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Original article

COMPARISON OF ANTIBIOTIC RESISTANCE AMONG BIOFILM-POSITIVE AND NEGATIVE *STAPHYLOCOCCUS AUREUS* MASTITIS ISOLATES

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Summary

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Because the success of treating *Staphylococcus aureus* (*S. aureus*) mastitis depends on numerous factors, different cure rates have been reported. Since biofilm production is one of the most critical factors for pathogenicity and antibiotic resistance, this study aimed to assess the ability of *S. aureus* to produce biofilm and compare antibiotic resistance in biofilm-positive and negative *S. aureus* strains. Milk samples (n=110) were collected from two herds of cows and subjected to bacteriological analysis. PCR (*nucA* gene) was used to detect accurately the cause of mastitis. Colorimetric microtiter plate assay was used to evaluate the biofilm formation capacity of the strains. The agar disk diffusion technique was utilised to specify the susceptibility to common antibiotics, including ampicillin, enrofloxacin, tylosin, penicillin, tetracycline, lincomycin, erythromycin, trimethoprim/sulfamethoxazole, oxacillin, ceftriaxone, methicillin, vancomycin, and cefazolin. From isolated *S. aureus* strains in this study, 31.8% were able to produce biofilm. In general, compared to biofilm-negative isolates, a higher percentage of biofilm-positive strains showed antibiotic resistance to antibiotics used, except for tylosin, cefazolin, and enrofloxacin. Resistance or susceptibility to penicillin, lincomycin, trimethoprim/sulfamethoxazole, oxacillin, methicillin and vancomycin was significantly distinct ($P<0.05$) between biofilm-positive and negative strains. Positive and negative biofilm groups demonstrated the highest sensitivity against enrofloxacin and cefazolin - most of biofilm-positive (85.7% and 77.1%, respectively) and biofilm-negative isolates (75.4% and 63.1%, respectively) were susceptible to these antibiotics. The present investigation revealed that a high percentage of *S. aureus* isolates causing bovine mastitis in Iran can form biofilms, so a practical therapeutic approach should be considered.

Key words: antibiotic resistance, biofilm, mastitis, *Staphylococcus aureus*

INTRODUCTION

Mastitis is a condition typified by the inflammatory response in the mammary tissue due to either infections or physical trauma. It is the most costly disease influencing the dairy industry resulting in sustained economic losses and reduced milk output and quality (Lasagno *et al.*, 2012). Ruminant mastitis research focuses on investigating pathogen and cow factors involved in the host response to the intramammary bacterial invasion, which may affect treatment options and effective preventative strategies. Besides, the treatment and prevention of mastitis with antimicrobial agents have introduced concerns about the safety and quality of dairy products. Specifically, the sustained use of antibiotics in animal food can result in drug residues in edible tissues and milk products, producing resistant strains of bacteria (Treiber & Beranek-Knauer, 2021).

S. aureus is a Gram-positive bacterium that can endure and multiply in different conditions. It provokes many infections in humans and animals (Cramton *et al.*, 1999; Cucarella *et al.*, 2001). *S. aureus,* known as an antibiotic-resistant pathogen, is one of the most crucial agents of infectious mastitis (Rainard *et al.*, 2018; Sadiq *et al.*, 2019). Intramammary infections, which often lead to chronic mastitis in lactating animals, are commonly caused by *S. aureus*. Bacteria from the infected epidermis, or bacteria that enter the gland by the influx of contaminated milk due to improper milking, begin to colonise the teat canal, leading to *S. aureus* mastitis. By reaching the subepithelial layers and binding to receptor proteins like fibrinogen, an infection ultimately is established that usually evolves into a chronic form (Foster & Höök, 1998). The successful treatment is usually interrupted due to the antibiotic inefficacy against the bacteria important in chronic infections and a high prevalence of methicillin-resistant strains. The only effective way to manage this condition seems to be premature culling of animals whose milk production has been significantly reduced, as antibiotic therapy of this type of mastitis is challenging (Cheng & Han, 2020).

Many chronic infections are associated with the formation of biofilms that result from the growth of bacteria in colonies encircled by an exopolysaccharide matrix. The bacteria attach to the surface, grow, and then accumulate to form multilayered cell clusters that are subsequently encased in a slimy matrix to form the biofilm (Anwar *et al.*, 1992; Amorena *et al.*, 1999). Biofilms become resistant to some antibiotics because of their aggregate size, which yields insensitivity to macrophage phagocytosis (Monzón *et al.*, 2001, 2002; Cucarella *et al.*, 2004). Delay in the penetration of antimicrobial agents into the biofilm matrix and altered growth rate of biofilm organisms are other mechanisms that make biofilms resistant to antimicrobial agents. Major study on animal biofilm formation has been conducted on *S. aureus* strains, notably those isolated from bovine mastitis, because biofilm formation is crucial in the pathogenesis of staphylococcal diseases (Conlon, 2014; Khoramian *et al.*, 2015).

One of the topics on which biomedical science focuses is the capacity of bacteria to build biofilms and the identification of adaptive genetic pathways involved in biofilm formation. The issue importance is also due to the resistance that biofilms show against common and otherwise ef-

fective antimicrobials. In veterinary medicine, the study of biofilms is also crucial in that the mastitis pathogens, with the formation of biofilms, can affect the therapeutic results. However, essential biofilm-producing genes have already been identified in *S. aureus* (Vasudevan *et al.*, 2003; Melchior *et al.*, 2006). Given that antibiotic resistance has increased, it is essential to find effective antibiotics for biofilm-positive and negative strains of *S. aureus*. To the best of our knowledge, no report has compared antibiotic susceptibility differences between biofilm-positive and negative *S. aureus* strains. The present study aimed to evaluate 110 strains of *S. aureus* isolated from bovine mastitis in terms of the number of biofilm-producing strains and then compare antibiotic response between biofilm-positive and biofilm-negative strains.

MATERIALS AND METHODS

Animals and sampling

The cows selected for the study were large Holstein dairy cows from Khorasan Razavi, Iran, healthy in clinical examinations without mastitis. The cows had high *S. aureus* (50 to 60 CFU/mL) counts in their milk. They were not treated with antibiotics or anti-inflammatory drugs in the previous two weeks. Immediately after being collected, milk samples were taken to the laboratory.

Laboratory bacteriological culture and identification of S. aureus isolates

Blood agar and McConkey medium (Merck, Germany) were used for culture. Samples were incubated aerobically at 37 °C for 48 hours. According to the National Mastitis Council (Adkins et al., 2017) guidelines, *S. aureus* was identified using the haemolytic pattern, morphology by Gram-staining features, positive catalase reaction, positive mannitol agar reaction, negative CAMP tests, and positive tube coagulase test. The isolates underwent Voges Proskauer (VP) and coagulase assays, and a quarter was considered contaminated if more than 100 CFU/mL of *S. aureus* had been isolated.

The *nucA* PCR was used to amplify the gene and confirm *S. aureus* isolates. DNA was extracted using the boiling extraction technique (Abdelhai *et al.*, 2016). The nucleotide sequences of the used primers were as followed: Forward – CTG GCA TAT GTA TGG CAA TTG TT and Reverse – TAT TGA CCT GAA TCA GCG TTG TCT (Bahraminia *et al.*, 2017). PCR product size was 613 bp.

Quantification of biofilm production

A colorimetric microtiter plate assay was performed to gauge each strain's ability to create biofilms (Peeters *et al.*, 2008). *S. aureus* isolates were cultured in trypticase soy broth (TSB) (Merck, Darmstadt, Germany) at 37 °C overnight.

The cultures were then diluted 1:100 in TSB medium. After that, sterile 96-well polystyrene microtiter plates were inoculated with 150 µL of this culture. After 24 hours of incubation at 37 °C without shaking, the wells were gently washed three times with 200 µL of phosphate-buffered saline (PBS; Denazist, Iran) and dried inverted. One hundred µL of 99% methanol was applied to fix the biofilms, supernatants were removed after 15 minutes, and the plate was air-dried. The following stage involved adding 100 µL of 1% crystal violet to each well. The excess crystal violet was removed after 20 minutes by washing the plate under running tap water. Finally, by adding 150 µL of 33% acetic acid, the bound crystal violet was dis-

solved. A microtiter plate reader was used to measure the optical density (OD) of each well at 590 nm. All tests were repeated four times.

In the current study, the uninoculated medium was used as a control to determine the background OD. The cut-off OD (ODc) was defined as three standard deviations above the negative control's mean OD, and the final OD value of a tested isolate was calculated as the average OD of the strain minus the ODc value. Strain'ss adherence capability was classified into four categories (Stepanović *et al.*, 2007): non-adherent (OD<ODc), weakly adherent (ODc<OD<2×ODc), moderately adherent (2×ODc<OD<4× ODc), and strongly adherent (4×ODc<OD).

Antimicrobial susceptibility test

Antibiotic susceptibility of *S. aureus* isolates was evaluated by the agar disk diffusion method (ADD). Antibiotics included ampicillin (10 µg), enrofloxacin (5 µg), tylosin (30 µg), penicillin (10 µg), tetracycline (30 µg), lincomycin (2 µg), erythromycin (15 µg), trimethoprim/sulfamethoxazole (1.25 µg+23.75 µg), oxacillin (1 µg), ceftriaxone (30 µg), methicillin (5 µg), vancomycin (30 µg), and cefazolin (30 µg), (Padtan Teb, Iran). The Clinical and Laboratory Standards Institute (CLSI) guidelines were used for setting the standards (James *et al.*, 2022). *S. aureus* ATCC 29213 was used as the control strain for antibiotic susceptibility testing. Antimicrobial susceptibility has been described as "susceptible," "intermediate," or "resistant" based on a standardised, threshold-based assessment as shown in Table 1 (James *et al.*, 2022; CLSI, 2018).

Statistical analysis

SPSS 16 (USA) was utilised for statistical analysis. The chi-square test was used to determine the differences in antibacterial resistance between *S. aureus* strains. A P value of ≤ 0.05 was supposed to be statistically significant.

Table 1. Interpretative criteria and zone diameters for each antibiotic (CLSI, 2018)

Antibiotics	Interpretive categories and zone diameter breakpoints, nearest whole mm		
	Sensitive	Intermediate	Resistant
Ampicillin	≥ 29	27–28	≤ 26
Enrofloxacin	≥ 23	17–22	≤ 16
Tylosin	≥ 29	27–28	≤ 26
Penicillin	≥ 28	20–27	≤ 19
Tetracycline	≥ 19	15–18	≤ 14
Lincomycin	≥ 21	15–20	≤ 14
Erythromycin	≥ 23	14–22	≤ 13
Trimethoprim/sulfamethoxazole	≥ 16	11–15	≤ 10
Oxacillin	≥ 13	11–12	≤ 10
Ceftriaxone	≥ 21	14–20	≤ 13
Methicillin	≥ 14	10–13	≤ 9
Vancomycin	≥ 17	15–16	≤ 14
Cefazolin	≥ 18	15–17	≤ 14

RESULTS

The *nucA* PCR was used to amplify the gene and confirm *S. aureus* isolates. The PCR product size was 613 bp (Bahraminia *et al.*, 2017) (Fig. 1).

Approximately 68.2% (75/110) of *S. aureus* isolates did not produce biofilms, 20% (22/110) were weak adherent biofilm producers, while 10% (11/110) and 1.8% (2/110) produced moderate and vigorous biofilms, respectively.

Disc agar susceptibility examination was conducted on 110 *S. aureus* isolates using antimicrobial agents commonly used *in vivo* to treat mastitis in cows. The results of behaviour of biofilm- positive and negative *S. aureus* isolates to 13 antimicrobial drugs are shown in Table 2. Biofilm-positive strains were more sensitive to enrofloxacin (85.7%) and cefazolin (77.1%); while biofilm-negative strains showed higher sensitivity to enrofloxacin (75.4%), trimethoprimsulfa (72.3%), oxacillin (64.6%), methicillin (63.1%), vancomycin (61.5%) and cefazolin (63.1%). Biofilm-positive and biofilm-negative groups were the most sensitive to enrofloxacin and cefazolin. This was demonstrated in most biofilm-positive (85.7% and 77.1%, respectively) and biofilm-negative isolates (75.4% and 63.1%, respectively) were susceptible.

The biofilm-positive isolates showed the highest antibiotic resistance to ampicillin (82.9%), penicillin (77.1%), lincomycin (68.6%), oxacillin (65.7%), methicillin (65.7%), tetracycline (62.9%) and vancomycin (62.9%). In the biofilm-negative isolates, the highest antibiotic resistance was related to ampicillin (78.5%) and penicillin (58.5%).

Generally, higher resistance percentages were seen in biofilm-positive isolates than in biofilm-negative isolates, excluding tylosin, cefazolin, and enrofloxacin. The differences between biofilm-positive and negative isolates were significant for penicillin, lincomycin, trimethoprimsulfa, oxacillin, methicillin, and vancomycin ($P<0.05$). No significant differences between biofilm-positive and bio-

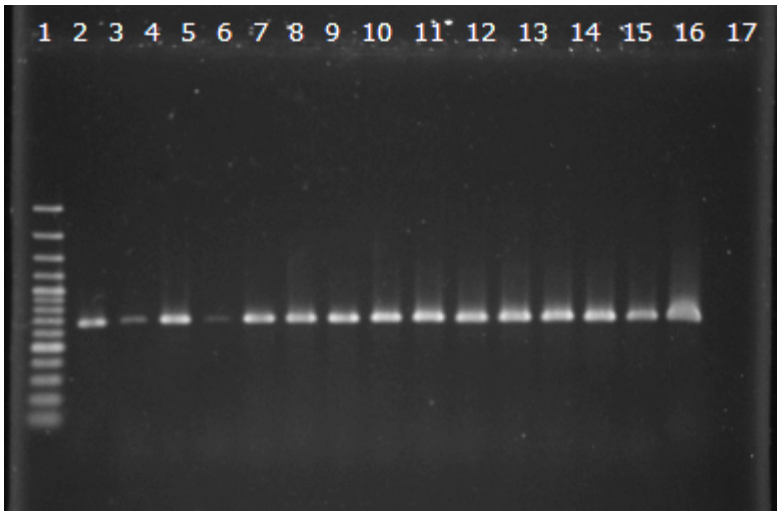

Fig. 1. Agarose gel electrophoresis of *S. aureus nucA* gene PCR product. Lane 1: 100 bp DNA ladder, lanes 2–16: positive samples, lane 17: negative sample.

Table 2. Results of antimicrobial susceptibility in biofilm-positive and -negative *S. aureus* isolated from bovine mastitis

Antibiotics	Biofilm-positive			Biofilm-negative		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Ampicillin	14.3% (n=5)	2.9% (n=1)	82.9% (n=29)	18.5% (n=12)	3.1% (n=2)	78.5% (n=51)
Enrofloxacin	85.7% (n=30)	0% (n=0)	14.3% (n=5)	75.4 % (n=49)	3.1% (n=2)	21.5% (n=14)
Tylosin	17.1% (n=6)	40% (n=14)	42.9% (n=15)	21.5% (n=14)	27.7% (n=18)	50.8% (n=33)
Penicillin	14.3% (n=5)	8.6% (n=3)	77.1% (n=27)	38.5% (n=25)	3.1% (n=2)	58.5% (n=38)
Tetracycline	37.1% (n=13)	0% (n=0)	62.9% (n=13)	52.3% (n=34)	3.1% (n=2)	44.6% (n=29)
Lincomycin	25.7% (n=9)	5.7% (n=2)	68.6% (n=24)	55.4% (n=36)	7.7% (n=5)	36.9% (n=24)
Erythromycin	22.9% (n=8)	22.9% (n=8)	54.3% (n=19)	46.2% (n=30)	16.9% (n=11)	36.9% (n=24)
Trimethoprim/ sulfamethoxazole	48.6% (n=17)	0% (n=0)	51.4% (n=18)	72.3% (n=47)	0% (n=0)	27.7% (n=18)
Oxacillin	31.4% (n=11)	2.9% (n=1)	65.7% (n=23)	64.6% (n=42)	6.2% (n=4)	29.2% (n=19)
Ceftriaxone	28.6% (n=10)	14.3% (n=5)	57.1% (n=20)	49.2% (n=32)	13.8% (n=9)	36.9% (n=24)
Methicillin	34.3% (n=12)	0% (n=0)	65.7% (n=23)	63.1% (n=41)	0% (n=0)	36.9% (n=24)
Vancomycin	37.1% (n=13)	0% (n=0)	62.9% (n=22)	61.5% (n=40)	0% (n=0)	38.5% (n=25)
Cefazolin	77.1% (n=27)	2.9% (n=1)	20% (n=7)	63.1% (n=41)	1.5% (n=1)	35.4% (n=23)

film-negative isolates were observed with respect to ampicillin, enrofloxacin, tylosin, tetracycline, erythromycin, ceftriaxone, and cefazolin.

DISCUSSION

Bacteria are the leading cause of mammary gland infection in cows, but more important is the ability of bacteria to produce biofilms, which is a crucial malignant property in the pathogenesis of mastitis (Felipe *et al.*, 2017). Since mastitis has a significant effect on milk production in cows, it can directly affect the profits and losses of the dairy industry.

Genotypic and phenotypic techniques are used to identify bacteria. However, because genotypic methods are more sensitive and specific (Kateete *et al.*, 2010), in this study *S. aureus* was confirmed by positive *nuc* gene amplification. *Nuc* gene is present in coagulate-positive staphylococci such as *S. aureus* (Sasaki *et al.*, 2010). This gene encodes a specific thermostable nuclease and has already been used to identify S. *aureus* (Palomares *et al.*, 2003; Costa *et al.*, 2005; Rusenova *et al.*, 2013).

One of the primary strategies for bacterial survival among infected host organisms is biofilm formation. Studies have shown that 35% of the isolates from the total number of isolated microorganisms have the ability to biofilm formation (Rudenko *et al.*, 2021). Darwish & Asfour (2013) investigated the ability of biofilm formation in *staphylococci* to cause bovine mastitis by plate microtiter method in Egypt. They reported that 20%, 27.5% and 52.5% of *S. aureus* isolates as producers of weak, moderate and strong biofilms, respectively. Using the tissue culture plate method, a study conducted in India showed that 29.41% of *S. aureus* were biofilm producers (Dhanawade *et al.*, 2010). This rate is relatively consistent with the result observed in the present study, in which approximately 31.8% of the isolates produced biofilms.

Today, many antibiotics are used to treat mastitis on farms. Microbiological studies show that the study of the biofilm-forming ability of pathogenic agents of bovine mastitis can help select an appropriate treatment regimen for mastitis in cattle farms and to produce new antimastitic drugs that work on both biofilm-positive and negative strains (Horiuk *et al.*, 2019). In this study, biofilm-positive isolates showed higher resistance than biofilm-positive isolates against used antibiotics. tylosin, cefazolin, and enrofloxacin were the exceptions in this regard. As reported previously, the minimum inhibitory concentration (MIC) of linezolamid in *S. aureus* was 16-fold higher for biofilm cells than for planktonic cells (De Oliveira *et al.*, 2016). A 64-fold increase in the minimum biofilm eliminating concentration (MBEC)/MIC ratio of vancomycin in *S. epidermidis* and *S. aureus*, strong biofilm producers, was observed by Antunes *et al.* (2011). Bacteria grown in a biofilm form can develop resistance to antimicrobial treatments 10–1000 times higher than bacteria growing in a plankton form (Mah & O'Toole, 2001; Olson *et al.*, 2002; Conley *et al.*, 2003).

One feature that distinguishes biofilm-positive from biofilm-negative isolates is the production of an exopolysaccharide matrix. This matrix impairs the access of antibiotics to the bacterial cells (Stewart, 1996). Adsorption of an antimicrobial agent to the biofilm matrix components or reaction with matrix compounds can also restrict their transport within the biofilm.

In this study the most effective antibiotics were enrofloxacin and cefazolin,

because most biofilm-positive and negative *S. aureus* isolates were susceptible to them. Horiuk *et al.* (2019) found that enrofloxacin can completely inactivate streptococci and staphylococci in biofilms due to its low molecular weight and the ability to penetrate through biofilm pores and channels into microbial cells. Ceftriaxone and doxycycline also affected the bacteria in the biofilm. This is in good accordance with the observation in this study in a way that biofilm-positive strains were more sensitive to enrofloxacin and cefazolin. In the current study, the highest antibiotic resistance was related to ampicillin (82.9%) and penicillin (77.1%) in the biofilm-positive group. The difference in the behaviour of *S. aureus* strains to ampicillin and penicillin can be most probably due to the Eagle effect described for *S. aureus* against penicillin and other beta-lactams (Rusenova *et al.*, 2022). It has been discovered that the bacteria in mature biofilms grow slowly (Costerton *et al.*, 1999). Because antimicrobial agents require growing organisms to exert bactericidal effects, this phenomenon reduces bacterial susceptibility to antimicrobial agents in biofilms. Penicillins and cephalosporins, for instance, kill bacteria at a rate proportionate to bacterial growth rate, and they have little to no effect on non-growing cells. Studies have shown that the biofilm produced by *K. pneumoniae* does not allow ampicillin to penetrate it. That ampicillin is ineffective even on β-lactamase-deficient *K. pneumoniae* mutants produced in the biofilm (Anderl *et al.*, 2000; Melchior *et al.*, 2006).

The results show high resistance to methicillin (65.7%) in biofilm-positive strains. One of the resistant forms of *S. aureus* is methicillin-resistant *S. aureus* (MRSA). MRSA is considered one of the main bacteria causing human infections in hospitals and community settings and the leading cause of mastitis (in some areas). Genetic mutation and resistance of MRSA to antibiotics commonly used in this field increase the challenges for healthcare workers (Shrestha *et al.*, 2021). The presence of high occupational risk for milkers, farmers, and veterinarians, close contact with MRSA-infected cows should also be considered. Therefore, revealed the prevalence and resistance characteristics of *S. aureus* and MRSA in Razavi Khorasan cattle farms in Iran, this study helps the rational use of antibiotics.

CONCLUSION

The treatment approach to mastitis can change with a better understanding of biofilm formation. In the current investigation, a significant portion (31.8%) of *S. aureus* isolates from cases of bovine mastitis in Khorasan Razavi, Iran, were found capable of developing biofilms. Since the antibiotic susceptibility pattern in *S. aureus* was different in biofilm-positive and biofilm-negative isolates, it is concluded that determining the biofilm-producing ability is necessary to select the most effective antibiotic. If this ability cannot be determined, enrofloxacin and cefazolin appear more appropriate for treating mastitis. They are highly effective on both biofilm-positive and negative *S. aureus*. Ampicillin and penicillin are not suitable options for treating *S. aureus* mastitis because they showed the highest resistance in both groups of isolates.

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