A comparison of the frequency of biofilm-forming genes (icaABCD) in Methicillin-Resistant S. aureus strains isolated from Human and Livestock

Mohammadi Mollaahmadi, C. 1, Anzabi, Y. 2,3 *, Shayegh, J. 4

1. Faculty of Basic Sciences, Department of Microbiology, Tabriz Branch, Islamic Azad University
2. Department of Pathobiology, Faculty of Veterinary Medicine, Tabriz branch, Islamic Azad University, Tabriz, Iran
3. Biotechnology Research Center, Tabriz Branch, Islamic Azad University, Tabriz, Iran
4. Department of Veterinary Medicine, Islamic Azad University, Shabestar Branch, Islamic Azad University, Shabestar, Iran

Corresponding author: anzabi@iaut.ac.ir

Abstract

MRSA can cause infections in both human and animal groups, which is a serious threat to public health worldwide. Attachment and colonization are the first step for Staphylococcus aureus pathogenesis, and biofilm mediated infections have a significant negative impact on human and animal health. Methicillin-resistant Staphylococcus aureus can adapt to different environments and give rise to different strains of human and animal methicillin-resistant Staphylococcus aureus, causing transmissions of the disease between humans and animals. This study is aimed at the investigation of biofilm production in vitro, and the presence of icaABCD genes in methicillin-resistant S. aureus (MRSA) isolates in both human and livestock isolates were evaluated by the Congo Red Agar method. The presence of mecA and icaABCDR genes were assessed by PCR, and Finally, PCR products were examined by agarose gel electrophoresis. The results showed that the mecA gene frequency in human and animal isolates was 64.1% and 36.1%, respectively, and there was a significant relationship between mecA and icaAD in human isolates. In addition, significant relationships were found between icaA and Rifampicin and also between icaC and...
Chloramphenicol and Penicillin in human isolates. In animal isolates, there is a significant relationship between mecA and Trimethoprim and between icaR and Rifampicin. It was concluded that all operon ica genes are involved in biofilm production, but icaA and icaD genes in methicillin-resistant Staphylococcus aureus were more closely associated with mecA. Both animal and human strains can be involved in disease transmission, but this conclusion should be made cautiously.

Keywords: Staphylococcus aureus, MRSA, icaABCD, Clinical, Bovine mastitis

Introduction:

Staphylococcus aureus is a gram-positive bacterium colonized in the skin (as normal human flora) and the mucous membranes of humans and animals. Upon overcoming the skin barrier, the bacteria can cause multiple systemic infections with fever, acute and chronic infections, and various syndromes (DeLeo and Chambers, 2009). In Livestock bacterial contamination, depending on the sanitary conditions of the environment and the equipment used, the bacterial contamination often occurs during the milking process. Bovine mastitis is caused by a variety of microorganisms such as S. aureus and Escherichia coli. This infection can be controlled by improving farm management practices (Ateba et al., 2010). Many factors are involved in this bacterium's pathogenicity, including the ability to produce biofilms and antibiotic resistance. A biofilm can be defined as an aggregate of microorganisms stuck to biotic or abiotic surfaces. Its phenotypic and genotypic structures adapt themselves to the environmental conditions (Fischetti et al., 2019). Biofilm expansion enhances bacterial viability in the environment and an important factor in the failure of antibiotics. Bacterial resistance to antibiotics has now turned into a serious challenge and has adverse effects on therapeutic interventions. These traits are carried by specific genes on bacterial chromosomes, plasmids, transposons and/or integron gene cassettes and can be transferred from one bacterium to another one (Turlej et al., 2011). The present research investigated the icaABCD and icaR genes involved in biofilm formation and the resistance and susceptibility of Staphylococcus aureus to antibiotics used to control it. The icaABCD genes are synthetic genes, and synthesis of polysaccharide intercellular adhesion (PIA) occurs after the related enzyme is expressed by the icaA and the icaD genes. The icaB gene is responsible for the
deacetylation of polysaccharides before they bind to cell membranes, and the icaC gene encodes a membrane protein. Moreover, the icaR gene plays a regulatory role and inhibits expression of the icaABCD genes and prevents biofilm formation. However, a protein named Rbf protein prevents this process by suppressing the icaR gene (Arciola et al., 2002; Fitzpatrick et al., 2005). In addition, it identified methicillin-resistant Staphylococcus aureus (MRSA) isolates in human and animal populations through detecting the presence of the mecA genes that are a component of SCCmec (a mobile genetic element of Staphylococcus bacterial species) (Deurenberg et al., 2007; Pontes et al., 2009; Sabath et al., 1977). This research aims to study biofilm production in vitro and the presence of icaABCD genes in methicillin-resistant S. aureus (MRSA) Staphylococcus aureus isolates in both human and animal groups and investigation of disease transmission between human and animal strains.

Material and Methods:

Sampling: In this study, 85 animal and 80 human samples were obtained. Livestock samples were collected from cows suspected of mastitis, and human samples were collected from blood, infectious secretions, and endotracheal tubes, etc. The bacterial samples were first enriched by culturing them in BHI broth. They were then transferred to blood agar medium and incubated for 24 hours at 37°C. Biochemical tests such as Gram test, catalase, coagulase, mannitol salt agar and DNase tests were then performed. Several samples were discarded.

DNA Extraction

Extraction of the Staphylococcus aureus genome was performed by the extraction kit (Geneall, South Korea) according to the manufacturer's protocols. At the end of the extraction, we measured the DNA concentration with a nanodrop device.

Identification of Staphylococcus aureus in humans and animals by genotypic method

The identities of the samples were confirmed by using the nucA gene (Figure 1-a) by PCR with forward and reverse primer sequences of F: CTGGCATATGTATGGCAATTGTT and R: TATTTGACCTGAATCAGCGTTGTCT and a number of isolates were removed. Staphylococcus aureus ATCC25923 was used as the positive control for identification nucA.
gene and Staphylococcus epidermidis ATCC12228 was used as the negative control (De Almeida et al., 2018).

**Phenotypic investigation of biofilm formation using Congo red agar:**

For this experiment, the powder formulation of Congo red agar medium was obtained from the (Merck - Germany) after preparing the medium on plates, single colonies of the bacteria were cultured using the streak plate technique and incubated aerobically in an oven at 37˚C each for 48 hours. The matte black colonies produced strong biofilms and the reddish transparent black colonies moderate biofilms. The red colonies were considered biofilm-negative strains (Arciola et al., 2002).

**Genotypic evaluation for identifying the mecA gene in human and livestock isolates**

The presence of mecA gene was assessed by PCR using the specific primer for each gene as specified in Table 1. PCR (final volume 25 μl) was performed for each tube in a PCR device (Eppendorf, Germany). Each Tube contained 10x PCR buffer (2.5 μl), dNTP (150 μmol), MgCl₂ (2 mmol), 10 pmol of F and R paired primers, Tag DNA polymerase (1 unit) and DNA (2 μl). The thermocycler temperature regime is shown in Table 1 and Finally, PCR products were examined by agarose gel electrophoresis.

**Genotypic evaluation for identifying the icaABCD and icaR genes involved in biofilm formation**

All isolates were evaluated by PCR to examine the presence of biofilm forming genes. Specialized primers were used for each ica genes. Each PCR reaction solution was 25 μl that of this amount, buffer (2.5 μl), dNTP (150 μmol), MgCl₂ (2 mmol), 10 pmol of F and R paired primers, Tag DNA polymerase (1 unit) and DNA (2 μl). The thermocycler temperature regime is shown in Table 1 and Finally, PCR products were examined by agarose gel electrophoresis.

**Antimicrobial susceptibility assay:**

Antibiotic susceptibility was determined using the standardized Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Figure 3). The antimicrobial agents tested included
Chloramphenicol (30μg), Ciprofloxacin (5 μg), Erythromycin (15μg), Gentamicin (10 μg), Oxacillin (1 μg), Penicillin (10 Units), Rifampin (5 μg), Trimethoprim (5 μg), Vancomycin (30 μg), Nitrofurantoin (300 μg). S. aureus ATCC 25923 is used for controlling the sensitivity of the test.

Statistical analysis

The data were statistically analyzed using Crosstabulation and the Chi-square tests in SPSS.

Results

The mecA gene frequency was observed to be 64.1% and 36.8% out of 39 human and 35 animal isolates, respectively (Figure 1-f). Based on the PCR results, the frequency of ica operon genes is presented in Table x and (Figure 1- b, c, d, e). There is a significant relationship between icaAD and mecA genes (P-value ≤ 0.05) based on statistical analysis. In the phenotypic study of the biofilm production by congo red agar method, 69.2% of the human isolates resulted in strong biofilm production, 15.4% in moderate, and 15.4% in weak biofilm production. In livestock isolates, 57.9% resulted in strong, 21.1% in moderate, and 21% in weak biofilm production. The results of the antibiogram for both human and animal groups are shown in Table y. Based on these results, there is a significant relationship between icaA in rifampicin antibiotic susceptibility in human isolates (P-value= 0.016). Also, a significant relationship was observed between icaC and chloramphenicol (P-value = 0.046) and penicillin (P-value = 0.016). In animal isolates, there is a significant relationship between mecA and the sensitivity to trimethoprim (P-value = 0.047), and also there is a significant relationship was observed between icaR and the sensitivity to rifampicin (P-value= 0.033).

Discussion

MRSA’s widespread prevalence is one of the most important factors that has turned Staphylococcus aureus into a dangerous pathogen that poses a serious threat to health care worldwide. MRSA can easily adapt to and evolve in different environments, just as the CA-MRSA and LA-MRSA strains are the results of HA-MRSA evolution. Epidemiologically, HA-MRSA was first reported from hospitals and is endemic to hospitals, CA-MRSA is also prevalent in the general population, and LA-MRSA is endemic to farms (Salas et al., 2020).
LA-MRSA and HA-MRSA are drug-resistant pathogens and cover a wide range of infections. High prevalence of LA-MRSA infection has been reported in farmers, ranchers, and people in direct contact with animals, with symptoms such as sepsis, pneumonia, and joint infections (Pirolo et al., 2019). Staphylococcus aureus is currently one of the leading causes of infection in cattle and causes severe economic damage to the dairy industry. A report states that certain strains of Staphylococcus aureus such as CC130, ST425, etc (Bardiau et al., 2013). Have been observed only in cows. In farms, most antibiotics are used as feed additives to enhance livestock growth, which increases antibiotic resistance and the development of LA-MRSA strains. According to a report, in the United States, approximately 80% of all antibiotics produced are used in agriculture, and a significant portion of this amount is used for non-therapeutic purposes and to enhance livestock growth (Martin et al., 2015). On the other hand, antibiotics used for livestock are from the same group of human antibiotics, which can transmit the antimicrobial resistance created in livestock to the human population (Smith et al., 2009). In a study conducted on veterinarians, 12.5% were infected with LA-MRSA strains (Wassenberg et al., 2010). The spread of contamination may occur through slaughterhouse workers, farmers, butchers, etc., who have direct contact with contaminated meat, through hospitals and health centers, or through the environment, such as water, air, and so on (Smith, 2015). The occurrence of common human-animal infections can be directly related to the prevalence of antibiotic-resistant bacteria in animals used for food (Smith et al., 2002). According to research, the mecA (Wu et al., 1996) and mecC (García-Álvarez et al., 2011; Shore et al., 2011) resistance genes are of animal origin. The domestication of animals, as well as the globalization of the livestock industry, have facilitated and significantly increased the exchange of bacteria between humans and animals (Lowder et al., 2009; Sakwinska et al., 2011). A report by (Weese et al., 2006) stated that MRSA can be transmitted between horses and humans and that veterinary hospital staff are more exposed to infection. There is also evidence that livestock can act as a reservoir for S. aureus's emergence in humans (Spoor et al., 2013). On the other hand, several studies show that MRSA is more of a human origin in pets (Haenni et al., 2012). Price et al., 2012 demonstrated in their study that a human-derived MSSA strain could spread to livestock and induce antibiotic resistance to methicillin and tetracycline in livestock. A study by Boer et al.
Aimed to isolate MRSA from animal feed in several countries. In the results, the highest contamination of meat products was reported from the Netherlands, so that the contamination of raw meat were 11.9% in retail stores, 6.10% in beef, 2.15% veal, 6.2% in lamb and mutton, 10.7% in pork, 16% in chicken and the last but not least, 35.3% in turkey (de Boer et al., 2009).

Animal source foods are usually prepared for human consumption, and LA-MRSA isolates found in live animals may also be detected on animal carcasses. In addition, slaughterhouse staff may contaminate the carcass with CA-MRSA and HA-MRSA during slaughter or processing. In a study in the United States, out of 120 meat samples prepared from 30 meat retail centers, 22 samples were reported to be CA-MRSA and HA-MRSA infected (Pu et al., 2009). In one study, fish meat was reported to be infected with MRSA (Hammad, Watanabe, Fujii, & Shimamoto, 2012). This means that the global fish trade could increase the possibility of intercontinental transmission of multidrug-resistant and enterotoxigenic Staphylococcus aureus (Chon et al., 2017). Biofilm is one of the most crucial pathogenicity factors in Staphylococcus aureus. When bacterium attaches to surfaces and accumulates, they form biofilms, which is one of the key and most essential factors in spreading infectious diseases. The ability of bacteria to produce biofilms and adhesions makes them more resistant to antibiotics. According to various theories, if antibiotics penetrate the biofilm, the biofilm can inactivate the antibiotics by producing enzymes. The effect of antibiotics is only on growing bacteria. Bacteria in biofilms grow more slowly, reducing the effect of antibiotics on biofilms (Gebreyohannes et al., 2017). Also, the high density of bacterial populations in biofilms increases the likelihood of genetic exchange in the bacterial population, which leads to the transfer of resistance genes between bacteria, resulting in increased antibiotic resistance due to horizontal gene transfer. Also, once the biofilm is formed, it will be easy for it to escape the immune system and cause chronic infections (Costerton et al., 2005). Although some genes and other conditions are responsible for biofilm production, research by Arciola et al., 2001, shows that icaD and fnbA genes play a key role in biofilm formation. In a study by Piechota et al., 2018, there is a significant relationship between ica operon and MRSA strains that is closely consistent with our study. In Bimanand et al., 2018's study, 95.8% of the isolates formed biofilms, and a significant relationship was found between icaD
and fnbA genes. Ghasemian et al., 2016 reported that the prevalence of icaABCD genes in isolates was high, but there was no significant relationship between ica operon genes, MRSA and MSSA, and that all MRSA strains contained icaABCD genes. However, in our study, in addition to the presence of ica genes in MRSA strains, there is a significant relationship between the frequency of icaAD and mecA genes. Serray et al., 2016 found a significant association between MRSA and the icaD gene. In a study by Nourbakhsh and Momtaz, 2016 the frequency of icaC and icaB genes was 67.3% and 63.2%, respectively, and 92.2% of 188 isolates contained the mecA gene, but no significant relationship was found. A study by Moghadam et al., 2014 reported that all MRSA isolates contained icaA and icaD genes. According to the results of the study performed by Mirzaee et al., 2014 the frequency of icaABCD genes was 51.6%, 45.1%, and 77.4% and 80.6%, respectively, only 38.7% of the samples contained all four genes, and no significant relationship was found. Based on the studies, the expression of 100% of all operon ica genes does not prove biofilm production. However, this does not mean that it is not important in biofilm production. Each icaABCD gene has a different role in the biofilm production process and the amount of gene expression in different samples of Staphylococcus aureus can be different. According to the results of our study, icaAD genes were significantly associated with mecA gene expression. Also, icaR gene expression was the exact opposite of icaABCD genes, which could indicate the importance of icaR gene in inhibiting biofilm production. That is, the lower the icaR expression, the higher the biofilm production in the bacterium. Based on our study results on the transmission of Staphylococcus aureus strains between humans and animals, it is not possible to express a definite conclusion, but recent research shows that bilateral transmission of S. aureus strains between humans and animals is not rare (T. C. Smith, 2015). Livestock-Associated staphylococcus aureus is an emerging group of S. aureus worldwide, and it seems that these strains cause less infection in humans and spread from person to person than typical familiar human strains. However, this conclusion should be made with caution because good prospective studies have not been performed so far, and more extensive and accurate studies are needed in both human and animal populations.
REFERENCES


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### Table 1: Sequence of specific primers and thermal cycler temperature

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature</th>
<th>PCR Product Size</th>
<th>Reference</th>
</tr>
</thead>
</table>
| mecA        | F: AAAATCGATGGTAAAGGTTGCC  
R: AGTTTCTGCAGTACC GGATTTGC | 52°- 30s | 553bp | (García-Álvarez et al., 2011) |
| icaA        | F: AAAATCGATGGTAAAGGTTGCC  
R: AGTTTCTGCAGTACC GGATTTGC | 55°- 60s | 188bp | (Rohde et al., 2001) |
| icaB        | F: AGAATCGTGAAATAGA AATTTTTCTTTTTATTGGAATCCGT  
R: TCTAATCTTTTTTCTTGGAATCCGT | 52°- 30s | 900bp | (Sabath et al., 1977) |
| icaC        | F: ATGGGACGCGGATCCATGGAAAAAGA  
R: TAAATAACATTATTGTTCAATT | 52°- 60s | 1100bp | (Sabath et al., 1977) |
| icaD        | F: ATGGTCAACCCGACACAGAG  
R: AGTATTTTCAATGTTAAAGCAA | 55°- 30s | 198bp | (Rohde et al., 2001) |
| icaR        | F: TACTGTCCTCAATAATTCGCCGA  
R: GGTACGATGGTACTACACTTGATG | 54°- 30s | 453bp | (Deurenberg et al., 2007) |

### Table 2. Frequencies of the icaABCD and icaR Gene in MRSA

<table>
<thead>
<tr>
<th>Gene Frequency</th>
<th>Clinical (S)%</th>
<th>Livestock(S)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>icaA</td>
<td>64.1%</td>
<td>36.8%</td>
</tr>
<tr>
<td>icaB</td>
<td>64.1%</td>
<td>31.6%</td>
</tr>
<tr>
<td>icaC</td>
<td>30.8%</td>
<td>26.3%</td>
</tr>
<tr>
<td>icaD</td>
<td>64.1%</td>
<td>36.8%</td>
</tr>
<tr>
<td>icaR</td>
<td>10.3%</td>
<td>10.5%</td>
</tr>
</tbody>
</table>
### Table 3. Antibiogram results by disk diffusion method

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Clinical (%)</th>
<th>Livestock (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamycin</td>
<td>48.7%</td>
<td>52.6%</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>38.5%</td>
<td>52.6%</td>
</tr>
<tr>
<td>Penicillin</td>
<td>43.6%</td>
<td>52.6%</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>56.4%</td>
<td>47.4%</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>58.2%</td>
<td>89.5%</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>59%</td>
<td>63.2%</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>41%</td>
<td>68.2%</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>56.4%</td>
<td>52.6%</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>71.8%</td>
<td>84.2%</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>61.5%</td>
<td>73.7%</td>
</tr>
</tbody>
</table>

Figure 1. Genetic profile of the nucA (664bp)
Figure 2. Genetic profile of the icaA (188bp), icaB (198bp)
Figure 3. Genetic profile of the icaB (900bp)
Figure 5. Genetic profile of the icaR (453 bp).

Figure 6. Genetic profile of the mecA (533 bp)
Figure 7. Biofilm formation assay on Congo Red Agar

Figure 8. Disk diffusion test for Staphylococcus aureus