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Characterization of Biofilm Formation Ability, Virulence Factors and Antibiotic Resistance Pattern of *Staphylococcus aureus* Isolates from Subclinical Bovine Mastitis

Saeideh Foroutan[1], Mohammad Amin Eslampour[1*], Mohammad Emaneini[2], Fereshteh Jabalameli[2], Ghasem Akbari[1]

1. *Department of Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, Iran*
2. *Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

Abstract

BACKGROUND: Mastitis is an important disease that affects dairy herds worldwide. The *Staphylococcus aureus* (*S. aureus*) is the causative pathogen for mastitis. This pathogen has the tendency to biofilm forming, and may happen to antibiotic resistance.

OBJECTIVES: The aim of this study was to characterize the biofilm formation of different genotypes and antibiotic resistance pattern of *S. aureus* isolated from the subclinical bovine mastitis in Tehran province.

METHODS: The lactating dairy cows were screened for the subclinical mastitis. The isolates were identified by phenotypic method and the presence of the *nuc* gene. The biofilm forming and quantification was characterized using colorimetric assay. The *S. aureus* biofilm gene was evaluated using PCR assay. The antimicrobial susceptibility of the isolates was assessed using DAD method. The lowest antimicrobial concentration preventing the visible growth was construed by MIC_{50}. The antibiotic susceptibility and MBECs for the bacteria embedded in the biofilms were determined by XTT method.

RESULTS: The antimicrobials susceptibility test showed penicillin and ceftiofur to be less and more effective in vitro, respectively. The genotypic characterization showed that the highest and the lowest frequencies for *icaD* (75%) and *fnbB* (31.2%) genes, respectively. The biofilm formation was also characterized. The MBEC results for the bacterial biofilm showed resistance to ceftiofur in the biofilm state; however, these strains were susceptible to this agent in the planktonic state.

CONCLUSIONS: The biofilm formation is a significant virulence factor that was detected at a high rate. It is antibiotic-resistant and responsible for the subclinical bovine mastitis that does not respond to the routine treatments.

In order to control the infection achieve the effective treatment, and prevent the emergence of antibiotic-resistant bacteria, it is necessary to isolate the causative agent and determine the antimicrobial susceptibility.

KEYWORDS: Antibiotic resistance, Biofilm forming, Bovine mastitis, *Staphylococcus aureus*, Subclinical mastitis

Correspondence

Mohammad Amin Eslampour, Department of Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, Iran. Tel: +98 (021) 44865136, Fax: +98 (021) 42914200, Email: maeslampour@gmail.com

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Introduction

Bovine mastitis causes an economic loss to the dairy industry and *Staphylococcus* spp. play an important role in this etiology (Pacha *et al*., 2020). Of these, *S. aureus*, stands out among the prevalent etiological agents in this type of infection with subclinical prevalence and poor response to the treatments (Pacha *et al*., 2020). The improper use of antimicrobials and formation of biofilms undermines the effectiveness of mastitis therapy. The biofilm structures are made up of surface attached bacteria in the organic matrix (Bolte *et al*., 2020). The *Staphylococcus aureus*, can producea series of virulence factors that contribute to the bacterium invading the host's phagocytic defense, facilitating its adherence to the epithelial cells and colonization in the tissue, favoring its extracellular persistence and thus guaranteeing its successful installation and maintenance in the host tissues (Bolte *et al*., 2020). Among these factors is the production of a mucopolysaccharide extracellular "slime", which seems to help the adherence and colonization of the microorganism to the mammary glandular epithelium. The ability of *S. aureus* to adhere to the surface of the epithelium has been associated with the production of biofilms, which are described as agglomeration of the cells embedded in an extracellular heterogeneous matrix, resulting in three-dimensional structures with specific physiological characteristics (Hathroubi *et al*., 2017). Several researches have studied on *S. aureus* mastitis.

Biofilm is a multi-step process involved in the formation and adherence to the host surface by adhesion factors, followed by the growth to form a matrix (Schiffer *et al*., 2019). The microbial surface components recognizing the adhesive matrix molecules (MSCRAMMs) are adhesion proteins of the staphylococcal families, such as fibronectin-binding proteins (*FnbA* and *FnbB*), and biofilm-associated protein (*Bap*) (Kıvanç, 2018; Schiffer *et al*., 2019). An intercellular polysaccharide adhesion molecule has been found that mediates the intracellular adhesion (*icaADBC*) and controls the biofilm production (Uribe-García *et al*., 2019; Zhao *et al*., 2021). Previous studies have not evaluated the antibiotic resistance in planktonic and biofilm conditions in the subclinical mastitis of bovine *S. aureus*, which can detect the trend in the biofilm formation ability, and the genes encoding biofilm and antibiotic resistance pattern. Thus, due to the necessity of this research, data obtained from the pattern of antibiotic resistance and virulence genes can gather more information in this regard for the possibility of developing more effective strategies for the treatment and control strategies. This study aimed to characterize the biofilm formation ability in the antibiotic resistance pattern of *S. aureus* isolates from the subclinical bovine mastitis.

Materials and Methods

Phenotyping *S. aureus*

Forty primary samples of the cows' milk belonging to the five farms located in the Tehran province were collected. The samples were subjected to the primary isolation and subsequent experiments for the phenotypic identification of the species. The 1-9 parities of lactating dairy cows were screened for the subclinical mastitis using the CMT and SCC determinations. The SCC cutoff value (200.000<SCC>–500.000 cells/mL) of the diagnostic subclinical mastitis was appointed on the herd prevalence of *S. aureus*. The positive quarters were defined; sampling was done and the samples were transported to the laboratory on ice-pack. Classical microbiological, biochemical, and coagulase tests were conducted using the methods described previously by Hogan (Hogan *et al*., 1986). The isolates were confirmed as *S. aureus* by PCR on the *nuc* gene. The genomic DNA was extracted as described before (Fatholahzadeh *et al*., 2009). The primers sequences were synthesized according to Sahebekhtiari and colleagues study (Sahebekhtiari *et al*., 2011). The *S. aureus* ATCC 29213 was included as control strain. Finally, a total of 30 isolates were defined as *S. aureus*. For the next experiments, *S. aureus* inoculum was prepared from each isolate in TSB (MERCK, Germany) including 1% glucose broth (Baldassarri *et al*., 2001). All assays were performed in triplicate (Figure 1 Step-A).

Biofilm Formation Study

The *S. aureus* biofilm forming and quantification was described before (Stepanović *et al*., 2007). Each *S. aureus* inoculum was diluted 2:200 in TSB + 1% glucose and poured into the wells of the sterile tray (Tissue culture 96-wells plate, JET BIOFIL, Canada) and incubated aerobically for 24 h (37°C); after which the supernatant was discarded, and the wells were washed thrice. The precipitates were fixed by Bouin's reagent, dried by air (60°C, 1 h), and stained with crystal violet. The bound dye was re-solubilized with 95% ethanol. The *S. aureus* ATCC 25923 and broth (TSB + 1% glucose) were used as positive and negative controls, respectively. The optical density was measured at 570 nm by a microplate reader (Epoch™ Microplate Spectrophotometer, BioTek). The cut-off value was established as ODc= average OD of negative control + (3SD of negative control). The biofilm formation was categorized as follow: OD≤ODc = no; ODc<OD≤2ODc = weak; 2ODc<OD≤4ODc = moderate; 4ODc<OD = strong. All assays were performed in triplicate (Figure 1 Step-B).

Biofilm-Encoding Genes Detection

The *S. aureus* biofilm genes, *icaAD*, *fnbAB*, and *bap*, were targeted by PCR. The primers sequences and amplification cycles were described before (Vancraeynest *et al*., 2004). The *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 were included as positive and negative reference strains, respectively (Figure 1 Step-C).

Antimicrobial Susceptibility/ Disk Diffusion Agar (DAD)

The Antimicrobial susceptibility of the isolates was performed by DAD method (Pfaller *et al*., 2001; Weinstein & Lewis, 2020). Briefly, the assay was done with, penicillin, gentamicin, ceftiofur, ampicillin, erythromycin, trimethoprim/sulfamethoxazole, tetracycline, chloramphenicol, ciprofloxacin, and enrofloxacin (Mastdiscs®, UK), on Mueller59 Hinton BBLII agar (Becton Dickinson, Heidelberg, Germany). The *S. aureus* ATCC 25923 was included as quality control (Figure 1 Step-D).

Antimicrobial Susceptibility/ Broth Microdilution (MIC)

The Antimicrobial susceptibility of the isolates was also evaluated using designation of MIC method (Pfaller *et al*., 2001; Weinstein & Lewis, 2020). Briefly, Mueller-Hinton broth containing ceftiofur was poured into a 96-well tray. Half McFarland density of *S. aureus* isolates were diluted to 5 x 10^5 CFU/mL, inoculated to the 96-well tray, and incubated for 24 h at 37°C. The MIC was construed as the lowest antimicrobial agent preventing the visible growth. The susceptibility thresholds and resistance breakpoints were based on the CLSI guidelines as ≤2 and ≥8 µg/mL for ceftiofur, respectively. The *S. aureus* ATCC 29213 was included as quality control (Figure 1 Step-E).

Determination of the Minimum Biofilm Eliminating Concentrations (MBECs)

All isolated strains were susceptible to ceftiofur in the planktonic state, thus, the antibiotic susceptibility and MBECs for the bacteria embedded in biofilms were determined by colorimetric assay according to (Amorena *et al*., 1999) study. The biofilms formation was performed as described previously; After biofilms formation in the 96-well tray, with 100 µL of ceftiofur serial dilutions for 20 h (37°C) incubation, 50 µL XTT (Roche, Germany) was added, then tray was covered, and incubated for 1 h at 37°C (Pettit et al., 2005). The MBECs values were construed as the lowest antimicrobial agent preventing the visible growth (Sepandj *et al*., 2004). These assays were performed in triplicate (Figure 1 Step-F).

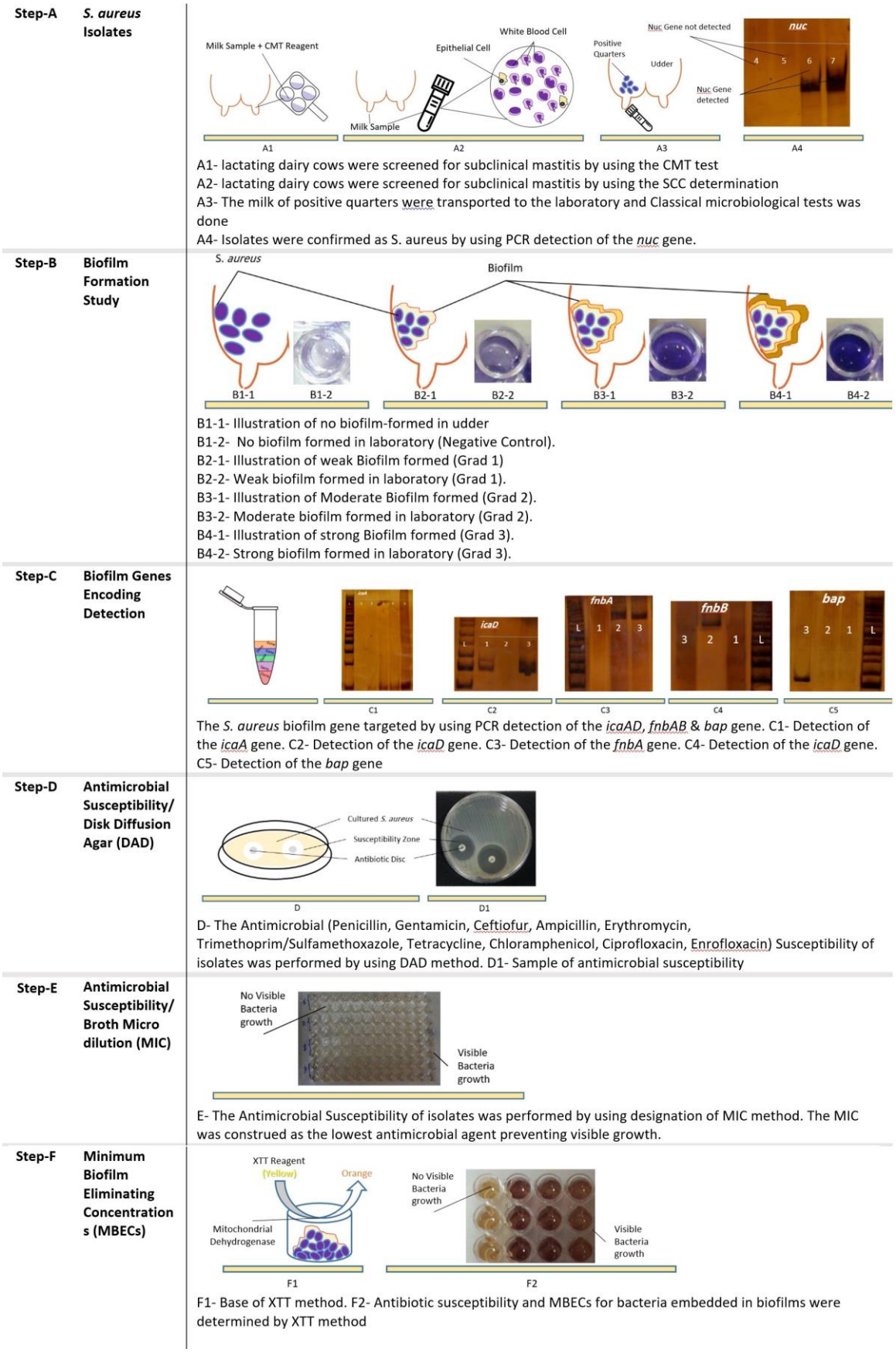

Figure 1. The total of 30 isolates of *S. aureus* were introduced into the experiment A to F (Application Source: Paint)

Results

A total number of 30 *S. aureus* isolates from the subclinical mastitis were studied to estimate the role and ability of biofilm formation in the antibiotic resistance pattern. The results of biofilm formation demonstrated that all isolates (100%) were biofilm producers, in which 77.4% of them produced strong biofilms, 12.9% and 9.7% produced moderate and weak biofilms, respectively. The biofilm-encoding genes frequency were as; *bap* (25%), *icaA* (9.4%), *icaD* (75%), *fnbA* (43.8%) and *fnbB* (31.2%) (Table 1). The rate of resistance to penicillin (74.4%), gentamicin (2.3%), ceftiofur (0%), ampicillin (57.5%), erythromycin (33.3%), trimethoprim/sulfamethoxazole (10%), tetracycline (70.3%), chloramphenicol (2.30%), ciprofloxacin (0%), and enrofloxacin (6.6%) were detected by DAD test. The highest resistance rate was detected against ceftiofur and ciprofloxacin; and the penicillin had the lowest resistance rate (Table 1). The MIC_{50} of ceftiofur was found 1 and 2 µg/mL for ATCC 29213 and isolated strains, respectively. Based on the CLSI guidelines, the percentages of sensitive, intermediate, and resistant *S. aureus* to ceftiofur were 96.67, 3.33, and 0%, respectively (Table 1). The MBEC results for the bacterial biofilm are listed in Table 1. Among the isolates, 28 strains were resistant to ceftiofur in biofilm state; however, these strains were susceptible to this agent in the planktonic state.

Table 1. *S. aureus* Isolates Frequency of The Genotypic Patterns, Biofilm Formation Type and Antimicrobial Susceptibility

Gen Profile	Farm no**	Biofilm Formation Grade*	Antibiotic Resistant (DAD Test)										Ceftiofur MIC+	Ceftiofur MBECs++
			Penicillin	Gentamicin	Ceftiofur	Ampicillin	Erythromycin	Tetracycline	Chloramphenicol	Ciprofloxacin	Enrofloxacin	Trimethoprim/ Sulfamethoxazole		
icaD, nuc	F1	S	+	-	-	-	+	+	-	-	-	-	Se	Re
icaD, nuc	F1	S	+	-	-	-	-	+	-	-	-	-	Se	Re
icaD, nuc	F1	S	+	-	-	-	-	+	-	-	-	-	Se	Re
icaD, nuc	F1	S	+	-	-	-	-	+	-	-	-	-	Se	Re
icaD, nuc	F1	S	+	-	-	+	-	+	-	-	-	-	Se	Re
icaD, fnbA, bap, nuc	F1	S	+	-	-	+	+	+	-	-	-	-	Se	Re
icaD, fnbA, bap, nuc	F1	S	+	-	-	+	+	+	-	-	-	-	Se	Re
icaD, fnbA, bap, nuc	F1	S	+	-	-	+	+	+	-	-	-	-	Se	Re
icaD, fnbA, nuc	F1	S	+	-	-	+	-	+	-	-	-	-	Se	Re
icaD, fnbA, nuc	F1	S	+	-	-	-	-	+	-	-	-	-	Se	Re
icaD, fnbB, nuc	F2	S	+	-	-	+	-	+	-	-	-	-	Se	Re
icaD, fnbB, nuc	F2	S	+	-	-	-	-	+	-	-	-	-	Se	Re
icaD, fnbB, nuc	F2	S	+	-	-	+	-	+	-	-	-	-	Se	Re
icaD, fnbA, nuc	F2	S	+	-	-	-	-	+	-	-	-	-	Se	Re
fnbA, bap, nuc	F3	M	-	-	-	+	-	-	-	-	-	-	Se	Re
fnbA, bap, nuc	F3	M	-	-	-	+	-	+	-	-	-	-	Se	Re

Gen Profile	Farm no**	Biofilm Formation Grade*	Antibiotic Resistant (DAD Test)										Ceftiofur MIC+	Ceftiofur MBECs++
			Penicillin	Gentamicin	Ceftiofur	Ampicillin	Erythromycin	Tetracycline	Chloramphenicol	Ciprofloxacin	Enrofloxacin	Trimethoprim/ Sulfamethoxazole		
bap, nuc	F3	W	+	-	-	-	-	-	-	-	-	-	Se	Su
nuc, fnbB	F3	W	-	-	-	-	-	-	-	-	-	-	Se	Su
icaA, icaD, fnbA, nuc	F4	S	-	-	-	+	+	-	-	-	-	+	Se	Re
icaD, nuc	F4	S	+	-	-	+	-	+	-	-	+	-	Se	Re
icaD, nuc	F4	S	+	-	-	-	-	-	-	-	-	+	Se	Re
icaD, nuc	F4	S	+	-	-	-	-	+	-	-	-	-	Se	Re
icaA, icaD, fnbB, nuc	F4	S	+	+	-	+	+	+	+	-	+	+	In	Re
icaD, bap, nuc	F5	S	-	-	-	+	+	-	-	-	-	-	Se	Re
icaD, fnbA, bap, nuc	F5	S	+	-	-	+	+	-	-	-	-	-	Se	Re
icaD, fnbA, fnbB, nuc	F5	S	+	-	-	+	+	-	-	-	-	-	Se	Re
icaD, fnbA, fnbB, nuc	F5	S	+	-	-	+	+	-	-	-	-	-	Se	Re
icaA, icaD, nuc	F5	S	+	-	-	-	-	+	-	-	-	-	Se	Re
fnbA, nuc	F5	M	-	-	-	+	-	+	-	-	-	-	Se	Re
fnbA, nuc	F5	M	-	-	-	-	-	+	-	-	-	-	Se	Re

* S: Strong, M: Moderate and W: Weak; ** F1: Farm1, F2: Farm2, F3: Farm3, F4: Farm4 and F5: Farm5; + Se: Sensitive and in: Intermediate; ++ Su: Susceptible and Re: Resistant.

Discussion

Studies have shown that *S. aureus* is the most important microorganism in the bovine subclinical mastitis. In this study, primary milk samples from the subclinical mastitis collected from the five farms in Tehran province were tested for the *S. aureus* phenotypic identification. For the sensitivity and specificity of the genotypic techniques, *S. aureus* was confirmed by *nuc* gene amplification (Fatholahzadeh *et al*., 2009).

Improper usage of antimicrobials to combat mastitis leads to the selection of resistant strains and undermines the effectiveness of therapies (Pacha *et al*., 2020). In this study, the isolates showed high resistance rate to tetracycline (70.3%) and penicillin (74.4%). The high resistance rate of *S. aureus* to penicillin and tetracycline was reported before (Gao *et al*., 2012), Aslantaş & Demir, (2016), and Jamali *et al*., (2014). The penicillin resistance rate in this study and Jamali's *et al*., (2014) study was similar. The tetracycline resistance rate (70.3%) was higher than Aslantaş & Demir, (2016), and Ren *et al*., (2020) studies and lower than (Jamali et al., 2014) findings. Similarly, erythromycin-resistance (33,3%) was found by Ren *et al*., (2020) study. The present study showed full susceptibility to ceftiofur (100%) and ciprofloxacin (100%). The rate of resistance to trimethoprim/sulfamethoxazole (10%) was higher than Aslantaş & Demir, (2016) study. Resistance prevalence against enrofloxacin (6.6%) was higher than Aslantaş & Demir, (2016) study. The gentamicin-resistance rate (2.3%) was inconsistent with Ren *et al*., (2020) study. Our finding of ampicillin-resistance rate (57.5%) was in agreement with Moroni *et al*., (2006) results. In contrast to these studies, high levels of chloramphenicol-resistance (2.3%) were

reported by Liu *et al*., (2017). The resistance rate to erythromycin (33%) was lower than those from the findings of Liu *et al*., (2017). According to the multidrug-resistant isolates and inconsistency in the antimicrobial resistance rate in numerous studies, suitable antimicrobial should be district-based.

The rise in multidrug resistant isolates of *S. aureus* is an important issue in mastitis control and the ability of biofilm formation is a potential role as a virulence factor (Notcovich *et al*., 2018). The *S. aureus* ability to produce biofilm is responsible for the establishing a persistent infection (Vasudevan et al., 2003). In *S. aureus*, the *icaA* and *icaD* genes have a significant character in the biofilm formation (Vancraeynest *et al*., 2004). This study reported the prevalence rate of *icaD, fnbA, fnbB, bap* and *icaA* genes at 75, 43.8, 31.2, 25, and 9.4%, respectively. Similarly, the highest frequency of the *ica* gene was identified in Ahmed *et al*., (2019); *icaA*: 58% and *icaD*: 60% and Salina *et al*., (2020) studies. However, the prevalence rates of the *icaA* and *icaD* genes vary greatly among different studies (Águila-Arcos *et al*., 2017; Kot *et al*., 2018; Mahmoudi *et al*., 2019) and others who found that biofilm formation can be influenced by several aspects (Demir *et al*., 2020). The *icaD* gene was the most prevalent among all detected genes, like in the study of Costa *et al*., (2018), which is in agreement with our study; whereas, these were inconsistent with Ghasemian *et al*., (2016) finding.

This study expressed that 25% of *S. aureus* isolates were positive for *bap* gene, whereas, this was lower than Salina *et al*., (2020) result. The moderate *fnbA* gene frequency was reported by Khoramian *et al*., (2015) and Ghasemian *et al*., (2016), which were higher compared to our results (43.8%). Zuniga *et al*., (2015) observed a high frequency of the *fnbA* gene (87.5%) from the caprine subclinical mastitis. Our reported prevalence rate of *fnbB* gene was lower than Khoramian *et al*., (2015) and Ghasemian *et al*., (2016) studies.

Conclusion

In conclusion, all the strong biofilm-producing isolates were positive for *ica* gene. The *fnbA, fnbB*, and *bap* (MSCRAMM) genes had prevalence in all types of biofilms (strong, moderate, and weak). It may make clear that detection of *ica* gene is much more important for the biofilm grade prediction than biofilm formation.

The MIC values of the ceftiofur were evaluated on the planktonic cells of *S. aureus*. The results showed sensitive (96.67%), intermediate (3.3%) and resistant (0%) breakpoints. In conclusion, all isolated *S. aureus* strains were found biofilm producers and most of them were positive for *icaA*, and *icaD* virulence genes; most of the isolated *S. aureus* strains were sensitive to ceftiofur.

The *S. aureus* is the most important microorganism in the bovine subclinical mastitis. The high frequency of *ica* gene, the strong biofilm formation and antibiotic resistance of most of the isolates were related to the antibiotics that are routinely used in the veterinary medicine. Therefore, in order to control, achieve the effective treatment, and prevent the emergence of antibiotic-resistant bacteria, it is necessary to isolate the causative agent and determine the antimicrobial susceptibility.

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Conflict of Interest

The authors declared no conflict of interest.

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شناسایی توانایی تولید بیوفیلم، عوامل حدت و الگوی مقاومت آنتی‌بیوتیکی *استافیلوکوکوس اورئوس* جدا شده از ورم پستان تحت بالینی

سعیده فروتن[۱]، محمد امین اسلامپور[۱*]، محمد ایمان عینی[۲]، فرشته جبل عامل[۲]، قاسم اکبری[۱]

[۱] *گروه علوم درمانگاهی، واحد علوم و تحقیقات، دانشگاه آزاد، تهران، ایران*

[۲] *گروه میکروب شناسی، دانشکده پزشکی، دانشگاه علوم پزشکی تهران، تهران، ایران*

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زمینه مطالعه: ورم پستان بیماری مهمی در گله های شیری دنیا است. *استافیلوکوکوس اورئوس* پاتوژن شایع ورم پستان است. این پاتوژن تمایل به تشکیل بیوفیلم دارد، که متعاقب آن مقاومت آنتی‌بیوتیکی ایجاد می‌گردد.

هدف: از این مطالعه توصیف تشکیل بیوفیلم ژنوتیپ‌های مختلف و الگوی مقاومت آنتی‌بیوتیکی *استافیلوکوکوس اورئوس* جداشده از ورم پستان تحت بالینی گاو در استان تهران است.

روش کار: گاوهای شیری از نظر ابتلا به ورم پستان تحت بالینی غربالگری شدند. ایزوله‌ها با روش فنوتیپی و حضور ژن *nuc* شناسایی شدند. امکان تشکیل و کیفیت بیوفیلم با استفاده از روش رنگ‌سنجی مشخص شد. ژن‌های وابسته به بیوفیلم در *استافیلوکوکوس اورئوس* با استفاده از روش PCR شناسایی شد. حساسیت ضد میکروبی ایزوله‌ها در حالت پلانکتونی با استفاده از روش DAD انجام شد. حداقل غلظت ممانعت‌کننده از رشد توسط MIC_{50} تعیین گردید. حساسیت آنتی‌بیوتیکی و MBEC برای ایزوله‌ها در بیوفیلم با استفاده از روش XTT تعیین شد.

نتایج: نتایج بالاترین میزان مقاومت آنتی‌بیوتیکی را در برابر پنی‌سیلین و کمترین میزان مقاومت را در برابر سفتیوفور و سیپروفلوکساسین نشان داد. MIC_{50} سفتیوفور ۲ µg/mL تعیین شد. ۱۰۰٪ ایزوله‌ها توانایی تولید بیوفیلم را داشتند و اکثر آنها بیوفیلم قوی تشکیل دادند. فراوانی ژن‌های عوامل حدت *icaA,D*، *fnbA,B* و *bap* کدکننده بیوفیلم هستند، شناسایی شد. بیشترین و کمترین فراوانی را ژن‌های *icaD*، و *fnbB* به ترتیب داشتند. نتایج MBEC برای برای ایزوله‌ها محاصره شده در بیوفیلم مقاومت به سفتیوفور را نشان داد، در حالی که، این ایزوله‌ها در حالت پلانکتونی به سفتیوفور حساس بودند.

نتیجه‌گیری نهایی: تشکیل بیوفیلم فاکتور حدت قابل توجهی است که با نرخ تشکیل بالا و ایجاد مقاومت در برابر آنتی‌بیوتیک، مسبب ورم پستان تحت بالینی گاو است که به درمان‌های معمول پاسخ نمی‌دهد. بر اساس این نتایج، کنترل، دستیابی به درمان موثر و جلوگیری از ظهور باکتری‌های مقاوم به آنتی‌بیوتیک، نیازمند جداسازی عوامل ایجادکننده و تعیین حساسیت ضد میکروبی است.

واژه‌های کلیدی: *استافیلوکوکوس اورئوس*، تشکیل بیوفیلم، مقاومت آنتی‌بیوتیکی، ورم پستان گاو، ورم پستان تحت بالینی

نویسندهٔ مسئول: محمد امین اسلامپور، گروه علوم درمانگاهی، واحد علوم و تحقیقات، دانشگاه آزاد، تهران، ایران ایمیل: maeslampour@gmail.com