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 (1). In Livestock bacterial contamination, depending on the sanitary conditions of the environment and the equipment used, 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 (2). Many factors are involved in the pathogenicity of this bacterium, 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 environmental conditions (3).

Biofilm expansion enhances bacterial viability in the environment and is 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 (4).

The present research investigated the icaABCD and icaR genes involved in biofilm formation and the resistance and susceptibility of S. aureus to antibiotics used to control it. The icaABCD genes are synthetic genes, and the synthesis of polysaccharide intercellular adhesion 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 the expression of the icaABCD genes, and prevents biofilm formation. However, a protein named Rbf protein prevents this process by suppressing the icaR gene (5, 6). In addition, it identified methicillin-resistant S. 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) (7-9). This research aimed to study biofilm production in vitro and the presence of icaABCD genes in MRSA S. aureus isolates in both human and animal groups and investigate disease transmission between human and animal strains.

2. Materials and Methods
2.1. 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. The bacterial samples were first enriched by culturing them in Brain Heart Infusion Broth. They were then transferred to a blood agar medium and incubated for 24 h at 37°C. Biochemical tests, such as Gram test, catalase, coagulase, mannitol salt agar, and deoxyribonuclease tests were then performed. It should be mentioned that several samples were discarded.

2.2. DNA Extraction
    Extraction of the S. aureus genome was performed by the extraction kit (GeneAll, South Korea) according to the protocols of the manufacturer. At the end of the extraction, the DNA concentration was measured with a nanodrop device.

2.3. 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) by polymerase chain reaction (PCR) with forward and reverse primer sequences of F: CTGGCATATGTATGGCAATTGTT and R: TATTGACCTGAATCAGCGTTGTCT and a number of isolates were removed. S. aureus ATCC25923 was used as the positive control for the identification of the nucA gene and Staphylococcus epidermidis ATCC12228 was used as the negative control (10).

![Figure 1. Genetic profile of the nucA (664 bp).](image-url)
2.4. Phenotypic Investigation of Biofilm Formation Using Congo Red Agar
For this experiment, the powder formulation of Congo red agar medium was obtained from 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 h. The matte black colonies produced strong biofilms and the reddish transparent black colonies produced moderate biofilms. The red colonies were considered biofilm-negative strains (5).

2.5. Genotypic Evaluation for Identifying the meca Gene in Human and Livestock Isolates
The presence of the meca gene was assessed by PCR using the specific primer for each gene as specified in table 1. The 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.

Table1. 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>
<tbody>
<tr>
<td>meca</td>
<td>F: AAAATCGATGGTTAAAGGTGGGC R: AGTTCTGCAGTACCGGATTGC</td>
<td>52°C-30s</td>
<td>553 bp</td>
<td>(11)</td>
</tr>
<tr>
<td>icaA</td>
<td>F: AAAATCGATGGTTAAAGGTGGGC R: AGTTCTGCAGTACCGGATTGC</td>
<td>55°C-60s</td>
<td>188 bp</td>
<td>(12)</td>
</tr>
<tr>
<td>icaB</td>
<td>F: AGAATCGTGAAGTATAGAAAATT R: TCTAATCTTTTCATGGAATCCGT</td>
<td>52°C-30s</td>
<td>900 bp</td>
<td>(9)</td>
</tr>
<tr>
<td>icaC</td>
<td>F: ATGGGACGGATTTCCATGAAAAAGA R: TAAATAAGCATTAATGTTCAATT</td>
<td>52°C-60s</td>
<td>1100 bp</td>
<td>(9)</td>
</tr>
<tr>
<td>icaD</td>
<td>F: ATGGTCAAGCCCGAGACAGAG R: AGTATTTTCAATGTTTAAAGCA</td>
<td>55°C-30s</td>
<td>198 bp</td>
<td>(12)</td>
</tr>
<tr>
<td>icaR</td>
<td>F: TACTGTCCTCAATAATCCCGGA R: GGTACGATGGTACTACACTTGATG</td>
<td>54°C-30s</td>
<td>453 bp</td>
<td>(7)</td>
</tr>
</tbody>
</table>

2.6. 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 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 PCR products were examined by agarose gel electrophoresis, and the thermocycler temperature regime is summarized in table 1.

2.7. Antimicrobial Susceptibility Assay
Antibiotic susceptibility was determined using the standardized Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Figures 2 and 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), and Nitrofurantoin (300 μg). The S. aureus ATCC 25923 was used for controlling the sensitivity of the test.

2.8. Statistical Analysis
The data were statistically analyzed using cross-tabulation and the Chi-square tests in SPSS software.
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3. Results

The *mecA* gene frequency was observed to be 64.1% and 36.8% in 39 human and 35 animal isolates, respectively (Figure 4). Based on the PCR results, the frequency of *ica* operon genes is presented in Table 2 and Figures 2, 3, 5 and 6. There is a significant relationship between *icaAD* and *mecA* genes ($P \leq 0.05$) based on statistical analysis. In the phenotypic study of the biofilm production by congo red agar method, as shown in Figure 7 69.2%, 15.4%, and 15.4% of the human isolates resulted in strong, moderate, and weak biofilm productions, respectively. In livestock isolates, 57.9%, 21.1%, and 21% resulted in strong, moderate, and weak biofilm productions, respectively.

Table 2. Frequencies of the *icaABCD* and *icaR* Gene in Methicillin-resistant Staphylococcus aureus.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Clinical(S)%</th>
<th>Livestock(S)%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>icaA</em></td>
<td>64.1%</td>
<td>36.8%</td>
</tr>
<tr>
<td><em>icaB</em></td>
<td>64.1%</td>
<td>31.6%</td>
</tr>
<tr>
<td><em>icaC</em></td>
<td>30.8%</td>
<td>26.3%</td>
</tr>
<tr>
<td><em>icaD</em></td>
<td>64.1%</td>
<td>36.8%</td>
</tr>
<tr>
<td><em>icaR</em></td>
<td>10.3%</td>
<td>10.5%</td>
</tr>
</tbody>
</table>

The results of the antibiogram for both human and animal groups are summarized in Table 3 and presented in Figure 8. Based on these results, there was a significant relationship between *icaA* in rifampicin antibiotic susceptibility in human isolates ($P=0.016$). Moreover, a significant relationship was observed among *icaC*, chloramphenicol ($P=0.046$), and penicillin ($P$-value = 0.016). In animal isolates, there was a significant relationship between *mecA* and the sensitivity to trimethoprim ($P=0.047$), and also a significant relationship was observed between *icaR* and the sensitivity to rifampicin ($P=0.033$).

Table 3. Antibiogram results by disk diffusion method.

<table>
<thead>
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<th>Gene Frequency</th>
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<td><em>icaA</em></td>
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</tr>
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</table>
Discussion

The widespread prevalence of MRSA is one of the most important factors that has turned \textit{S. aureus} into a dangerous pathogen that poses a serious threat to healthcare worldwide. The MRSA can easily adapt to and evolve in different environments, just as the community-associated MRSA (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; moreover, CA-MRSA is also prevalent in the general population, and LA-MRSA is endemic to farms (13).

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 (14). \textit{S. aureus} is currently one of the leading causes of infection in cattle and causes severe economic damage to the dairy industry.

A report stated that certain strains of \textit{S. aureus}, such as CC130, ST425 (15) 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 produced antibiotics
are used in agriculture, and a significant portion of this amount is used for non-therapeutic purposes and to enhance livestock growth (16).

The 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 (17). In a study conducted on veterinarians, 12.5% of them were infected with LA-MRSA strains (18). The spread of contamination may occur through slaughterhouse workers, farmers, and butchers, who have direct contact with contaminated meat, or through hospitals and health centers, or the environment, such as water and air (19).

The occurrence of common human-animal infections can be directly related to the prevalence of antibiotic-resistant bacteria in animals used for food (20). According to research, the mecA (21) and mecC (11, 22) 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 (23, 24).

A report by Weese, Caldwell (25) stated that MRSA can be transmitted between horses and humans and that veterinary hospital staff is more exposed to infection. There is also evidence that livestock can act as a reservoir for the emergence of S. aureus in humans (26). Moreover, the results of several studies have shown that MRSA is more of a human origin in pets (27). Price, Stegger (28) 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 performed by de Boer, Zwartkruis-Nahuis (29) aimed to isolate MRSA from animal feed in several countries. Based on their results, the highest contamination of meat products was reported from the Netherlands; accordingly, the contamination rates of raw meat were 11.9%, 6.10%, 2.15%, 6.2%, 10.7%, 16%, and 35.3% in retail stores, beef, veal, lamb and mutton, pork, chicken, and turkey, respectively.

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 conducted in the United States, 22 out of 120 meat samples prepared from 30 meat retail centers were reported to be infected by CA-MRSA and HA-MRSA (30).

In a study, fish meat was reported to be infected with MRSA (31). This means that the global fish trade could increase the possibility of intercontinental transmission of multidrug-resistant and enterotoxigenic S. aureus (32). Biofilm is one of the most crucial pathogenicity factors in S. 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 (32). Furthermore, 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.

In addition, once the biofilm is formed, it will be easy for it to escape the immune system and cause chronic infections (33). Although some genes and other conditions are responsible for biofilm production, results of a study performed by Arciola, Baldassarri (34) showed that icaD and fnba genes play key roles in biofilm formation. In a study conducted by Piechota, Kot (35), there was a significant relationship between ica operon and MRSA strains, which is consistent with the findings of the present study.
In a study carried out by Bimanand, Taherikalani (36), 95.8% of the isolates formed biofilms, and a significant relationship was found between icaD and fnbA genes. Ghasemian, Najar Peerayeh (37) reported that the prevalence of icaABCD genes in isolates was high, but there was no significant relationship among ica operon genes, MRSA, and MSSA. They also found that all MRSA strains contained icaABCD genes. However, in the present study, in addition to the presence of ica genes in MRSA strains, there was a significant relationship between the frequency of icaAD and mecA genes.

Serray, Oufrid (38) found a significant association between MRSA and the icaD gene. In a study conducted by Nourbakhsh and Montaz (39), the frequency of icaC and icaB genes were 67.3% and 63.2%, respectively, and 92.2% of 188 isolates contained the mecA gene, but no significant relationship was found. Results of another study carried out by Ohadian Moghadam, Pourmand (40) reported that all MRSA isolates contained icaA and icaD genes. According to the results of a study performed by Mirzaee, Najar-Peerayeh (41), the frequency of icaABCD genes was 51.6%, 45.1%, 77.4%, and 80.6%, respectively. Moreover, they found that only 38.7% of the samples contained all four genes and that there was no significant relationship.

Based on previous 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 plays a different role in the biofilm production process and the amount of gene expression in different samples of S. aureus can be different. According to the results of the present study, icaAD genes were significantly associated with mecA gene expression.

Besides, icaR gene expression was the exact opposite of icaABCD genes, which could indicate the importance of the icaR gene in inhibiting biofilm production. That is, the lower the icaR expression, the higher the biofilm production in the bacterium. Based on the results of the present study performed on the transmission of S. aureus strains between humans and animals, it is not possible to express a definite conclusion. However, recent research shows that bilateral transmission of S. aureus strains between humans and animals is not rare Smith (19).

Livestock-associated S. 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 since good prospective studies have not been performed so far, and more extensive and accurate studies are needed in both human and animal populations.

**Authors' Contribution**

Study concept and design: Y. A.
Acquisition of data: C. M. M.
Analysis and interpretation of data: J. S.
Drafting of the manuscript: Y. A.
Critical revision of the manuscript for important intellectual content: Y. A.
Statistical analysis: J. S.
Administrative, technical, and material support: C. M. M.

**Ethics**

The present study was approved by the Ethics Committee of the Tabriz Branch, Islamic Azad University, Tabriz, Iran.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

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