Biofilm formation and antimicrobial resistance in methicillin-resistant Staphylococcus aureus isolated from burn patients, Iran

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Abstract
Introduction: Burns are the most serious forms of trauma and a major cause of mortality worldwide. Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most common pathogens of burn wound infections; treatment has faced serious problems due to antibiotic resistance in these strains. Biofilm formation, which increases antibiotic resistance capabilities and is considered to be a virulence factor, also causes treatment failure and recurrent staphylococcal infections in burn patients.

Methodology: A total of 135 pus/wound swabs were collected; S. aureus was identified by confirmatory tests. The icaA/D and mecA genes were detected in DNA extracts by polymerase chain reaction assay separately. To determine the prevalence of biofilm formation, a modified Congo red agar and the microtiter plate method were used. Investigation of antibiotic resistance was performed using the disk diffusion method.

Results: S. aureus (48.87%) was identified in 65 (48.87%) samples, of which 40 (61.53%) were confirmed to be MRSA. Among MRSA and methicillin-sensitive S. aureus (MSSA) isolates, 97.5% and 60% produced biofilm, respectively. Resistance of MRSA isolates to amikacin, ceftriaxone, ciprofloxacin, gentamicin, mupirocin, rifampin, tetracycline, and tobramycin was 64.1%, 76.92%, 51.28%, 87.18%, 71.8%, 10.26%, 5.13%, and 89.74%, respectively. All MRSA and MSSA isolates were susceptible to fusidic acid, linezolid, teicoplanin, tigecycline, and vancomycin.

Conclusions: The high prevalence of biofilm-producing, drug-resistant S. aureus isolates in our study suggests that epidemiological studies on the characteristics of common strains found in burn centers and a definition of their antibiotic resistance pattern would be helpful for therapeutic decisions.

Key words: biofilm; antimicrobial resistance; MRSA; burn patients.


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Introduction
Burns are the most common and serious form of trauma [1] and are still a major public health problem and a leading cause of mortality worldwide [2,3]. Due to the loss of protective barriers and consequently a reduction of cellular and humoral defense, microbial colonization and infection risk in patients hospitalized with burns is very high [3]. Staphylococcus aureus is one of the most common pathogens of burn wounds. It has the ability to generate a large number of virulence factors, which are important in pathogenesis and infectious invasion [4]. The global emergence of organisms with multiple drug resistances (MDRs), such as methicillin-resistant Staphylococcus aureus (MRSA), is an important factor in acute and chronic infections that leads to increased mortality rates and increased healthcare costs [5]. Difficulty in choosing appropriate antibiotics due to antibiotic resistance has limited the treatment of burn wound infections [6,7].

In addition to antibiotic resistance, the other factor that causes treatment failure and chronic and recurrent staphylococcal infections in burn patients is biofilm formation in these strains [8,9]. Since the ability of biofilm production by MRSA increases antibiotic resistance, hospital patients infected with these strains are at serious risk for treatment failure [10]. Biofilm formation is considered to be a virulence factor because the microorganisms that establish in a burn wound biofilm fundamentally differ from suspended populations [11]. Biofilm acts as a barrier to antimicrobial agents and the host immune system that assists sustained bacterial colonization [12].

Biofilms are organized communities of bacterial cells that are accumulated in a polymeric matrix produced by bacteria and can be attached to living or inanimate surfaces [12,13]. Researchers have demonstrated that the first stage of staphylococcal infections is attachment to surfaces such as host
tissues[14]. Recently, studies about genes involved in biofilm formation and their role in staphylococcal infections have received a great deal of attention [15]. Molecular studies have shown that during the late stages of attachment, organisms are attached to each other to form biofilms. This is done through polysaccharide intercellular adhesin (PIA), which is synthesized by products of the icaABC operon [16].

Choosing an appropriate antibiotic for treatment of a bacterial infection mainly depends on knowledge about the characteristics of resident strains and their antibiotic susceptibility profiles. Thus, determination of the antibiotic susceptibility patterns in prevalent strains of burn units leads to appropriate treatment and a decrease in economic costs. Biofilm formation followed by development of higher antibiotic resistance is more crucial in burn patients than in other patients. The aim of this study was to determine the prevalence of biofilm formation among MRSA and methicillin-sensitive S. aureus (MSSA) isolated from burn wound infections and their antibiotic susceptibility patterns. MRSA and MSSA isolates were examined for production of slime by Congo red assay for the presence of the icaA/D genes required for the production of PIA or polymeric N-acetyl glucosamine, and for their ability to attach to substrate in the microtiter plate assay for biofilm development.

Methodology

Bacteria isolates

Between December 2012 and March 2013, a total of 135 pus/wound swabs from skin and soft tissue infections (SSTIs), were collected from patients in the Motahari burn care center affiliated with Tehran University of Medical Sciences (TUMS). The samples were immediately sent to the TUMS laboratory in the microtiter plate assay for biofilm development. All isolates were incubated for 72 hours under aerobic conditions at 37°C using the modified Congo red agar medium; this was performed three times for all isolates. The morphology of colonies was then interpreted based on colony color as red, almost black, black, and very black. Strains with red colonies were considered to be non-slime-producing, and strains almost black, black, and very black colonies were considered to be slime-producing [19].

Molecular studies have shown that during the late stages of attachment, organisms are attached to each other to form biofilms. This is done through polysaccharide intercellular adhesin (PIA), which is synthesized by products of the icaABC operon [16].

Confirmation of methicillin-resistant S. aureus (MRSA)

Bacterial suspension of all S. aureus isolates equivalent to 0.5 McFarland was prepared and cultured on Mueller-Hinton agar medium containing 2% NaCl and oxacillin antibiotics (6 µg/mL), then incubated for 24 hours at 30°C. The growth inhibition zones were interpreted using Clinical Laboratory Standards Institute (CLSI) guidelines [17]; thus methicillin-resistant isolates were screened.

Bacterial DNA was extracted using a DNeasy kit (Qiagen, Valencia, USA) according to the manufacturer's instructions; lysostaphin enzyme was used in the first stage. The mecA gene was then detected in DNA extracts by PCR assay [18].

Slime assay on modified Congo red agar (MCRA)

All isolates were incubated for 72 hours under aerobic conditions at 37°C using the modified Congo red agar medium; this was performed three times for all isolates. The morphology of colonies was then interpreted based on colony color as red, almost black, black, and very black. Strains with red colonies were considered to be non-slime-producing, and strains almost black, black, and very black colonies were considered to be slime-producing [19].

Microtiter plate assay

The microtiter plate method was conducted as previously described [13]. Briefly, 200 µl of bacterial suspension grown in trypticase soy broth (TSB), supplemented with 1% glucose and diluted. A total of 1:100 was poured into the wells of sterile flat-bottomed 96-well polystyrene tissue culture plates (200 µl of TSB supplemented with 1% glucose was used as the negative control) and incubated 24 hours at 37°C. All tests were performed in triplicate. Washing was then performed three times for each well with sterile phosphate-buffered saline (PBS; pH 7.2). After that, the fixation step was done by air drying. Subsequently, the adherent biofilm layer was stained by crystal violet for 15 minutes at room temperature. This was followed by the washing steps. Then the plates were air dried and resolubilized with ethanol (95%) for 30 minutes. Finally, the optical density (OD) of each well was measured at 570 nm, and average OD value of negative controls and samples was calculated. Interpretation of results was described as follows:

ODs ≤ ODc = no biofilm producer; ODc ≤ ODs ≤ 2 × ODc = weak biofilm producer; 2 × ODc ≤ ODs ≤ 4 × ODc = moderate biofilm producer; 4 × ODc < ODs = strong biofilm producer (ODc = OD control negative, ODs = OD samples). Staphylococcus epidermidis ATCC 35984 was used as the biofilm producer control strain.

Identification of icaA/icaD genes

PCR assays were used to detect the mecA, icaA, and icaD genes separately. The genes were amplified
on an Eppendorf (Hamburg, Germany) thermocycler with a final volume of 20 μl containing 10 μl of Qiagen HotStarTaq master mix (Invitrogen, Carlsbad, USA, containing PCR buffer with 3 mM MgCl2, 400 μM of each dNTP and 2.5 units HotStarTaq DNA polymerase), 1 μl of each primer (20 pMol, MecA, ICAA and ICAD) (Table 1) [11,18], 6 μL of RNase-free water, and 2 μl of DNA template. The products were then electrophoresed on agarose gel and the presence or absence of any resulting bands was evaluated.

**Antibiotic susceptibility test**

The antibiotic susceptibility patterns were determined by the disk diffusion method (Kirby-Bauer test). This was carried out on Mueller-Hinton agar medium, and growth inhibition zones were measured and interpreted according to the CLSI guidelines [17]. The antibiotics used in this study included amikacin (30 μg), ciprofloxacin (5 μg), ceftriaxone (30 μg), erythromycin (30 μg), fusidic acid (5 μg), gentamicin (10 μg) linezolid (30 μg), mupirocin (5 μg), rifampin (5 μg), tetracycline (30 μg), teicoplanin (30 μg), tigecycline (15 μg), tobramycin (10 μg), and vancomycin (30 μg) (MAST Diagnostics, Merseyside, UK). *S. aureus* ATCC29213 was used as a standard strain.

**Microtiter plate method**

Using the microtiter plate method due to the negative control OD (ODn = 0.08), the majority of MRSA strains (62.5%) were moderate biofilm producers (0.16 < ODs ≤ 0.32), and only one isolate (the same isolate that had produced red colony on MCRA medium previously) was considered to be a non-biofilm-producing strain (OD ≤ 0.08). It should be mentioned that, according to the mean OD570 of the negative control (0.08), values between 0.08 and 0.160 (2 × 0.08) were considered to be weak biofilm producers, those between 0.160 and 0.320 (4 × 0.08) to be moderate biofilm producers, and values higher than 0.320 were considered biofilm producers (Table 3).

**Antibiotic susceptibility**

The antimicrobial susceptibility patterns of MRSA and MSSA isolates are presented in Table 4. The highest resistance rate was observed for both tetracycline and gentamicin among the MRSA isolates and for ceftriaxone among MSSA isolates. Resistance to rifampin and tobramycin was the lowest among MRSA and MSSA isolates, respectively.
Table 3. Results of biofilm production for MRSA and MSSA isolates using the microtiter plate method

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Biofilm former</th>
<th>Non-biofilm former</th>
<th>Total</th>
<th>Biofilm former</th>
<th>Non-biofilm former</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MRSA N = 25</td>
<td>MSSA N = 25</td>
<td></td>
<td>MRSA N = 40</td>
<td>MSSA N = 40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S N (%)</td>
<td>R N (%)</td>
<td></td>
<td>S N (%)</td>
<td>R N (%)</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>12 (80)</td>
<td>3 (20)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>28 (70)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>13 (86.67)</td>
<td>2 (13.33)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>12 (80)</td>
<td>3 (20)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>12 (80)</td>
<td>3 (20)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>14 (93.33)</td>
<td>1 (6.67)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Linezolid</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Mupirocin</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>13 (86.67)</td>
<td>2 (13.33)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>14 (93.33)</td>
<td>1 (6.67)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>25 (62.5)</td>
<td>7 (17.5)</td>
<td>32 (80)</td>
<td>2 (8)</td>
</tr>
</tbody>
</table>

1. ODs ≤ ODnc 2. ODnc < ODs ≤ 2.ODnc; 3. ODnc < ODs ≤ 4.ODnc; 4. ODnc < ODs; *: OD570nm of sample; **: OD570nm of negative control

Table 4. Frequency of antibiotic resistance of MRSA and MSSA strains

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MSSA N = 25</th>
<th>MRSA N = 40</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S N (%)</td>
<td>R N (%)</td>
</tr>
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<td>13 (86.67)</td>
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<td>Ceftriaxone</td>
<td>12 (80)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>12 (80)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>15 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>14 (93.33)</td>
<td>1 (6.67)</td>
</tr>
<tr>
<td>Linezolid</td>
<td>15 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mupirocin</td>
<td>15 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>15 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>15 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>13 (86.67)</td>
<td>2 (13.33)</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>15 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>15 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>14 (93.33)</td>
<td>1 (6.67)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>15 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
All MRSA and MSSA in this study were susceptible to fusidic acid, linezolid, teicoplanin, tigecycline, and vancomycin. Additionally, 66% of biofilm producer strains (MRSA and MSSA) were resistant to more than five antibiotics.

Distribution of icaA/icaD genes

All MRSA strains analyzed in this study were positive for the presence of icaA/icaD genes. The existence of the icaA/icaD genes in all biofilm producer MSSA isolates (15 isolates) was seen by PCR as well. These genes were absent among MSSA non-biofilm formers. The mecA gene was observed in all 40 MRSA isolates by PCR assay.

Discussion

Despite the development of control and prevention methods, bacterial infections are still the leading causes of death among burn patients [20]. Studies indicate that infections in burns lead to increased average lengths of stay in hospital that cost more than 800 dollar per case [21] and impose a heavy cost to health systems worldwide [22]. Observance of infection control measures reduces infection, and this leads to reduction of diagnosis and treatment costs and to the emergence of drug resistance in clinical isolates [2]. Recently, several studies announced the increasing prevalence of MRSA [23]. Prevalence of MRSA varies in various burn units, which may be due to the implementation of different infection control policies. In the present study, S. aureus was among the most common pathogens isolated from SSTIs infections. This result is consistent with the results of other studies [24-26]. The prevalence rate of MRSA is different in various regions within a burn center. For example, this rate varies from a low of 33% in the United States [27] to a high of 98% in Korea [28]. This study showed that S. aureus was the causative agent of 48.14% of SSTIs, among which 61.54% were MRSA.

Challenges in the treatment of staphylococcal infections are accompanied by several mechanisms of biofilm formation in this bacterium [11]. There are several methods for the quantification of biofilm formation [29], but there is still no standard protocol for the detection of biofilm production. Although various methods are currently used for this purpose, the most widely used assay for evaluation of biofilm formation is the microtiter plate method [13].

In this study, morphology of colonies on MCRA was used to assess slime production. The microtiter plate method was used to evaluate and quantify the attachment ability of these strains. Microbial cell attachment to surfaces and the formation of multicellular communities is a key step in infection, and this characteristic is considered to be a virulence factor for the organism [30]. Using the microtiter plate method, all of the MRSA strains studied were found to produce biofilm except for one isolate; this rate was much lower (60%) in MSSA isolates.

Slime production is characterized by diffusion of black pigment in MCRA, so strains with red or black colonies were interpreted as being non-biofilm producers and biofilm producers, respectively. Results of culturing on MCR to determine the prevalence of biofilm formation among MRSA and MSSA isolates were similar to the results of the microtiter plate method.

Our results demonstrated that all MRSA strains studied were positive for icaA/icaD genes. One of the MRSA isolates was positive for icaA/D genes but did not produce slime in the MCRA and microtiter plate methods. It would be interesting to follow up on this isolate, which may carry a mutation in one or more of the icaA/D genes or in the upstream region from which these genes are transcribed since icaA/D have been implicated as being necessary for biofilm formation in some studies. All MSSA isolates that formed biofilm were positive for the presence of icaA/D genes as well. Our results were consistent with some studies that emphasized the important role of icaA/D in biofilm formation [19]. Since the icaA/D gene is required for PIA, and PIA is required for attachment and biofilm production, it seems logical that all but one of the MRSA isolates produced biofilm. Perhaps there is a mutation in the icaA/D locus in the strain that has the genes but does not make PIA or biofilm. MRSA isolates that were resistant to multiple antibiotics were mostly biofilm producers, so it can be concluded that the majority of MDR pathogens are likely biofilm producers or vice versa. This result is in accordance with other studies [31,32]. Most hospital-acquired MRSA are also resistant to numerous other antibiotics. Furthermore, it is known that growth in biofilm makes any organism more tolerant to antibiotics to which they are susceptible as planktonic cells. Greatly enhanced tolerance to antibiotics is a characteristic of bacteria present in biofilms. MRSA strains are also resistant to numerous other antibiotics, especially when the strains are acquired in a hospital setting. This is a concern in the treatment of patients who are infected by such strains. All MRSA and MSSA in this study were susceptible to fusidic acid, linezolid, teicoplanin, tigecycline, and vancomycin. This finding
is in agreement with other studies that have reported that MRSA is almost always susceptible to these antibiotics [33,34]. Therefore, these antibiotics can still be used for staphylococcal infections in burn patients in Iran. It seems that misuse and overuse of some antibiotics including tetracycline, gentamicin, and ceftriaxone have caused a high prevalence of resistance to them in this region; appropriate measures are needed to prevent treatment failures in burn patients. Thus, the presence of resistant strains and their antibiotic susceptibility patterns should be considered to plan serious procedures for their treatment and eradication. However, the tolerance of a strain in biofilm means that a different approach to therapy is needed, such as topical antibiotics, debridement to remove biofilm followed by more topical treatment, and systemic antibiotics to kill any planktonic cells dislodged. In conclusion, a high prevalence of biofilm-producing isolates involved in burn infections may indicate a potential outbreak of MRSA in the burn center, which is a problem that makes the treatment difficult and complicated. This emphasizes the necessity of antibiotic susceptibility monitoring of MRSA because knowledge about the epidemiology, characteristics of MDR strains, and spreading of such strains in a hospital setting, particularly in burn care facilities whose patients are immunodeficient, leads to the foundation of prevention and control programs that minimize the prevalence of subsequent infections due to such resistant strains.

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References


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