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Several Virulence Factors of Multidrug-Resistant *Staphylococcus aureus* Isolates From Hospitalized Patients in Tehran

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Background: Biofilm formation plays an important role in resistance of *Staphylococcus aureus* isolates; especially multidrug-resistant isolates are a threat to healthcare settings.

Objectives: The aims of this study were to detect biofilm formation and presence of several related genes among multidrug-resistant (MDR) isolates of *Staphylococcus aureus*.

Patients and Methods: A total Of 209 S. *aureus* strains were isolated from patients and identified by conventional diagnostic tests. The multidrug-resistant MRSA isolates were detected by antibiotic susceptibility test. The phenotypic biofilm formation was detected by microtitre tissue plate assay. The polymerase chain reaction (PCR) was performed to detect the *mecA*, Staphylococcal Cassette Chromosome *mec* (*SCCmec*) types, accessory gene regulatory (*agr*) genes, the *icaADBC* and several genes encoding staphylococcal surface proteins including *clfAB*, *fnbAB*, *fib*, *eno*, *can*, *ebps* and *bbp* genes with specific primers.

Results: Sixty-four (30.6%) isolates were methicillin-resistant, among which thirty-six (56.2%) were MDR. These isolates were resistant to amoxicillin, tetracycline, ciprofloxacin, gentamicin, erythromycin and trimethoprim-sulfamethoxazole (except to 6 isolates). All the isolates were susceptible to vancomycin and linezolid. All the MDR-MRSA harbored *SCCmec* type III. All the MDR- MRSA isolates were strong biofilm producers in the phenotypic test. The majority of MDR- MRSA was belonged to *agrI* (67%, n = 24), followed by *agr II* (17%, n = 6), *agrIV* (11%, n = 4) and *agrIII* (5.5%, n = 2). The frequency of *icaADBC* genes were 75% (n = 27), 61% (n = 22), 72% (n = 26) and 72% (n = 26), respectively. Furthermore, the prevalence of *clfA*, *clfB*, *fnbA*, *fnbB*, *fib*, can, eno, ebps and *bbp* genes was 100%, 100%, 67%, 56%, 80%, 63%, 78%, 7% and 0%, respectively. Furthermore, approximately all the MRSA was strong biofilm producers.

Conclusions: Multidrug-resistant isolates produced biofilm strongly and the majority harbored most of biofilm related genes, suggesting that biofilm formation is associated to the presence of these genes, and also biofilm production can increase the antibiotic resistance as have demonstrated in MDR-MRSA isolates.

Keywords: Staphylococcus aureus; MRSA; Multidrug-Resistance

1. Background

The development of multidrug resistance by *Staphylococcus aureus* (SA) especially due to MRSA isolates in nosocomial settings is a public health concern. In the hospital milieu, infected and colonized patients contribute to the transmission and spreading of *S. aureus* and hospital personnel, serving as reservoirs, facilitate further dissemination (1, 2). Infections caused by MRSA often prove difficult to treat because of high levels of resistance to multiple antibiotics as a result of both intrinsic and acquired mechanisms (3). Moreover, in recent years vancomycin resistance have made treatment of these isolates very difficult (4). Drug resistance in *S. aureus* is occurred by complex genetic arrays such as the staphylococcal cassette chromosome *mec* (*SCCmec*) elements for methicillin-resistance and likewise all the beta-lactam antibiotics via production of new Penicillin Binding Protein2a (PBP2a) (5). MRSA isolates that are acquired from nosocomial milieu are referred as Healthcare Associated (HA)-MRSA. Various nosocomial infections such as those associated with the use of central venous catheters, prosthetic heart valves, urinary catheters and orthopedics devices contribute to the biofilm formation and persistent infections that culminate in evading immune system responses and antibiotic resistance (6). Susceptibility tests with in- vitro biofilm models have depicted the survival of bacteria in biofilms after treatment with antibiotics with even much more than the minimum inhibitory concentrations. Moreover, in the body, chemotherapy dose not kill bacteria in biofilms, and when stopped, culminates in growing and spreading of the bacteria from biofilms (7). Biofilm formation also mediates the spreading of the antibiotic resistance traits in nosocomial pathogens by developing mutation rates and the exchange of genes responsible for antibiotic resistance (8). Biofilms are closely involved in higher antibiotic resistance because of several conditions including lower penetration of antibiotics, lower

growth rate of bacteria in biofilms and altered metabolic requirements. Eventually, the surviving isolates in the inner portion of biofilms are likely to possess a higher probability of acquiring the ability to develop biofilms as well as multidrug resistance in clinical settings (9). Biofilm forming strains are more frequently isolated from non-fluid tissues, in particular bone and soft tissues, and also MDR pathogens are more often biofilm formers. Several studies have shown that methicillin-resistance can alter the ability of the *S. aureus* isolates regarding biofilm production (10). To our knowledge, previous studies on the biofilm formation among MDR isolates and the relationship to clinical manifestations are scarce (11).

2. Objectives

The aim of this study was to detect the biofilm production and the biofilm encoding genes in MDR *S. aureus* isolates.

3. Patients and Methods

3.1. Bacterial Isolates

We evaluated a total of 36 MDR MRSA clinical isolates from different clinical sites of patients including 14 males and 22 females from July 2012 to January 2013. The *S. strains* were isolated from ICU, infectious diseases, inpatient and pediatrics settings. In addition, the isolates were identified with catalase, coagulases, acid production from mannitol on mannitol salt agar and DNase tests.

3.2. The Antibiotic Susceptibility Test

The antibiotic susceptibility pattern of *S. aureus* isolates was drawn according to Clinical and Laboratory Standards Institute (CLSI, Kirby Bauer assay) that detected MDR- MRSA isolates. The phenotypic detection of MRSA was conducted with oxacillin (1 μg) disk (MAST, UK).

3.3. The Biofilm Formation in the Phenotypic Test

The phenotypic biofilm production was performed by Microtitre-tissue plate (Mtp) assay.

3.4. Genomic DNA Extraction

Total genomic DNA was extracted by preparation of a suspension of bacterial isolates in 200 μL of TE buffer and lysostaphin (comprising 200 μL of TE buffer and 20 μL of lysostaphin [2 μg/mL, Sigma]). The DNA was isolated according to Straubinger method (12).

3.5. DNA Amplification

DNA was amplified with specific primers (shown in Table 1) to detect the *mecA* gene, *SCCmec* types, *agr* specific groups and biofilm related genes, including the *icaADBC*, *clfAB*, *fnbAB*, *cna*, *ebps*, *eno*, *fib* and *bbp* genes among the clinical isolates. The annealing temperature was 55°C (30 seconds) for *mecA* gene and 51°C (1 minute) for *SCCmec* types, according to Zhang study (13). Primers for *mecA* gene and *SCCmec* types have been shown in Table 1. For observation of the PCR products by electrophoresis, 5 μL of each product was mixed with 1 μL of each gel red and loading buffer dyes, and were run in 1% agarose gel electrophoresis and was observed by transluminator uv.

3.6. Data Analysis

The relationship between multidrug resistance and biofilm formation was evaluated by the Pearson Chi-Square test where any difference less than 0.05 was considered significant.

4. Results

4.1. Bacterial Isolates

The bacterial isolates were collected from different hospital settings and clinical sites, according to Figures 1 and 2.

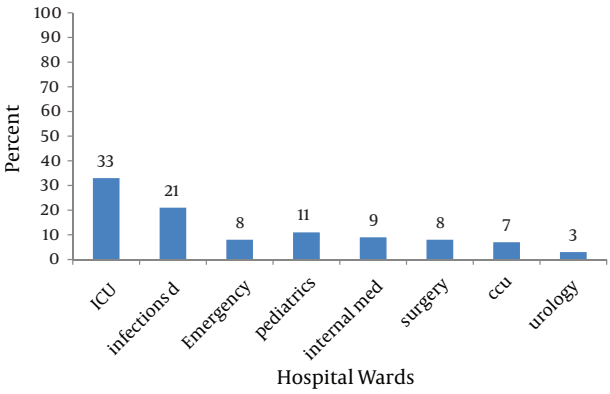

Figure 1. The Hospital Settings From Which the Bacterial Isolates Were Collected

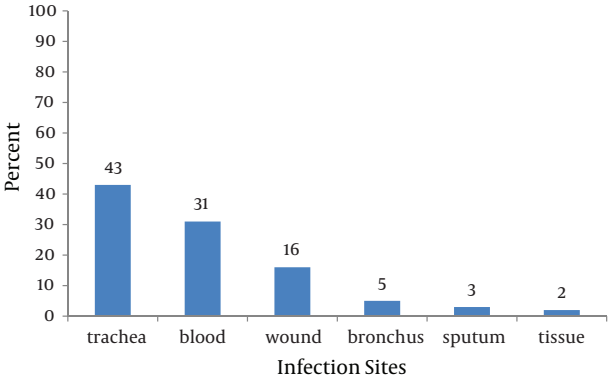

Figure 2. Isolation of *S. aureus* From Different Clinical Specimens

4.2. The Phenotypic Tests

Among the total of 209 *S. aureus* isolates, 30.6% (n = 64) were methicillin-resistant with oxacillin disk and in addition by detection of *mecA* gene with 147 bp size. Of 64 MRSA, 56% (n = 36) were resistant to all the used antibiotics (MDR MRSA) including amoxicillin, tetracycline, erythromycin, clindamycin, trimethoprim-sulfamethoxazole (except for 6 isolates), gentamicin and ciprofloxacin. Also, 24 isolates in this study were multidrug-resistant and methicillin susceptible *S. aureus* (MDR-MSSA). All these isolates were resistant to amoxicillin, erythromycin and tetracycline, and the majority was resistant to ciprofloxacin (79%) and clindamycin (62.5%). The majority of MDR isolates were belonged to ICU setting. The resistance to trimethoprim- sulfamethoxazole and gentamicin between MDR-MRSA and MDR- MSSA were significant. All the MDR-MRSA and the majority of MDR- MSSA isolates were strong biofilm producers in the Mtp assay.

4.3. The SCCmec Elements

All the MDR- MRSA carried *SCCmec* type III with a 280 bp size PCR product. The rate of antibiotic resistance among these isolates was significantly higher than MSSA (P value = 0.012). Six isolates were susceptible to co- trimoxazole (SXT). In our study, isolates with *SCCmec* type III were resistant to more variety of antibiotics compared to other types.

4.4. The agr Groups

The majority of MDR- MRSA was belonged to *agrI* (67%, n = 24), followed by *agr II* (17%, n = 6), *agrIV* (11%, n = 4) and *agrIII* (5.5%, n = 2). The relation between the agr specific groups and the antibiotic resistance was not confirmed. There was not a confirmed relationship between the agr groups and antibiotic susceptibility pattern or biofilm production by the isolates. Among the biofilm related genes, the frequency of icaADBC genes in MDR-MRSA were 75% (n = 27), 61% (n = 22), 72% (n = 26) and 72% (n = 26), respectively. Furthermore, the prevalence of *clfA*, *clfB*, *fnbA*, *fnbB*, *fib*, *can*, *eno*, *ebps* and *bbp* genes was 100%, 100%, 67%, 56%, 80%, 78%, 7% and 0%, respectively. No relation between each of the *agr* specific groups and biofilm encoding genes among these isolates was confirmed. The high prevalence of these associated genes was correlated with strong biofilm production in the isolates. The differences among MSSA, MRSA and MDR isolates regarding biofilm formation and the presence of biofilm related genes has been depicted in Figure 3. Furthermore, the relationships of the virulence or biofilm genes and the *agr* genes have exhibited in Table 1.

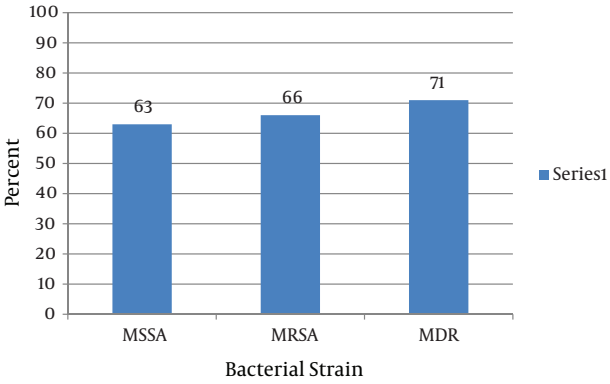

Figure 3. The Differences Among MSSA, MRSA and MDR Isolates Regarding Biofilm Formation and the Presence of Biofilm Related Genes

5. Discussion

In the present study, the prevalence of MRSA was 30.6%. In our previous studies also it was not high (14-16). However several other previous published surveys from Iran have determined that MRSA frequency is relatively high. For example a systemic review by-results that it is more than 50%. The difference in these results originates mainly from differences in the genetic background of strains, clinical origins and epidemiological areas of studies. In the present study, the MRSA isolates exhibited significantly more resistance to the used antibiotics, except to vancomycin and linezolid. Among the 64 MRSA 36 isolates (56%) were resistant to the all the antibiotics and contained *SCCmec* type III. Although 6 isolates with *SCCmec* type III were only susceptible to SXT, vancomycin and linezolid. Moreover, 24 MSSA isolates were MDR *S. aureus*. The majority of MDR isolates in this study belonged to ICU patients, indicating that the origin of the isolates may be the same. In the phenotypic biofilm production, the MDR isolates produced biofilms strongly; exhibiting that biofilm production can highly affect the extent of antibiotic resistance among multidrug-resistant isolates.

Table 1. The Relation Between the *Agr* Specific Groups and the Presence of Each Virulence Genes in MDR-MRSA Isolates

Agr	**Biofilm Genes, %**									
	clfA	*clfB*	*fnbA*	*fnbB*	*fib*	*eno*	*cna*	*ebps*	*bbp*	*icaADBC*
agrI	100	100	73	63	77	72	57	11	0	67
agrII	100	100	67	46	73	72	64	9	0	66
agrIII	100	100	66	46	76	66	54	4	0	57
agrIV	100	100	64	57	78	68	55	6	0	63

The majority of healthcare associated MRSA harbor the *SCCmec* type III, according to previous results (17-19). Also these MRSA were significantly more resistant to antibiotics, enhancing the idea that these isolates have caused hospital acquired infections (20, 21). This study depicts the importance of *SCCmec* type III in the multidrug antibiotic resistance of MRSA. The majority (73.2%, n = 26) of multidrug MRSA was belonged to *agrI* (67%, n = 24) Followed by *agrII* (19.5%, n = 7), *agrIV* (8%, n = 3) and *agrIII* (5.5%, n = 2). Our previous studies also exhibited that *agrI* was the predominant specific group (22). The *agr* groups play an important role in the regulation of several virulence factors of *S. aureus*. However, the relation between each specific group and pathogenesis, clinical manifestations and drug resistance of MRSA isolates has not certainly been determined. In this study, all the MDR-MRSA strains were capable of producing biofilm strongly by attachment into wells of micro-titre tissue plates. This result emphasizes the role of biofilm formation in various persistent and chronic infections caused by MDR-MRSA that do not response to antimicrobial therapy. Furthermore, the majority (72%) of multidrug MRSA harbored icaAD genes necessary for biofilm formation. As mentioned, these gene form biofilm via a synthesis of a Polysaccharide Inter-cellular Adhesion (PIA). The frequency of *icaADBC* genes were 75% (n = 27), 61% (n = 22), 72% (n = 26) and 72% (n = 26), respectively. The high prevalence of the *ica* genes alongside with strong biofilm formation justifies the resistance of MRSA isolates to a myriad of adverse conditions in addition to antibiotics. Besides this, in our previous study, we demonstrated that all these isolates can express all the biofilm related genes in Real time PCR assay (manuscript submitted). PIA related biofilms are mainly acquired from medical devices and catheters and culminate in systemic infections, and also make the treatment very more difficult (23, 24). In this study several patients had died albeit antibiotic therapy. Previous studies also have determined the high prevalence and importance of these genes in biofilm producing isolates (25). In the study of Semczuk, all the isolates producing biofilm phenotypically harbored *icaAD* genes. In Hou's study, among 55.56% of isolates that produced biofilm in phenotypic test, 11.11% contained the *icaA* gene (26), but the other genes has not been investigated. In this study, methicillin resistant isolates harbored higher rate of *icaADBC* genes compared to MSSA, but no significant difference was confirmed, similar to two other studies (27, 28). Moreover, Smith determined no significant relation between susceptibility to methicillin and biofilm formation (29). Likewise, Rasha detected the *icaAD* genes in 32% of blood and catheter isolates (37). In Zmantar's et al. study 36 of 46 Staphylococcal isolates harbored *icaA* and *icaD* genes (38); while Grinholc and coworkers did not detect *icaD* but all strains were *icaA* positive (39). Lachachi detected the *icaAD* genes in 17 (38.5%) of the 44 staphylococcal isolates from urinary tract (31). In the Wang study, biofilm formation in most of the isolates was PIA dependent (32). On the other hand, clinical origin of the isolates and infection site may be an important factor in the ability of the isolates to biofilm formation. for example, Smith depicted that isolates of *S. aureus* from infected skin lesions were significantly more capable of producing biofilms than those isolated from blood and other infected sites. Satorres suggests that the *ica* genes might be more prevalent in Staphylococcus strains isolated from the hospitalized patients or the staff, than healthy individuals or from the community (33). To our knowledge, the previous studies about biofilm production in MDR-MRSA isolates are scarce. However, we determined that there is a relationship between these isolates and biofilm formation. Furthermore, in our study the prevalence of *clfA*, *clfB*, *fnbA*, *fnbB*, *fib*, *eno*, *can*, ebps and *bbp* genes was 100%, 100%, 67%, 56%, 80%, 78%, 65%, 7% and 0%, respectively. Similarly, in Atshan's study, all MRSA and MSSA strains harbored *clfA,B* genes (34). However, Momtaz confirmed that nearly 20% of *S. aureus* isolates that caused mastitis contained *clfA* gene (35). It seems that the kind of clinical isolates (site of infections) may be important in the prevalence of these genes essential for colonization in addition to the epidemiological differences. Furthermore, we observed that all the MRSA and MSSA isolates harbored *clfAB* genes. In this study the prevalence of *fnbA* and *fnbB* was 60% and 47%, respectively. A study by Boden Wastfelt have detected the *fib* gene in all *S. aureus* strains (36). The prevalence of *eno* and *cna* genes was 78% and 63%, respectively, showing the important role of these genes in colonization of *S. aureus*. Because of the high presence of laminin and collagen in tissues, *S. aureus* isolates can easily and rapidly bind to the specific receptors. The limitation of this study was low number of MDR isolates, thus more studies are needed in future to detect biofilm formation in MDR nosocomial isolates. All the MRSA with multiple antibiotic resistances contained *SCCmec* type III and belonged to *agrI*. The frequency of different biofilm associated genes and likewise the amount of biofilm production was high in MDR-MRSA isolates. MDR isolates of *S. aureus* might be capable of strong biofilm formation that contributes to the higher antibiotic resistance.

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Authors' Contributions

Abdolmajid Ghasemian performed the clinical experiment, Dr. Shahin Najar Peerayeh advised the work, Bita Bakhshi and Mohsen Mirzaee provided purely technical help, copyediting, proofreading or translation assistance.

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