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Original Article

Comparison of the Frequency of Biofilm-Forming Genes (*icaABCD*) in Methicillin-Resistant *S. aureus* Strains Isolated from Human and Livestock

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Abstract

Methicillin-resistant *Staphylococcus aureus* (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 steps for *S. aureus* pathogenesis, and biofilm-mediated infections have a significant negative impact on human and animal health. The MRSA can adapt to different environments and give rise to different strains of human and animal MRSA, causing transmissions of the disease between humans and animals. This study aimed to investigate biofilm production in vitro, and the presence of *icaABCD* genes in MRSA isolates in both human as well as the disease transmission between human and animal strains. In total, 39 human and 35 livestock isolates were evaluated by the Congo Red Agar method. The presence of *mecA* and *icaABCDR* genes were assessed by polymerase chain reaction (PCR), and finally, the 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 was a significant relationship between mecA and Trimethoprim as well as between *icaR* and Rifampicin. It was concluded that all operon *ica* genes were involved in biofilm production, but *icaA* and *icaD* genes in MRSA 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: Bovine mastitis, Clinical, *icaABCD*, MRSA, *Staphylococcus aureus*

1. 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).

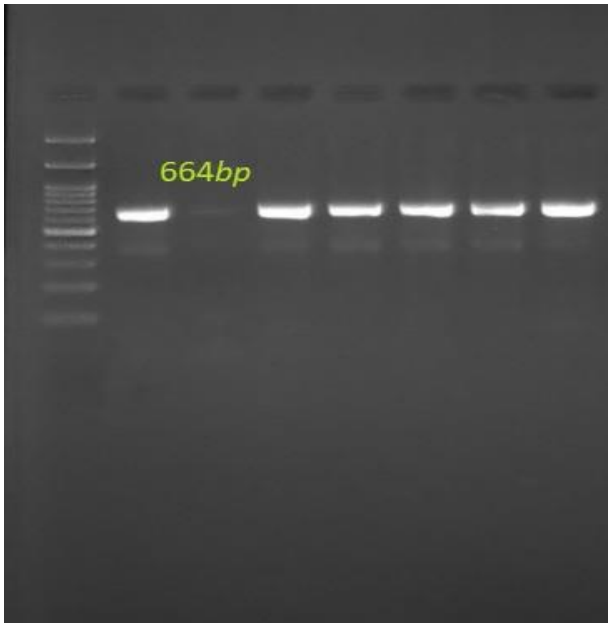

Figure 1. Genetic profile of the nucA (664 *bp*).

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 Eevaluation 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), $MgCl_2$ (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

Target Gene	Primer sequence	Annealing temperature	PCR product size	Reference
mecA	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTGC	52°- 30s	553 bp	(11)
icaA	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTGC	55°- 60s	188 bp	(12)
icaB	F: AGAATCGTGAAGTATAGAAAATT R: TCTAATCTTTTTCATGGAATCCGT	52°- 30s	900 bp	(9)
icaC	F: ATGGGACGGATTCCATGAAAAAGA R: TAATAAGCATTAATGTTCAATT	52°- 60s	1100 bp	(9)
icaD	F: ATGGTCAAGCCCAGACAGAG R: AGTATTTTCAATGTTTAAAGCAA	55°- 30s	198 bp	(12)
icaR	F: TACTGTCCTCAATAATCCCGAA R: GGTACGATGGTACTACACTTGATG	54°- 30s	453 bp	(7)

PCR: polymerase chain reaction

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), $MgCl_2$ (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. Statical Analysis

The data were statistically analyzed using cross-tabulation and the Chi-square tests in SPSS software.

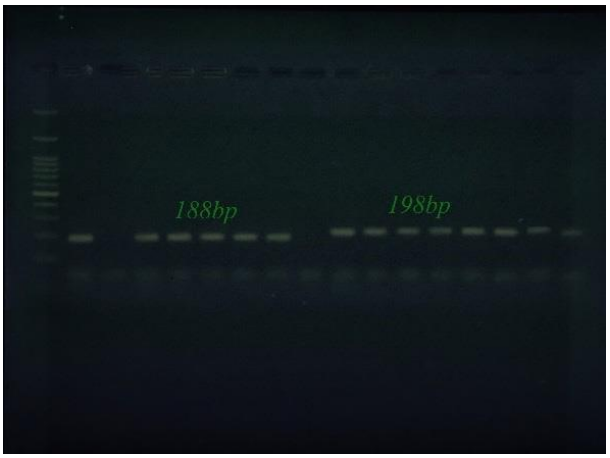

Figure 2. Genetic profile of the *icaA* (188*bp*), *icaB* (198 *bp*)

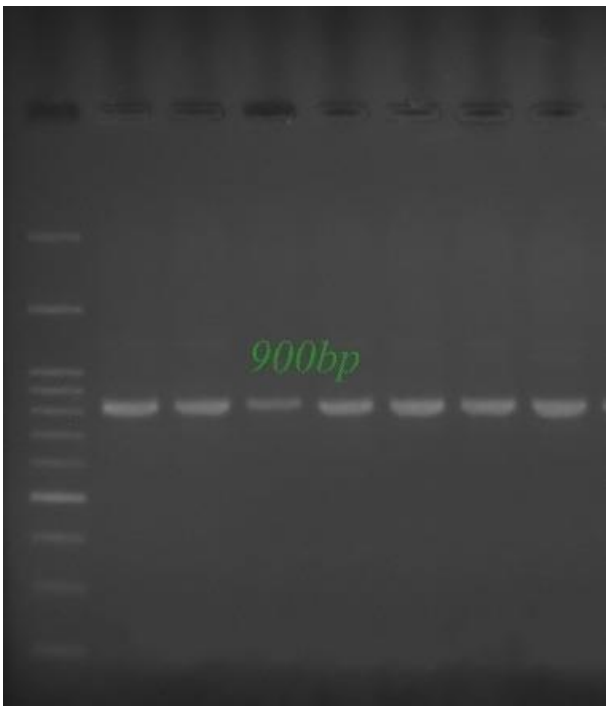

Figure 3. Genetic profile of the *icaB* (900 *bp*)

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.

Antibiotics	Clinical(S)%	Livestock(S)%
Gentamycin	48.7 %	52.6 %
Chloramphenicol	38.5 %	52.6 %
Penicillin	43.6 %	52.6 %
Rifampicin	56.4 %	47.4 %
Vancomycin	58.2 %	89.5 %
Ciprofloxacin	59 %	63.2 %
Nitrofurantoin	41 %	63.2 %
Oxacillin	56.4 %	52.6 %
Erythromycin	71.8 %	84.2 %
Trimethoprim	61.5 %	73.7 %

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.

Gene Frequency	Clinical (S)%	Livestock(S)%
*ica*A	64.1 %	36.8 %
*ica*B	64.1 %	31.6 %
*ica*C	30.8 %	26.3 %
*ica*D	64.1 %	36.8 %
*ica*R	10.3 %	10.5 %

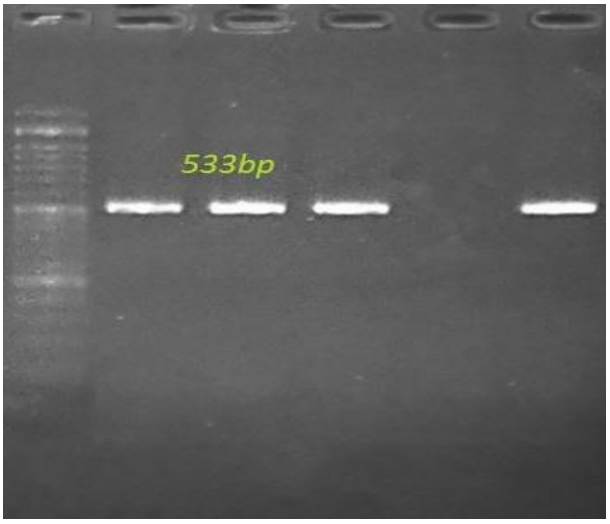

Figure 4. Genetic profile of the *mecA* (533 *bp*)

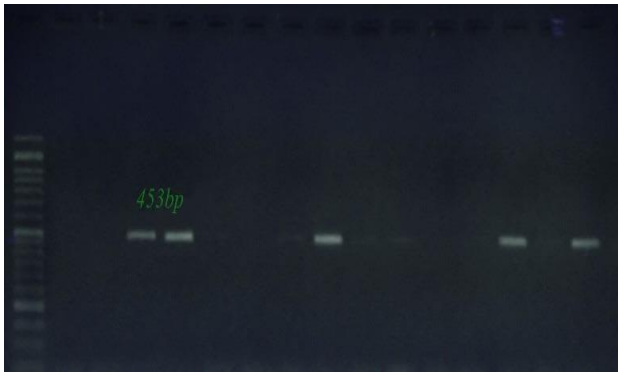

Figure 5. Genetic profile of the *icaR* (453 *bp*)

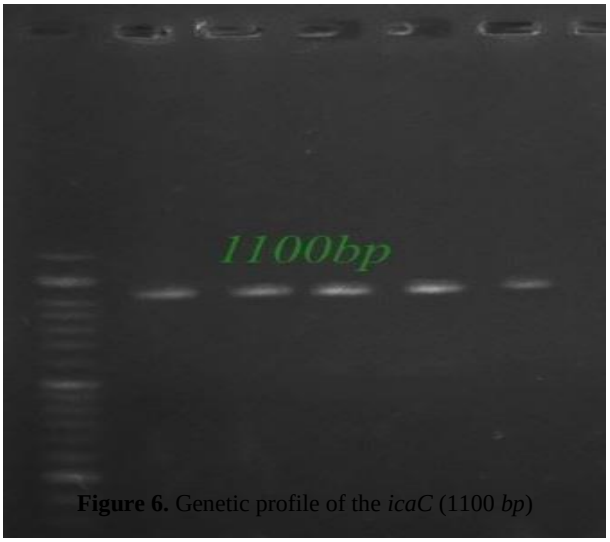

Figure 6. Genetic profile of the *icaC* (1100 *bp*)

Figure 7. Biofilm formation assay on Congo Red Agar

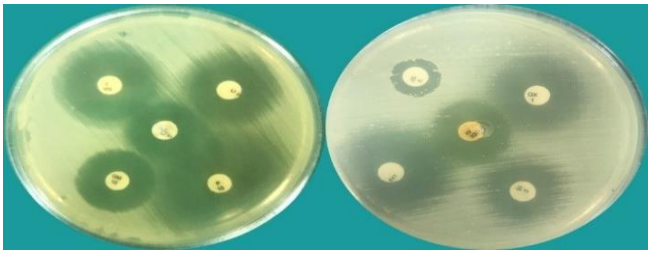

Figure 8. Disk diffusion test for *Staphylococcus aureus*

4. Discussion

The widespread prevalence of MRSA is one of the most important factors that has turned *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). *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 *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 Momtaz (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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