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Biofilm-forming ability and *Agr*-specific group of methicillin-resistant *Staphylococcus aureus* in Northern Iran

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Abstract

Background: *Staphylococcus aureus* is one of the most common agents of nosocomial infection worldwide. Methicillin-resistant and biofilm-associated infections of this bacterium have become a clinical concern in patients. This research aimed to identify biofilm-forming ability and *accessory gene regulator* (*Agr*) - specific group of clinical isolates of methicillin-resistant *S. aureus* (MRSA) in Northern Iran.

Methods: In 2021, a total of 200 clinical isolates were identified as *S. aureus* by biochemical tests. The disk diffusion method was used to examine the antibiotic resistance of isolates and the microplate method was applied to investigate the biofilm production capability. In addition, the PCR method was used to determine the frequency of biofilm-associated genes and *Agr* typing of MRSA isolates. $P \leq 0.05$ was considered significant.

Results: Overall, 62.5% of isolates were methicillin-resistant and 75% were multiple antibiotic-resistant. Biofilm-forming ability was detected in 99 (79.2%) methicillin-resistant isolates in which *ica*A and *ica*D were found in 85% and 78% of biofilm-producing isolates, respectively. Type 1 of the *Agr* gene was the most common type among methicillin-resistant isolates. The frequency of biofilm-associated genes showed a significant association with MDR phenotype and the presence of *Agr* locus ($P \leq 0.05$).

Conclusion: The present findings indicate a high frequency of biofilm and antibacterial resistance in methicillin-resistant *S. aureus* isolates in Guilan Province. These findings suggest reliable and rapid identification of biofilm-forming MRSA strains to prevent the spread of these bacteria.

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Introduction

Infections caused by *Staphylococcus aureus* are among the most common causes of nosocomial infections in both developed and developing countries (1). This bacterium has a broad spectrum of virulence agents that allow it to withstand antimicrobial drugs, including methicillin. Methicillin-resistant *S. aureus* (MRSA) remains a leading cause of severe infections, particularly in the healthcare industry, associated with mortality despite rising healthcare costs (2).

Virulence factors are expressed in MRSA infections in response to regulators of sub-genes (*Agr* genes) that encode an auto-inducing peptide (AIP) (3). The accessory gene regulator (*Agr*) is one of the major regulatory and control factors in the cell surface proteins and virulence gene expression of *S. aureus*. The *Agr* system is also essential for bacteria's capacity to form biofilms and control the production of binding and secretory proteins (3). Furthermore, *Agr* system is polymorphic and allows classification of *S. aureus* strains in four groups (*Agr* I, *Agr* II, *Agr* III, and *Agr* IV) according to the sequences diversity in the variable regions (4).

S. aureus strains are in planktonic and biofilm modes. The ability to form biofilms facilitates bacterial adhesion to various surfaces of medical devices, including catheters and artificial heart valves as the main mechanisms in the pathogenicity development of *S. aureus* strains. Biofilms can reduce bacterial drug susceptibility in *S. aureus*-related chronic diseases, including osteomyelitis, endocarditis, implant-related, and wound infections (5,6). Biofilm-producing bacteria adopt several mechanisms to be more resistant to antibiotics. These mechanisms included limited antibiotic diffusion into the biofilms, and transmission of resistance genes across the community (7). *S. aureus* express several proteins, including clumping factors A and B (ClfA and ClfB), which can bind specifically to fibronectin, and some of them, such as fibronectin-binding proteins A and B (FnBPA and FnBPB), can bind to fibronectin, fibrinogen, and collagen. They are associated with the biofilm formation (8). The operon *ica* in *S. aureus* produces an intercellular adhesion molecule, which regulates biofilm formation and facilitates cell junctions. These molecules allow *S. aureus* to attach to host cell surfaces, invade, damage tissue, and form biofilms, which protect the internal bacteria inside biofilms, hiding from immune system defense mechanisms and failure of antibiotic therapy (9).

Due to the clinical importance of MRSA, the role of biofilm formation in antibacterial drug resistance, and regulatory effects of *Agr* genes in *S. aureus*, the present study aimed to investigate biofilm-forming ability and *Agr*-specific group of clinical MRSA in Northern Iran.

Methods

In this cross-sectional study, clinical samples were collected from patients' blood, urine, and skin lesions in Guilan province, Northern Iran in 2021. In total, 200 non-duplicate *S. aureus* isolates were included in this study and duplicate samples from patients were excluded. Isolation and identification of test bacteria were performed using biochemical and molecular methods as described previously (10). *S. aureus* ATCC43300 strain has been used as a positive control.

Antibacterial resistance of test isolates was investigated according to the CLSI (2020) guideline. The disks of antibiotics (High Media-India), including Cefoxitin (30 µg), Cephalexin (30 µg), Cephalothin (30 µg), Penicillin (10 µg), Amoxicillin (25 µg), Imipenem (10µg), Gentamicin (10 µg), Clindamycin (2 µg), Doxycycline (30 µg), Minocycline (30 µg), Tetracycline (30 µg), Nitrofurantoin (300 µg), Teicoplanin, Cotrimoxazole (23.75 µg), Azithromycin (15 µg), Erythromycin (15 µg), Clarithromycin (15 µg), Rifampicin (5 µg), Ciprofloxacin (5 µg) were used to determine the antibiotic sensitivity of methicillin-resistant isolates. The resistance of isolates to vancomycin was assessed by MIC measurement using the CLSI broth microdilution method. Isolates exhibiting resistance to at least one agent in three or more different antimicrobial categories were defined as multi-drug resistant (MDR) (11).

All the isolates were determined as MRSA following phenotypic (Cefoxitin disc screening) and genotypic (Amplification of the *mec*A gene) methods. The Gram-positive bacteria DNA extraction kit was used to extract the bacterial genome (Cinnagen, Iran). Previously published primers were used for *mec*A gene amplification (1). PCR reaction was performed in 25 µl, including 12.5 µl of PCR master mix (Cinnagen, Iran), 20 pmol of each primer, and 5 µL of template DNA. Thermocycler thermal treatment was as described previously (1). Eventually, PCR products were electrophoresed on 1.5% agarose gel, which was examined via UV transilluminator. *Agr* gene types were determined by *Agr* group-specific multiplex PCR using specific primers of the four types of this gene as described previously (3). PCR reaction was performed as described above and obtained products were detected by electrophoresis using a 1% agarose gel and confirmed by sequencing. Biofilm-forming assay was performed in a microtiter plate. In brief, standard overnight cultures (1.5×108 CFU/ml) were diluted 100 folds in Tryptic soy broth containing 1% glucose. From each culture dilution, 200 µl was transferred into individual wells of a 96-well flat-bottomed polystyrene plate and incubat¬ed overnight at 37 °C for 48 hours. Then, the wells were rinsed three times with PBS and subsequently fixed with methanol for 20 min, stained with 200 µl of 0.02% crystal violet and rinsed with distilled water for 5 minutes. Biofilm was quantitatively analyzed by adding 200 µl of 33% glacial acetic acid to each well after drying the plates, followed by measuring their OD at 492 nm as described previously (12). *Staphylococcus epidermidis* ATCC 35984 strains and *Staphylococcus epidermidis* ATCC 12228 strains were used as positive and negative biofilm formation controls, respectively. The frequency of eight genes associated with biofilm formation, including *ica*A, *ica*D, *bap*, *fnb*A, *fnb*B, *clf*A, *clf*B, and *cna*, was determined using previously published methods in a PCR

reaction (9), with the same PCR reaction parameters as the previous stage. PCR products were detected by electrophoresis using a 1% agarose gel. The correlation between antibiotic resistance and *Agr* locus with frequency of biofilm encoding genes of MRSA isolates was analyzed using SPSS software and Chi-square test. P≤0.05 was considered statistically significant.

Results

In total, 200 isolates of *S. aureus* were identified in clinical samples of urine (120 isolates), skin lesions (45 isolates), and blood (35 isolates). Among them, resistance to selected antibiotics was detected as: gentamicin 50 %, clindamycin 62.5%, cephalexin 50. %, cefoxitin 62.5%, cephalothin 62.5%, penicillin 91.5%, amoxicillin 71.5%, imipenem 12.5%, doxycycline 74%, minocycline 41.5%, tetracycline 75%, nitrofurantoin 55%, teicoplanin 9.5%, Co-Trimoxazole 38.5%, azithromycin 50%, erythromycin 62.5%, clarithromycin 66.5%, rifampicin 46%, ciprofloxacin 69.5%. In 10 isolates (8.3%), MIC values of vancomycin were ≥4 µg/ml and considered vancomycin-non-susceptible isolates. Moreover, more than 75% of the isolates demonstrated multiple antibiotic resistance. Among 125 cefoxitin-resistant isolates, the *mec*A gene was detected in 120 (96 %) isolates using PCR reaction.

Agr types 1, 2, 3, and 4 were identified in 78, 15, 17, and five MRSA isolates, respectively, and ten isolates were non-typeable for *Agr* locus. Among 125 MRSA isolates, 79.2% were able to form biofilms, of which 19 (15.2%), 26 (20.8%), and 54 (43.2%) isolates produced weak, moderate, and strong biofilms, respectively. The *clf*A, *clf*B, and *fnb*A genes were found in all isolates that passed the biofilm-producing phenotypic test, and *ica*A and *ica*D were found in 85.85% and 78% of them, respectively. The frequency of all tested biofilm-associated genes was significantly higher in MDR isolates (P< 0.05). All of the biofilm-associated genes were identified in *Agr*-positive strains. Table 1 represents the frequency of biofilm production encoding genes of MRSA isolates in *Agr*-specific groups. Also, agarose gel electrophoresis of *mec*A, *Agr* and selected biofilm associated genes are shown in Figures 1.

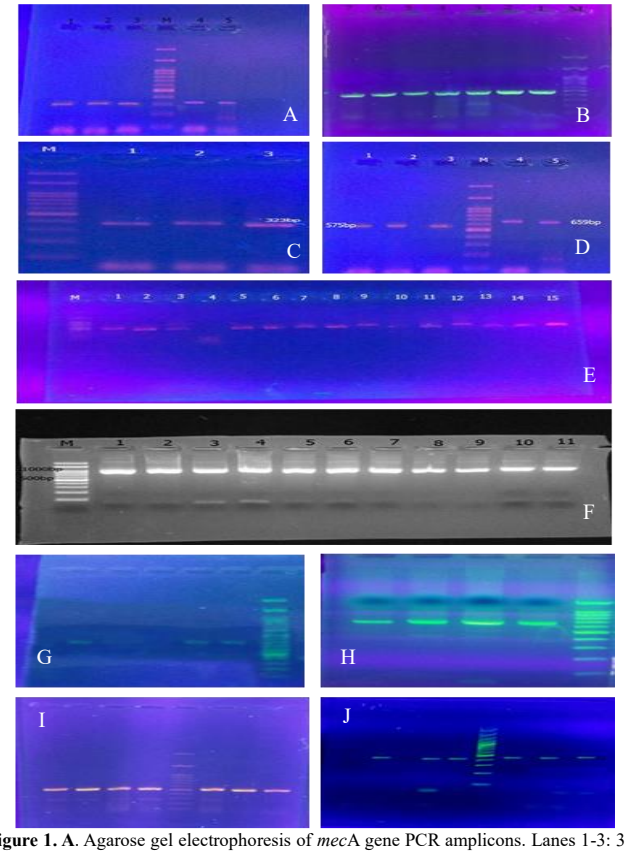

Figure 1. A. Agarose gel electrophoresis of *mec*A gene PCR amplicons. Lanes 1-3: 310 bp PCR amplicons of *mec*A; Lane M:100 bp DNA marker**; B.** Agarose gel electrophoresis of *Agr*I gene PCR amplicons. Lanes 1-7: 441 bp PCR amplicons of *Agr*I; Lane M:100 bp DNA marker; **C**. Agarose gel electrophoresis of *Agr*III gene PCR amplicons. Lanes 1-3: 323 bp PCR amplicons of *Agr*II; Lane M:100 bp DNA marker; **D**. Agarose gel electrophoresis of *Agr*II and *Agr* IV gene PCR amplicons. Lanes 1-3: 575 bp PCR amplicons of *Agr*II; Lane M:100 bp DNA marker; Lanes 4 and 5: 659 bp PCR amplicons of *Agr*IV; **E**. Agarose gel electrophoresis of *clf*B gene PCR amplicons. Lanes 1-3 and 5-15: 505 bp PCR amplicons of *clf*B; Lane M:100 bp DNA marker; **F**. Agarose gel electrophoresis of *bap* gene PCR amplicons. Lanes 1-11: 971 bp PCR amplicons of *bap*; Lane M:100 bp DNA marker; **G**. Agarose gel electrophoresis of *clf*A gene PCR amplicons. Lanes 1,2 and 5: 855 bp PCR amplicons of *clf*A; Lane M:100 bp DNA marker; **H**. Agarose gel electrophoresis of *fnb*A gene PCR amplicons. Lanes 1-4: 643 bp PCR amplicons of *fnb*A; Lane M:100 bp DNA marker; **I**. Agarose gel electrophoresis of *fnb*B gene PCR amplicons. Lanes 1-7: 524 bp PCR amplicons of *fnb*B; Lane M:100 bp DNA marker; **J**. Agarose gel electrophoresis of *cna* gene PCR amplicons. Lanes 1-6: 423 bp PCR amplicons of *cna*; Lane M:100 bp DNA marker.

Table 1. Frequency of biofilm production encoding genes of MRSA isolates in *Agr*-specific group

Gene	Frequency (Percentage)	*Agr* type			
		Agr IV	*Agr III*	*Agr II*	*Agr I*
*ica*A	85 (68)	2	8	5	70
*ica*D	78 (62.4)	3	4	3	68
bap	16 (12.8)	-	-	-	16
*fnb*A	101 (80.8)	2	14	11	74
*fnb*B	90 (72)	-	10	9	71
*clf*A	99 (79.2)	3	12	10	74
*clf*B	99 (79.2)	3	13	9	74
cna	23 (18.4)	-	-	1	22

Discussion

In the present study, a total of 200 *S. aureus* isolates were screened for methicillin resistance, biofilm formation and *Agr*-specific grouping. Among them, 62.5% were methicillin-resistant and 75% were MDR isolates. The frequency of MRSA may vary by region, indicating the rising trend over the years. According to a systematic review and meta-analysis, the overall prevalence of MRSA in Iran varied from 20% to 90% (13). In a study by Arabestani et al. (2016), more than 50% of *S. aureus* isolates were methicillin-resistant which is in line with those in this research (14). A 100% frequency of MRSA was also reported in an epidemiological investigation conducted in various teaching hospitals in Tehran (15). In our study, *mec*A was found in 96% of MRSA isolates. The absence of the *mec*A gene is common in cefoxitin-resistant strains. This finding may have resulted from a false-negative PCR reaction that can arise from point mutation or deletion in *mec*A gene or as a result of the non-*mec*A methicillin resistance mechanisms, such as the novel *mec*A homologous, *mec*C (16,17). The frequency of MDR isolates detected in the present study is higher than what was reported by Derakhshan et al., (2021) and is in accordance with different studies from Iran (3,18,19). Also, according to previous studies (3,20), the present *S. aureus* strains showed a high frequency of resistance to β-lactams, which can be due to the wide use of these antibacterials in the treatment of different infections. Additionally, 79.2% of MRSA isolates have the ability to generate biofilms with the frequency of *fnb*A (80.8%), *fnb*B (72%), *clf*A (79.2%) *clf*B (79.2%), *ica*A (68%), *ica*D (62.4%), *bap* (12.8%) and *cna* (18.4%). In addition, we found an association between the frequency of all tested biofilm-associated genes and MDR phenotype (P<0.05) and *Agr* type I was the most prevalent type (62.4%) in tested isolates, followed by types III (13.6%), II (12%), and IV (4%). Furthermore, all of the biofilm-associated genes were identified in *Agr*-positive strains. This finding is consistent with two different studies that found that the presence of the *Agr* operon was strongly associated with the carriage of virulence genes (9,18).

Conclusion

The findings of the current study indicate a significant relationship between the frequency of biofilm-associated genes, MDR phenotype, and the presence of *Agr* locus in MRSA. However, the correlation between antimicrobial resistance and biofilm production with *Agr* type is difficult to demonstrate and needs further investigations. The present study suggests that reliable and rapid identification of biofilm-forming MRSA strains and treatment of related diseases are required to prevent the spread of these bacteria.

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None.

Ethical statement

Since we did not use any animal models and patients and just used isolates that were previously obtained from clinical samples in laboratories, we did not have any ethical approval for this study, nevertheless, we confirm that the study complies with all regulations.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Mahsa Aghaei: Data curation, writing-original draft. Leila Asadpour: Writing-review and editing, supervision, and methodology. Amir Arasteh: Methodology.

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