, MRSA strains were classified into 7 different genotypes of HVR

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groups [9].

Also, *blaZ* gene was identified in 88.2 and 97.5% (39) of tested and *mecA*-positive strains, respectively. The results of different studies carried out in different parts of the world in many cases indicated an increase in the level of staphylococcal strains resistance to beta-lactam antibiotics, which is partly due to the excessive consumption of these antibiotics. Frequency of *blaZ* gene in clinical isolates of *S. aureus* has also increased in many cases. In a study by Ferriara *et al.* (2016), all tested *S. aureus* isolates were resistant to beta-lactams, and *blaZ* gene was detected in 82% of beta-lactam resistant isolates [5]. Yang *et al.* (2015) detected *blaZ* gene in 35 (94.6%) out of 37 penicillin-resistant *S. aureus* isolates [12]. Also, according to Soares *et al.*'s study, all *mec*A + coagulase-negative *Staphylococci* strains isolated from bovine mastitis were also positive for *blaZ* gene, and the presence of both genes was correlated with phenotypic beta-lactam resistance [14].

The present study also investigated nucleotide sequences of *mecA* promoter in 20 isolates. Among which in two samples, a missense mutation was identified in codon 226 of *mecA* gene, where the substitution of A to T resulted in the amino acid alteration (Arg> Ser). In a similar study by Djoudi *et al.* (2016) on MRSs strains, 9 mutations were reported in *mecA* gene, most of which were the repetitive point mutations at G246E [15]. Ender *et al.* (2008) identified spot mutations in around -10 *mecA* promoters, which are binding sites for *mecI* and *blaI* inhibitors. This mutation converted codon C to codon T, but its effect on resistance to beta-lactam was negligible in all instances [7]. Furthermore, in a study by Kobayashi *et al*., mutation was detected downstream of the *mecA* promoter sequence (-10) on a palindrome structure corresponding to the presumptive operator of the *mecA* gene [3].

Conclusion

The present study identified the most common dru types in mecA HVR of MRSA strains. Also, according to this study results, it was appeared that beta-lactamase enzyme production and antibiotic hydrolysis played an important role in the occurrence of resistance to beta-lactam antibiotics, and the effect of mutation in genes regulating *mecA* gene expression was negligible.

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Conflict of Interests: The authors declare that there is no conflict of interest.

Ethical Permissions: Since we did not use any animal models and we used isolates which were previously obtained from clinical samples, we have no ethical code for this study.

Authors' Contribution: Leila Asadpour (First author), Original researcher/ Introduction author/ Methodologist/ Statistical analyst / Discussion author; Saeed Veisi (Second author), Methodologist (20%)/ Original researcher/ Statistical analyst.

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