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Molecular investigation of biofilm-forming genes in staphylococci isolated from dogs in Ilam, Iran

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Abstract

Biofilm formation is a key virulence factor in *Staphylococcus aureus*, contributing to bacterial persistence, antimicrobial resistance, and chronic infections. This study aimed to investigate the presence of biofilm-associated genes (*fib, fnbA, fnbB, clfA*, and *clfB*) in *S. aureus* isolates from dogs in Ilam, Iran. From December 2022 to September 2023, 250 swab samples were collected from nasal, oral, and rectal sites of dogs, yielding 81 *S. aureus* isolates confirmed by PCR amplification of the *nuc* gene. The prevalence of biofilm-associated genes varied, with *clfA, clfB,* and *fnbA* detected in 98.80% of isolates, *fib* in 63.00%, and *fnbB* in 16.00%. Notably, *fnbA, clfA,* and *clfB* were present in all rectal isolates, while *fnbB* was absent in this group. The findings highlighted the widespread presence of biofilm-related genes in *S. aureus* from dogs, suggesting their potential role in colonization and zoonotic transmission. The high prevalence of adhesion-associated genes underscored the need for monitoring biofilm-forming *S. aureus* in companion animals to mitigate antimicrobial resistance and public health risks.

Introduction

Biofilm formation is a critical factor in bacterial persistence and virulence, enabling bacteria to survive in hostile environments, resist antimicrobial agents, and evade host immune responses. Biofilms are structured communities of bacteria that enhance their resilience against environmental stresses and immune defenses, contributing significantly to chronic infections and antibiotic resistance.[1] The formation of biofilms is regulated by various molecular mechanisms, including the stringent response mediated by alarmone molecules, such as guanosine penta/tetraphosphate, which help bacteria adapt to stress and promote persistence.[2] This adaptation allows bacteria to enter a dormant state, making them less susceptible to antibiotics that target actively growing cells.[3] Thus, biofilms promote bacterial survival and drive the pathogenesis of infections, underscoring their importance as a critical target for developing novel therapeutic strategies.[4]

Among bacterial pathogens, *Staphylococcus* species are of particular interest due to their association with infections in both humans and animals. In humans, *Staphylococcus aureus* is a major cause of skin and soft tissue infections and more severe conditions, such as bloodstream infections.[5] Methicillin-resistant *S. aureus* poses a significant challenge due to its resistance to common antibiotics, making it a critical concern in healthcare settings.[6] In veterinary medicine, *Staphylococcus pseudintermedius* is frequently isolated from canine infections, particularly skin infections, and has been shown to have a significant impact on animal health.[7] The overlap in antibiotic use between human and veterinary medicine raises concerns about the spread of antibiotic resistance, highlighting the importance of monitoring these pathogens in both contexts.[8]

The ability of *S. aureus* and coagulase-negative staphylococci to produce biofilms is mediated by several genes, including *fib* (fibrinogen-binding protein), *fnbA* and *fnbB* (fibronectin-binding proteins), and *clfA* and *clfB* (clumping factors). The *icaADBC* operon is particularly crucial for biofilm formation, especially in coagulase-negative staphylococci, where it is associated with virulence in clinical settings.[9] These genes facilitate bacterial adhesion to host tissues and surfaces, playing a central role in colonization and infection. For example, fibronectin-binding proteins, encoded by *fnbA* and *fnbB*, are essential for adherence to fibronectin-coated surfaces, promoting bacterial attachment to host tissues.[10] Similarly, clumping factors (ClfA and ClfB) enable binding

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to fibrinogen, enhancing the ability of staphylococci to adhere to blood clots and damaged tissues.[11] The *fib* gene encodes a fibrinogen-binding protein that also contributes to adhesion, facilitating colonization during infection. These adhesion factors are critical for establishing infections, as they allow staphylococci to persist on host surfaces and evade immune responses, ultimately leading to successful colonization and potential disease.[12]

In veterinary medicine, *Staphylococcus* species are commonly implicated in infections among companion animals, particularly dogs. The *S. aureus*, *S. intermedius*, and *S. schleiferi* are significant pathogens in dogs, often associated with skin and soft tissue infections.[13] Methicillin-resistant *S. aureus* has been identified in dogs, indicating that companion animals can act as reservoirs for this pathogen, potentially transmitting it to humans.[14] Infections caused by these staphylococci can lead to various clinical manifestations, ranging from superficial infections, like pyoderma, to more severe conditions, such as deep tissue infections. The emergence of multiple drug-resistant strains poses a significant challenge in treating these infections, necessitating careful antibiotic stewardship in veterinary practice.[15]

Dogs can serve as reservoirs for biofilm-producing staphylococci, which may be transmitted to humans, posing significant zoonotic risks. Studies have shown that various *Staphylococcus* species, including *S. pseudintermedius* and *S. schleiferi*, are commonly isolated from dogs and can produce biofilms, enhancing their virulence and resistance to treatment.[15] These biofilm-forming strains can be transmitted to humans, particularly through close contact, leading to potential infections. The presence of methicillin-resistant strains in dogs raises additional concerns, as they can carry resistance genes that may be transferred to humans.[16] Moreover, the close interaction between dogs and their owners facilitates the exchange of staphylococci, increasing the risk of zoonotic transmission.[17] Therefore, monitoring and managing these bacteria in companion animals are crucial for public health.

The prevalence and genetic characteristics of biofilm-forming *S. aureus* in dogs from Ilam, Iran, have been remained underexplored, despite the potential implications for both veterinary and public health. The *S. pseudintermedius* and *S. aureus* are recognized as significant pathogens in canine infections, with biofilm formation serving as a critical virulence factor, facilitating bacterial persistence and complicating therapeutic interventions.[15,18] Although biofilm-associated genes, such as *icaA*, *icaD*, *fib*, *fnbA*, *fnbB*, *clfA*, and *clfB*, have been identified in staphylococcal isolates from dogs in other regions, data specific to Ilam are scarce.[19] This study aimed to isolate and identify *S. aureus* from dogs in Ilam, Iran, and investigate the presence of biofilm-related genes to better understand their role in canine infections.

Materials and Methods

Sample collection and processing. From December 2022 to September 2023, 250 nasal, rectal, and oral swabs were collected from stray and owned dogs in Ilam, Iran. Within 2 hr, samples were transported in tryptic soy broth on ice to the Microbiology Laboratory at Ilam University, Ilam, Iran.

Bacterial isolation and phenotypic characterization. Samples were streaked onto mannitol salt agar and incubated at 37.00 ˚C for 24 - 48 hr. Presumptive *S. aureus* colonies (yellow, salt-tolerant) were sub-cultured on Columbia nalidix acid agar (CNA) with 5.00% sheep blood to obtain pure isolates. Gram-positive, catalase-positive cocci, exhibiting β-hemolysis on CNA and coagulase activity, were confirmed as *S. aureus*. The DNase activity was assessed using DNase agar with 1.00 N HCl. Isolates were stored at – 20.00 ˚C in tryptic soy broth with 20.00% glycerol. All media used in this study were purchased from Conda (Madrid, Spain).

DNA extraction and molecular identification. Genomic DNA was extracted from 48-hr cultures using a simple boiling method described earlier.[20] The PCR was performed to amplify specific genetic markers for the identification and characterization of *Staphylococcus* species. The *16S rRNA* gene (764 bp) was amplified by PCR to enable genus-level identification as described previously.[21] For species-specific confirmation of *S. aureus*, a 279 bp fragment of the *nuc* gene, a well-documented marker for *S. aureus*, was targeted.[22] Primer sequences used in this study are provided in Table 1. *Staphylococcus aureus* (ATCC 33591^{T}) and *S. aureus* (ATCC 25923) were employed as quality controls in all experiments.

Screening of biofilm-associated genes using PCR. The PCR assay was conducted to identify the presence of biofilm-associated genes, including *clfA* and *clfB* (encoding clumping factors A and B), *fnbA* and *fnbB* (encoding fibronectin-binding proteins A and B), and *fib* (encoding fibrinogen-binding protein), in all isolates. The detection of *clfB* and *fib* was performed using duplex PCR with the following thermal cycling conditions: Initial denaturation at 95.00 ˚C for 5 min, followed by 30 cycles of denaturation at 95.00 ˚C for 1 min, annealing at 59.00 ˚C for 1 min, and extension at 72.00 ˚C for 1 min, with a final extension step at 72.00 ˚C for 7 min. For the detection of *clfA*, *fnbA*, and *fnbB*, a multiplex PCR assay was employed under similar thermal cycling conditions, except for the annealing temperature, which was adjusted to 56.00 ˚C. Amplified products were electrophoresed on 1.20% agarose gels in sodium borate buffer (pH: 8.20) and visualized under ultraviolet light.

Statistical analysis. Prevalence rates of biofilm-associated genes (*clfA*, *clfB*, *fnbA*, *fnbB*, and *fib*) were calculated as proportions (%) with 95.00% confidence intervals (CIs) using the Wilson score interval method,

Table 1. List of PCR primers used in the study.

Target genes	Sequence (5'-3')	Amplicon size (bp)	References
16S rRNA	AACTCTGTTATTAGGGAAGAACA CCACCTTCCTCCGGTTTGTCACC	764	21
nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	279	22
fib	CTACAACTACAATTGCCGTCAACAG GCTCTTGTAAGACCATTTTCTTCAC	404	23
fnbA	CATAAATTGGGAGCAGCATCA ATCAGCAGCTGAATTCCCATT	127	24
fnbB	GTAACAGCTAATGGTCGAATTGATACT CAAGTTCGATAGGAGTACTATGTTC	524	23
clfA	ATTGGCGTGGCTTCAGTGCT CGTTTCTTCCGTAGTTGCATTTG	292	23
clfB	ACATCAGTAATAGTAGGGGGCAAC TTCGCACTGTTTGTGTTTGCAC	205	23

which is robust for small-to-moderate sample sizes and proportions near 0.00 or 100%. Analyses were performed in R (version 4.3.0; R Foundation for Statistical Computing, Vienna, Austria) and cross-verified with MedCalc (version 22.009; MedCalc Software Ltd., Ostend, Belgium).

Results

Bacterial isolation and phenotypic characterization. A total number of 250 nasal, oral, and rectal swabs from dogs in Ilam, Iran, yielded 82 *Staphylococcus* isolates. Nasal swabs accounted for the highest proportion of isolates (36/82; 43.90%), followed by oral (31/82; 37.80%) and rectal (15/82; 18.29%) samples (Table 2). All isolates were Gram-positive cocci in grape-like clusters, catalase-positive, coagulase-positive, and DNase-positive, confirming phenotypic consistency with *S. aureus*.

Genotypic confirmation. The PCR amplification of the *16S rRNA* gene confirmed all 82 isolates as *Staphylococcus* spp. Subsequent *nuc* gene-specific PCR identified 81/82 isolates as *S. aureus*, with one isolate (code 72) excluded due to the repeated *nuc* negativity.

Table 2. Frequency (number) and percentage of studied genes in bacteria isolated from different anatomical sites.

Body sites	Genes	Positive isolates (%)	Negative isolates (%)
Mouth	*fnbA*	30 (100)	0 (0.00)
	fnbB	6 (20.00)	24 (80.00)
	clfA	30 (100)	0 (0.00)
	clfB	30 (100)	0 (0.00)
	fib	21 (70.00)	9 (30.00)
Nose	*fnbA*	35 (97.22)	1 (2.78)
	fnbB	7 (19.44)	29 (80.56)
	clfA	35 (97.22)	1 (2.78)
	clfB	35 (97.22)	1 (2.78)
	fib	13 (36.11)	23 (63.89)
Rectum	*fnbA*	15 (100)	0 (0.00)
	fnbB	0 (0.00)	15 (100)
	clfA	15 (100)	0 (0.00)
	clfB	15 (100)	0 (0.00)
	fib	8 (53.33)	7 (46.67)

Prevalence of biofilm-associated genes. Among *S. aureus* isolates (n = 81), the *clfA*, *clfB*, and *fnbA* genes were detected in 98.80% (95.00% CI: 93.60 - 99.8%) of isolates, demonstrating near-ubiquitous presence. The *fib* gene was identified in 63.00% (95.00% CI: 51.80 - 73.00%) of isolates, while *fnbB* was rare (16.00%; 95.00% CI: 9.40 - 26.20%; Table 2). Duplex PCR targeting *fib* and *clfB* (Fig. 1) and multiplex PCR for *fnbA*, *fnbB*, and *clfA* (Fig. 2) confirmed these findings.

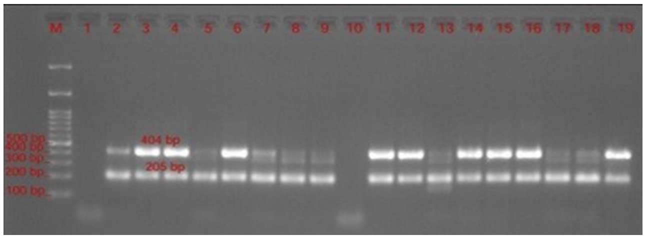

Fig. 1. Agarose gel electrophoresis of duplex PCR amplification targeting *cflB* and *fib* genes in selected isolates. Lane M: 100 bp molecular size marker; Lane 1: Negative control; Lanes 2-19: Representative isolates of this study.

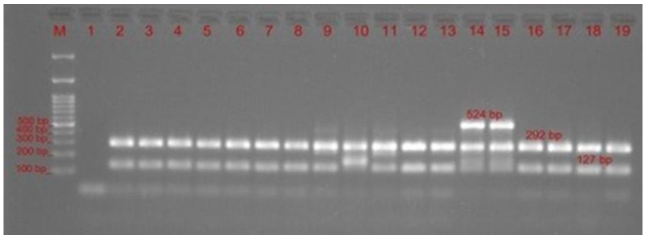

Fig. 2. Agarose gel electrophoresis of multiplex PCR amplification targeting *fnbA*, *fnbB*, and *clfA* genes in selected isolates. Lane M: 100 bp molecular size marker; Lane 1: Negative control; Lanes 2-19: Representative isolates of this study.

Site-specific gene distribution. Table 3 summarizes the distribution of adhesion-related genes across isolates. The *fnbA*, *clfA*, and *clfB* genes were present in 100% (95.00% CI: 81.60 - 100%) of rectal isolates (15/15), 97.20% (95.00% CI: 85.80 - 99.50%) of nasal isolates (35/36), and 100% (95.00% CI: 88.70 - 100%) of oral isolates (30/30). However, *fnbB* gene was absent in rectal isolates, but identified in 19.40% (95.00% CI: 9.40 -35.90%) of nasal

Table 3. Distribution of adhesion-related genes across isolates.

Genes	Rectal (%)	Nasal (%)	Oral (%)
fnbA	100 (15/15; 95.00% CI: 81.60 - 100%)	97.20 (35/36; 95.00% CI: 85.80 - 99.50%)	100 (30/30; 95.00% CI: 88.70 -100%)
clfA	100 (15/15; 95.00% CI: 81.60 - 100%)	97.20 (35/36; 95% CI: 85.80 - 99.50%)	100 (30/30; 95.00% CI: 88.70 - 100%)
clfB	100 (15/15; 95.00% CI: 81.60 - 100%)	97.20 (35/36; 95.00% CI: 85.80 - 99.50%)	100 (30/30; 95.00% CI: 88.70 - 100%)
fnbB	0.00 (0/15; 95.00% CI: 0.00 - 18.40%)	19.40 (7/36; 95.00% CI: 9.40 - 35.90%)	20.0 (6/30; 95.00% CI: 9.60 - 37.30%)

CI: Confidence interval.

(7/36) and 20.00% (95.00% CI: 9.60 - 37.30%) of oral isolates (6/30). Two oral and three nasal isolates harbored all five tested genes (*fnbA*, *fnbB*, *clfA*, *clfB*, and *fib*). Rectal isolates lacked *fnbB*, but universally carried *fnbA*, *clfA*, and *clfB*.

Discussion

The findings of this study underscored the significant prevalence of biofilm-associated genes, particularly *clfA*, *clfB*, and *fnbA*, in *S. aureus* isolates derived from dogs. These genes are crucial for bacterial adhesion and biofilm formation, which are key factors in the pathogenicity of *S. aureus*.

The distribution of *S. aureus* genes across various anatomical sites (nasal, oral, and rectal) highlighted the bacterium remarkable adaptive capabilities. Research indicates that *S. aureus* can colonize multiple sites, with a particularly high prevalence in the nasal cavity, serving as a primary reservoir for this pathogen. A study found that *S. aureus* was detected in 67.90% of nasal swabs compared to the 42.00% in rectal samples.[25]

The genetic diversity and virulence factors of *S. aureus* vary by anatomical site, suggesting that the bacterium adjusts its gene expression according to the different environments. Nasal carriers often harbor virulence genes associated with severe infections, while oral and rectal sites may exhibit different profiles of resistance and virulence.[26] This adaptability enables *S. aureus* to thrive in diverse environments and evade host immune responses.

The presence of specific genes (*fnbA*, *clfA*, and *clfB*) in rectal isolates suggests a strong association with gastrointestinal colonization. These genes encode proteins that facilitate adhesion to host tissues, being essential for colonization and persistence.[27,28] The absence of *fnbB* in rectal isolates reflects selective niche adaptation, as different adhesion mechanisms may be advantageous in the gastrointestinal environment.[29]

Identifying highly virulent *S. aureus* strains harboring multiple biofilm-related genes in nasal and oral samples raises concerns about zoonotic transmission. Biofilm formation enhances bacterial adhesion, persistence, and resistance to host immune responses.[30] The presence of biofilm-related genes in these strains increases the risk of transmission between species, complicating treatment and control measures.[31,32] The detection of virulent strains underscores the need for monitoring and controlling *S. aureus* in both human and animal populations.[33]

These findings align with prior research emphasize the significance of biofilm-related genes in both human and veterinary *S. aureus* isolates. Biofilm formation is a critical virulence factor, enhancing persistence and antimicrobial resistance.[34] In veterinary contexts, genes, such as *clfA*, *clfB*, and *fnbA*, contribute to biofilm production and increased virulence, suggesting a shared pathogenic potential with human strains.[35] The genetic similarities between human and animal isolates highlight the risk of zoonotic transmission.[36]

The prevalence of adhesion-related genes *clfA*, *clfB*, and *fnbA* in *S. aureus* is crucial for adhesion and infection processes. A meta-analysis revealed that *clfB* had a prevalence of 85.40%, while *fnbA* was found in 68.90% of isolates, underscoring their role in pathogenicity.[37] The *clfA* enhances bacterial adhesion under mechanical stress, while *fnbA* and *fnbB* contribute to host tissue adhesion and infection severity.[28,29] The high prevalence of these genes makes them potential therapeutic targets.

The variable presence of *fnbB* is strain-dependent and influenced by host and environmental factors. Some strains express this gene, while others do not.[38] External stimuli, such as antibiotic exposure, can induce the expression of fibronectin-binding proteins, including *fnbB*.[39,40] This variability highlights *fnbB* role in *S. aureus* adaptability and pathogenicity.

The strong association between nasal carriage and biofilm formation supports the hypothesis that nasal isolates serve as reservoirs for transmission.[41] Nasal carriage strains share genetic similarities with clinical isolates, suggesting their role in the spread of virulent strains.[42] These strains possess enhanced biofilm-forming capabilities, aiding survival, persistence, and antibiotic resistance.[43,44] This highlights the importance of nasal isolates in *S. aureus* epidemiology.

Despite valuable insights, this study had limitations. The absence of functional biofilm assays prevented direct confirmation of biofilm-forming capacity. Such assays are crucial for accurately assessing of biofilm production and structure, being essential for understanding *S. aureus* pathogenicity.[45] Indirect methods may underestimate biofilm formation, as some strains exhibit variability under different conditions.[46] The lack of comprehensive biofilm characterization may hinder effective treatment strategies against biofilm-associated infections.[47] Future research should incorporate functional assays to enhance the understanding of *S. aureus* biofilms and their clinical implications.

This study revealed the widespread prevalence of biofilm-associated genes (*clfA*, *clfB*, and *fnbA*) in *S. aureus* isolates from dogs in Ilam, Iran, underscoring their role in bacterial adhesion, colonization, and zoonotic potential. The near-ubiquitous presence of these genes (98.80%) contrasted with the niche-specific absence of *fnbB* in rectal isolates, highlighting adaptive strategies for gastro-intestinal persistence. Nasal and oral isolates harboring multiple virulence genes pose significant zoonotic risks, being exacerbated by biofilm-mediated antibiotic resistance and immune evasion. While genetic screening provides critical insights, the absence of functional biofilm assays limits direct pathogenicity correlations. Future studies should integrate phenotypic assays to validate biofilm formation and explore targeted interventions against adhesion mechanisms.

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Conflict of interest

The authors declare no conflict of interest.

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