Dicle Üniversitesi Veteriner Fakültesi Dergisi
Dicle University Journal of Faculty of Veterinary Medicine
e-ISSN:1308-0679
https://dergipark.org.tr/tr/pub/duvetfd

RESEARCH ARTICLE

Isolation of *Staphylococcus epidermidis* from Milk Samples of Cows with Mastitis and Investigation of Its Biofilm Formation Ability

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Received
18.08.2025

Accepted
14.05.2026

Published
30.06.2026

DOI

10.47027/duvetfd.1765192

How to cite: Aktepe B , Savaşan S, Nuhay Ç, Yaman Ş (2026). Isolation of *Staphylococcus epidermidis* from mastitic milk and investigation of its biofilm formation ability. *Dicle Univ Vet Fak Derg.*, 19(1):91-95.

Abstract

Mastitis is one of the most important infectious diseases in dairy cattle and is associated with considerable economic losses. Among the causative agents, *Staphylococcus epidermidis* is an opportunistic pathogen whose ability to form biofilm is considered an important virulence factor. The aim of this study was to evaluate the biofilm-forming capacity of *S. epidermidis* isolates recovered from bovine mastitic milk samples using phenotypic and genotypic methods. For this purpose, a total of 150 milk samples were collected from 150 different dairy cows with clinical mastitis from different farms in the provinces of İzmir and Aydın. The samples were cultured on 5% sheep blood agar, and the isolates were identified by conventional procedures and the VITEK® 2 Compact system. Phenotypic biofilm production was evaluated by the Congo Red Agar (CRA) method, and the presence of the *icaA* and *icaD* genes was investigated molecularly. As a result of bacteriological examination, 13 isolates were identified as *S. epidermidis*. Of these, 11 (84.6%) were found to be biofilm-positive by the CRA method, whereas 2 (15.4%) were biofilm-negative. Molecular analysis revealed that none of the isolates carried the *icaA* or *icaD* genes. In conclusion, most *S. epidermidis* isolates obtained from mastitic milk samples showed phenotypic biofilm-forming ability despite the absence of the investigated classical biofilm-associated genes. These findings suggest that biofilm formation in bovine *S. epidermidis* isolates may involve alternative molecular mechanisms and that phenotypic results should be interpreted together with genotypic analyses.

Key Words: Biofilm formation, cattle, mastitis, milk, *Staphylococcus epidermidis*

Mastitisli İneklerin Süt Örneklerinden *Staphylococcus epidermidis* İzolasyonu ve Biyofilm Oluşturma Yeteneğinin İncelenmesi

Öz

Mastitis, süt sığırlarında görülen en önemli enfeksiyöz hastalıklardan biridir ve önemli ekonomik kayıplarla ilişkilidir. Etkenler arasında yer alan *Staphylococcus epidermidis*, biyofilm oluşturma yeteneği önemli bir virülens faktörü olarak kabul edilen fırsatçı bir patojendir. Bu çalışmanın amacı, sığır mastitisli süt örneklerinden elde edilen *S. epidermidis* izolatlarının biyofilm oluşturma kapasitesini fenotipik ve genotipik yöntemler kullanarak değerlendirmektir. Bu amaçla, İzmir ve Aydın illerindeki farklı işletmelerde bulunan klinik mastitisli 150 farklı süt sığırından toplam 150 süt örneği toplanmıştır. Örnekler %5 koyun kanlı agara ekilmiş ve izolatlar konvansiyonel yöntemler ile VITEK® 2 Compact sistemi kullanılarak tanımlanmıştır. Fenotipik biyofilm üretimi Kongo Red Agar (CRA) yöntemi ile değerlendirilmiş, *icaA* ve *icaD* genlerinin varlığı ise moleküler olarak incelenmiştir. Bakteriyolojik inceleme sonucunda 13 izolat *S. epidermidis* olarak tanımlanmıştır. Bunların 11'i (%84,6) CRA yöntemi ile biyofilm pozitif, 2'si (%15,4) ise biyofilm negatif olarak değerlendirilmiştir. Moleküler analiz sonucunda ise izolatların hiçbirinde *icaA* veya *icaD* geni saptanmamıştır. Sonuç olarak, mastitisli süt örneklerinden elde edilen *S. epidermidis* izolatlarının büyük çoğunluğu, araştırılan klasik biyofilm ile ilişkili genlerin yokluğuna rağmen fenotipik olarak biyofilm oluşturma yeteneği göstermiştir. Bu bulgular, sığırlara ait *S. epidermidis* izolatlarında biyofilm oluşumunun alternatif moleküler mekanizmalar içerebileceğini ve fenotipik sonuçların genotipik analizlerle birlikte değerlendirilmesi gerektiğini düşündürmektedir.

Anahtar Kelimeler: Biyofilm oluşumu, mastitis, sığır, *Staphylococcus epidermidis*, süt

INTRODUCTION

Mastitis, defined as inflammation of the mammary tissue, is considered one of the most important infectious diseases in dairy cattle due to its association with reduced milk yield, impaired product quality, and significant economic losses (1,2). Globally, mastitis-related losses result in millions of dollars in economic damage for dairy farms each year. Mammary health issues rank among the leading causes of culling in dairy herds, with reported rates reaching up to 34.8% (3).

Among the causative agents of mastitis, *Staphylococcus* species particularly *Staphylococcus aureus* and *Staphylococcus epidermidis* are noteworthy due to their prevalence and antimicrobial resistance profiles (4). *S. epidermidis* is one of the most frequently isolated coagulase-negative staphylococci (CNS). Although it is a common component of skin flora, it acts as an opportunistic pathogen responsible for various infections (5,6). One of its key virulence factors is its ability to form biofilms (7).

Studies investigating the prevalence and genetic basis of biofilm formation in *S. epidermidis* isolates associated with mastitis have highlighted the necessity of using both phenotypic and molecular diagnostic methods (8,9).

Biofilm formation is an important virulence factor in *S. epidermidis* and involves adhesion, accumulation, maturation, and dispersal stages. The best-known genetic basis of this process in staphylococci is the *icaADBC* operon, which mediates the synthesis of polysaccharide intercellular adhesin (PIA/PNAG). In particular, *icaA* and *icaD* are widely investigated because *icaA* encodes the main transferase enzyme involved in PIA synthesis, while *icaD* enhances its activity and contributes to effective biofilm production. Since biofilm formation may also occur through ica-independent mechanisms and is influenced by environmental factors such as nutrient conditions, osmolarity, and oxygen availability, both phenotypic and genotypic approaches are recommended. Among the available phenotypic methods, the CRA method is frequently preferred as a simple, economical, and practical screening tool for detecting slime-associated biofilm production (10-12).

The aim of this study was to evaluate the biofilm-forming capacity of *S. epidermidis* isolates recovered from bovine milk samples using phenotypic and genotypic approaches. In this context, biofilm production was assessed by the CRA method, while the presence of the biofilm-associated *icaA* and *icaD* genes was determined by PCR.

MATERIAL AND METHODS

As part of this study, a total of 150 milk samples were collected from 150 different dairy cows with clinical mastitis from different farms in the provinces of İzmir and Aydın. The samples were collected aseptically under appropriate hygienic conditions and transported to the Routine Diagnostic Laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, under cold chain preservation. Clinical mastitis was determined on the basis of udder examination findings and observable alterations in milk secretion. Signs such as udder swelling, heat, pain, and the presence of clots, flakes, or abnormal milk consistency were taken into account during the sampling process.

Upon arrival at the laboratory, the milk samples were inoculated onto blood agar medium containing 5% sheep blood and incubated at 37 °C for 24–48 hours under aerobic conditions. The resulting colonies were evaluated for their morphological characteristics and purity. Colonies obtained in pure culture were subjected to Gram staining, and those identified as Gram-positive cocci and catalase-positive were further identified using the VITEK® 2 Compact system (bioMérieux, France) with the GP identification kit (13,14).

The biofilm-forming ability of the isolates was phenotypically evaluated using the CRA method (15). Isolates producing black and rough colonies were considered biofilm-positive. *Staphylococcus epidermidis* ATCC® 35984 and *Staphylococcus epidermidis* ATCC® 12228 were used as positive and negative control strains, respectively, for phenotypic biofilm evaluation. For genotypic evaluation, the presence of the icaA and icaD genes was investigated by PCR. A synthetic positive control was used for PCR amplification of the target genes. DNA extraction from the isolates identified as S. epidermidis was performed using a commercial DNA extraction kit (High Pure PCR Template Preparation Kit, Roche). Amplification was carried out in a Techne TC-412 thermal cycler (Keison Products) using Xpert Fast Hotstart Mastermix (2×, GRiSP). The PCR protocol included an initial denaturation step at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 15 s, with a final extension at 72°C for 3 min. The amplified products were separated by electrophoresis on 2% agarose gel stained with Xpert Green DNA Stain Direct (GRiSP) and visualized using a gel imaging system (Er Biotech Fx51/FxT). The primers used in the study are shown in Table 1.

Table 1. Primer Details for Molecular Detection of Biofilm-Associated Genes (*icaA* and *icaD*)

Gene	Primer Direction	Sequence (5'-3')	Annealing Temperature (°C)	Reference
icaA	Forward	CCT AAC TAA CGA AAG GTA G	55 °C	Arciola et al. (1)
	Reverse	AAG ATA TAG CGA TAA GTG C		
icaD	Forward	AAA CGT AAG AGA GGT GG	55 °C	Arciola et al. (1)
	Reverse	GGC AAT ATG ATC AAG ATA		

Descriptive statistics were expressed as numbers and percentages. The 95% confidence intervals (CI) for proportions were calculated using the Wilson score method (16). The discrepancy between phenotypic biofilm formation detected by the Congo Red Agar (CRA) method and molecular detection of the icaA and icaD genes was evaluated using McNemar's exact test. A P-value of <0.05 was considered statistically significant.

RESULTS

In this study, a total of 150 milk samples collected from cows with clinical mastitis were bacteriologically examined. Following cultural isolation, colony morphology, Gram staining, catalase testing, and identification using the VITEK® 2 Compact system, 13 isolates were identified as Staphylococcus epidermidis. Accordingly, the isolation rate of S. epidermidis among the examined mastitic milk samples was determined as 8.7% (13/150; 95% CI: 5.1–14.3%). The remaining 137 samples were negative for S. epidermidis, corresponding to 91.3% of the total samples (95% CI: 85.7–94.9%). The isolation findings are presented in Table 2.

Table 2. Isolation rate of *S. epidermidis* from mastitic milk samples

Parameter Examined	Number (n)	Percentage (%)	95% Confidence Interval
Total milk samples	150	100.0	-
***S. epidermidis*-positive samples**	13	8.7	5.1–14.3
***S. epidermidis*-negative samples**	137	91.3	85.7–94.9

Phenotypic biofilm-forming ability of the 13 S. epidermidis isolates was evaluated using the Congo Red Agar (CRA) method. Of these isolates, 11 were classified as biofilm-positive based on the formation of black and rough colonies, while two isolates were evaluated as biofilm-negative. Thus, the phenotypic biofilm positivity rate among S. epidermidis isolates was 84.6% (11/13; 95% CI: 57.8–95.7%), whereas the biofilm negativity rate was 15.4% (2/13; 95% CI: 4.3–42.2%). The distribution of CRA-based biofilm formation results is shown in Table 3 and illustrated in Figure 1.

Table 3. Phenotypic biofilm formation of *S. epidermidis* isolates by CRA method

CRA Result	Number (n)	Percentage (%)	95% Confidence Interval
Biofilm-positive	11	84.6	57.8–95.7
Biofilm-negative	2	15.4	4.3–42.2
Total	13	100.0	-

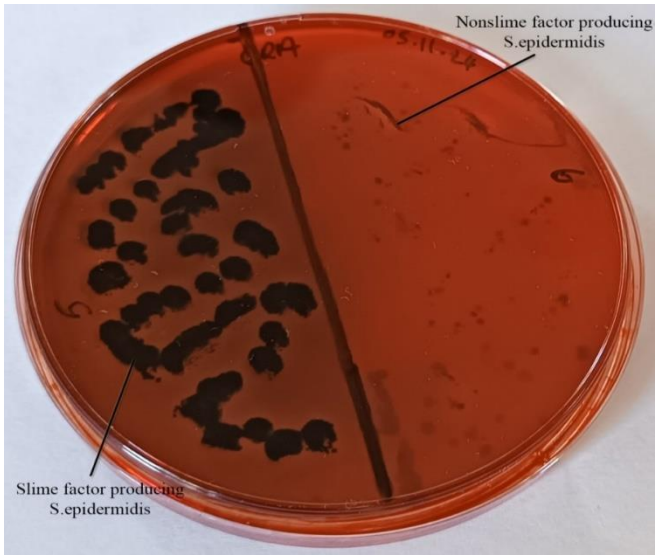

Figure 1. Evaluation of Biofilm Formation by *Staphylococcus epidermidis* Strains on Congo Red Agar

Molecular analysis was performed to determine the presence of the biofilm-associated icaA and icaD genes in all S. epidermidis isolates. PCR analysis revealed that none of the 13 isolates produced amplification products specific for either the icaA or icaD genes. Therefore, the molecular positivity rate for both genes was determined as 0.0% (0/13), while all isolates were negative for both target genes.

When phenotypic and genotypic findings were evaluated together, 11 isolates showed a CRA-positive/icaA-icaD-negative profile, whereas two isolates were negative by both CRA and PCR. No isolate was positive for icaA or icaD despite the high rate of phenotypic biofilm formation detected by the CRA method. Therefore, the overall phenotypic-genotypic discordance rate was 84.6% (11/13). McNemar's exact test demonstrated a statistically significant discrepancy between CRA-based phenotypic biofilm formation and PCR-based detection of icaA/icaD genes (P = 0.001). The comparison of CRA results with icaA/icaD gene detection is presented in Table 4.

Table 4. Comparison of phenotypic biofilm formation and icaA/icaD gene detection

CRA result	icaA positive	icaD positive	icaA/icaD negative	Total
CRA positive	0	0	11	11
CRA negative	0	0	2	2
Total	0	0	13	13

DISCUSSION AND CONCLUSION

Mastitis is one of the most significant infectious diseases affecting the mammary tissue in dairy cattle, leading to considerable economic losses due to reduced milk yield, increased treatment costs, a higher culling rate, and even mortality. In this study, the biofilm-forming capacities of *S. epidermidis* strains isolated from mastitic cattle were evaluated using both phenotypic and genotypic methods.

The findings revealed that a significant portion (84.6%) of the isolates demonstrated biofilm production based on the CRA test. This suggests that these strains have a high adhesion ability to surfaces and potentially possess mechanisms to evade host immune responses. Türkyılmaz et al. (9) reported biofilm positivity rates of 77.8% in coagulase-positive and 44.4% in coagulase-negative *Staphylococcus* strains isolated from various animal clinical samples. Compared to our findings, these rates are relatively lower, which could be attributed to differences in sample origin, methods used, and ecological characteristics of the strains. Similarly, Kord et al. (17) reported that biofilm formation in *S. epidermidis* clinical isolates could be demonstrated phenotypically and that the presence of ica genes was not always sufficient alone to explain biofilm production. These reported rates, especially among CNS strains, are notably lower than the phenotypic positivity observed in our study, indicating possible variability in genetic and physiological biofilm capacity depending on environmental conditions. In a study by Arciola et al. (8), 9 out of 15 clinical *S. epidermidis* isolates (60%) were found to be biofilm-positive, whereas none of the 10 isolates from skin and mucosal surfaces exhibited biofilm formation. These results highlight that biofilm production

may be closely associated with the source of isolation (8,9,17).

CRA has long been used as a phenotypic indicator of biofilm formation in *S. epidermidis*. CRA positivity is generally associated with the expression of the *icaADBC* operon, which plays a central role in the production of the biofilm matrix. However, in this study, none of the 11 CRA-positive isolates tested positive for *icaA* or *icaD* by PCR, suggesting that biofilm formation is not solely dependent on the *icaADBC* operon. This finding supports the existence of alternative molecular mechanisms involved in biofilm development. Similar discrepancies between phenotypic and genotypic findings have been reported in the literature. For instance, Mirzaei et al. (22) observed biofilm-positive phenotypes via CRA in clinical *S. epidermidis* isolates but could not detect *ica* genes at the molecular level. Additionally, Patel et al. (20) reported that some *S. epidermidis* strains lacking the *icaA* gene retained the ability to adhere to surfaces and exhibit intercellular adhesion. These findings indicate that *S. epidermidis* has a high level of plasticity and adaptability in biofilm formation (7,18,19,20-22).

In the present study, 11 of 13 *S. epidermidis* isolates were found to be biofilm-positive by the CRA method, whereas none of the isolates carried the *icaA* or *icaD* genes by PCR. Similar discrepancies between phenotypic and genotypic findings have been reported previously, indicating that phenotypic biofilm positivity may occur despite the absence of these genes. Moreover, biofilm formation in *S. epidermidis* is not exclusively dependent on the *ica* locus, and *ica*-independent mechanisms mediated by surface-associated proteins and matrix-related components have also been described. Likewise, strains with the *icaA/icaD* genotype may still produce protein- or protein/eDNA-based biofilms, supporting the role of alternative biofilm pathways. Therefore, the absence of *icaA* and *icaD* in the present isolates does not necessarily exclude biofilm-forming ability (10,23,24).

Biofilm formation is a complex, multi-stage process that is not solely dependent on PIA synthesis. Key proteins involved in PIA-independent biofilm formation include Aap, Embp, and Bhp. Aap plays a role in cell-to-cell adhesion during the accumulation phase, while Embp contributes to biofilm matrix stabilization. Embp has also been shown to confer resistance to phagocytic cells and can mediate biofilm formation independently. These mechanisms may explain the presence of genotypically *ica*-negative but phenotypically biofilm-positive isolates. Additionally, modified teichoic acids, eDNA, and surface proteins may also contribute to biofilm formation via *ica*-independent pathways. The genetic regulation of biofilm formation is strongly influenced by environmental factors. Variables such as pH, glucose concentration, iron availability, and oxygen levels can modulate the expression of the *icaADBC* operon. Thus, the culture conditions used in this study may have suppressed *ica* gene expression. Additionally, the repressor effect of the *icaR* gene may have prevented the transcription of *icaA* and *icaD* (7,17,18-20,25-27).

The absence of *ica* genes despite CRA-based biofilm positivity also raises the possibility of false-positive results with the CRA test, suggesting that this phenotypic method alone may not be sufficient for reliable biofilm detection. However, the lack of PIA does not necessarily imply a weak biofilm structure. On the contrary, PIA-independent biofilms may still contribute significantly to immune evasion and antimicrobial resistance. Indeed, *S. epidermidis* strains lacking PIA have been shown to effectively evade the immune system and cause persistent infections. Particularly in chronic infections, the biofilm layer acts as a physical barrier that limits the penetration and efficacy of antimicrobial agents, leading to treatment failure (7,19-21,28,29).

In conclusion, the findings of this study demonstrate that most *S. epidermidis* isolates from mastitic milk samples possess phenotypic biofilm-forming capacity, despite lacking classical biofilm marker genes *icaA* and *icaD*. This highlights that biofilm formation is not exclusively dependent on the *icaADBC* operon and that alternative molecular mechanisms may be involved. Moreover, considering the potential for false positives in phenotypic methods such as CRA, it is crucial to complement biofilm studies with genotypic analyses. Furthermore, CNS, particularly *S. epidermidis*, which are commonly isolated in mastitis cases, play a significant role in the chronicity of infection due to their ability to evade host defenses, adhere to surfaces, and resist antibiotics. Therefore, molecular characterization of CNS strains in dairy herds is essential for developing more effective control strategies against mastitis.

ACKNOWLEDGEMENT

This study is derived from the Master's thesis research conducted by Bayram Aktepe.

FINANCIAL SUPPORT

No funding or support was received from any institution for the conduct of this research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

BA and SS were responsible for the study design and sample collection. All authors contributed to the experimental procedures. The manuscript writing and initial review were carried out by ÇN, SS, and BA, with contributions from all authors.

ETHICAL STATEMENT

The authors affirm that the study was conducted in accordance with ethical standards, and no unethical procedures were involved.

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