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Detection of Biofilm-Associated icaA and icaD Genes in *Staphylococcus aureus* Isolated from Ovine and Caprine Mastitis in Diyala Governorate, Iraq

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Abstract **Background:** One of the primary causes of chronic mastitis in small ruminants globally is infection by *Staphylococcus aureus* which has biofilm formation in which helps it survive and resist antimicrobial treatment. **Objectives:** The purpose of this study was to assess the frequency of *S. aureus* in clinical cases of mastitis and examine the molecular frequency of the biofilm-related genes icaA and icaD in isolates obtained from infected mastitis sheep and goats in Diyala Governorate, Iraq. **Methods:** A cross-sectional study was conducted involving 100 mastitic milk samples (50 from goats and 50 from sheep). The samples were cultured on different selective media (Blood Agar, Mannitol Salt Agar and Baird-Parker Agar). The presumptive *S. aureus* isolates were confirmed via phenotypic methods (catalase and coagulase tests) and the VITEK-2 Compact system. After genomic DNA was extracted from the confirmed isolates, the icaA (151 bp) and icaD (211 bp) genes were analyzed using a polymerase chain reaction (PCR). **Results:** The total frequency of *S. aureus* was recorded as 69% (69/100). There was a statistically significant higher frequency ($\chi^2 = 6.73$, $p = 0.0094$) in sheep (82%, 41/50) than in goats (56%, 28/50). There was no statistically significant difference in icaA and icaD gene carriage between sheep and goats as all 69 isolates (100%) from the two species possessed both icaA and icaD genes. **Conclusion:** The investigation uncovers the existence of the severe impact of *S. aureus* mastitis, especially concerning sheep. The icaAD gene's omnipresence in all isolates indicates that the ability to form biofilms is most likely a conserved and important facet of virulence in *S. aureus* strains that are in circulation, increasing the difficulty of mastitis management and treatment in small ruminant herds.

Key Words *Staphylococcus aureus*, Mastitis, icaA, icaD, Biofilm-Associated Genes, Sheep and Goats

INTRODUCTION

Mastitis refers to inflammation of the mammary gland and economically, it is one of the most damaging diseases that affect dairy sheep and goats around the world. The impact on the economy is due to the loss of milk quantity and quality, increased expenses on veterinary services, the need to cull the affected animals and loss of the ability to trade internationally [1,2]. *Staphylococcus aureus* is one of the numerous bacterial pathogens associated with mastitis disease in small ruminants and is a major cause of both clinical and subclinical forms of mastitis [3]. This Gram-positive coccus is especially problematic in causing persistent recurrent intramammary infections that are extremely difficult to resolve with antibiotics, which creates a chronic problem for the entire herd [4,5].

Several factors contribute to the ability of *S. aureus* to persist within mammary tissue, the most important being its ability to develop biofilms [6]. Biofilms are highly organized communities of bacteria surrounded by a self-produced extracellular matrix composed of polysaccharides, proteins and extracellular DNA [7]. This matrix promotes adhesion to living (mammary epithelium), as well as non-living (milking machinery, implants) surfaces, pose a significant barrier to host immune defenses like phagocytosis and complement-mediated lysis and tremendously increases bacterial tolerance to antimicrobial agents, often by multiple logs [8,9]. Consequently, infections associated with biofilms are extremely hard to eliminate and are a significant cause of the chronicity and failure of treatment of mastitis.

The icaADBC (intercellular adhesion) operon controls PIA (Polysaccharide Intercellular Adhesin) or in other words, biofilm matrix polysaccharide, in *S. aureus*. icaA encodes for an enzyme called N-acetylglucosaminyltransferase that is located in the membrane and is able to polymerize PIA from

UDP-N-acetylglucosamine. In addition, icaD is important for the stabilization of IcaA and for the enhancement of the catalytic activity of IcaA, to an extent that is considered dramatic [10,11]. For this reason, the presence of icaA and icaD together is a significant predictor of biofilms. However, with regards to animal mastitis, there is extensive geographical variation of these virulence genes in *S. aureus*, depending on the type of bacterial strain, the structure of a particular farm and the usage of antibiotics [12].

The contribution of small ruminant's dairy production in Iraq and the Middle East as a part of the agricultural economy and food security is significant; however, there is a paucity of information regarding the molecular epidemiology of mastitis pathogens and more specifically, the [biofilm related] virulence factors [biofilm genes]. There are a few regional studies but they report varying prevalence, for example, in studies conducted in Iran, there was an 80.9% prevalence of icaA in bovine *S. aureus* and in studies carried in Egypt, icaA and icaD were found in 48 and 52% of the isolates, respectively [13,14]. In Iraq, the literature is very little and fragmented. Such gaps hinder the implementation of effective and focused control measures for mastitis.

The current study was, therefore, designed with two main goals; first, to assess the prevalence of *S. aureus* in clinical cases of mastitis in sheep and goats in the Diyala Governorate of Iraq and second, to conduct a molecular characterization of the isolates for the biofilm-forming genes icaA and icaD using PCR. The results will help to understand the local epidemiology of this key pathogen and the possible impact of biofilm in the persistence of mastitis in the study area [15].

METHODS

Study Design, Area and Sample Collection

A cross-sectional study was conducted from January to June 2023 in the eastern Iraqi Diyala Governorate on privately owned, semi-intensive dairy farms. Since the focus was on farms with a history of clinical mastitis, convenience sampling was used. From 50 sheep and 50 goats, 100 individual mammary quarter milk samples were aseptically collected. Clinical signs of mastitis included udder signs of swelling, heat, pain on palpation and milk abnormalities such as the presence of clots, flakes, discolored or watery secretion.

Before sampling, the orifice of the teat was cleaned with a 70% ethanol wipe and allowed to dry. To prevent contamination from the teat canal, the first three streams of milk were discarded. Approximately 10 ml of milk was collected into 15 ml conical tubes (Greiner Bio-One, Austria) which were sterile and labeled. The samples were placed in a cooler with ice packs (4°C) to remain cold and were transported to the Microbiology Laboratory of the College of Veterinary Medicine, University of Diyala within 4 hours of collection to be processed.

Bacteriological Isolation and Phenotypic Identification

Isolation streaking was performed for each milk sample (10 µL) for three selective and differential media via sterile bacteriological loop: 5% defibrinated sheep Blood Agar (BA), Mannitol Salt Agar (MSA) which has 7.5% NaCl and phenol red as a pH indicator and Baird-Parker Agar (BPA) with egg yolk tellurite emulsion (all media from HiMedia Laboratories, India). The plates were incubated at 37°C for 24-48 hours and analyzed for their colonial morphology.

Presumptive identifications of *S. aureus* were made by observing specific growth behaviours: Production of β-hemolysis (clear zones in the vicinity of colonies) on BA, acid production via mannitol fermentation resulting in a color shift on MSA to yellow and the surrounding colonies turned yellow (pH decrease) and a BPA black halo (lecithinase activity) (shiny colonies) opaque [16]. From each sample that exhibited growth of *S. aureus*, a single, isolated colony of each morphotype was subcultured on Nutrient Agar (HiMedia) to acquire pure isolates for additional examinations.

Standard biochemical tests were performed on pure cultures (such as catalase testing with 3% hydrogen peroxide (H_2O_2), Gram staining and tube coagulase assays). In the coagulase testing, 0.5 mL of rabbit plasma (Sigma-Aldrich, USA) was emulsified with one loop of bacteria in a small test tube and incubated in a 37°C water bath. If a clot formed in 2-4 hours, the test was classified positive. Identification of all phenotypically identified *S. aureus* isolates was done by the automated VITEK® 2 Compact (bioMérieux, France) with the GN (Gram-Negative) and GP (Gram-Positive) identification cards, following the manufacturer's guidance in accordance with the standard procedures. The system offers probability-based identification with confidence levels.

Molecular Detection of icaA and icaD Genes

DNA Extraction: Using the Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., Taiwan), genomic DNA was extracted from each VITEK-confirmed *S. aureus* isolate's single, fresh colony. The manufacturer's protocol for Gram-positive bacteria was followed and 50 µL was used for final DNA elution. The DNA concentration and purity (A260/A280 ratio) were analyzed using the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). All DNA samples were diluted to approximately 50 ng/µL and transferred to -20oC until PCR was performed.

PCR Amplification

Using multiplex PCR, the icaA and icaD genes were amplified with the use of gene-specific primers (see Table 1 for sequences and sizes of the amplicons). The South Korean company, Macrogen Inc., supplied the primers.

PCR reactions were completed with a final volume of 25 µL. Mixtures were composed of 12.5 µL of EmeraldAmp® MAX PCR Master Mix (2X Premix, includes Taq DNA polymerase, dNTPs, $MgCl_2$ and buffer; Takara Bio Inc., Japan), 1 µL of each of the forward and reverse primers (10 pmol/µL) for both icaA and icaD, 3 µL of DNA template (approx. 150 ng) and the rest volume with nuclease-free water. PCR cycling was performed with T100™ Thermal Cycler (Bio-Rad Laboratories, USA) at

Table 1: Oligonucleotide Primers Used for PCR Detection of icaA and icaD Genes in *Staphylococcus aureus*

Target Gene	Primer Sequence (5' → 3')	Amplicon Size (bp)	Reference
icaA	Forward: ACACTTGCTGGCGCAGTCAA Reverse: TCTGGAACCAACATCCAACA	151	Fowler *et al.* [19]
icaD	Forward: ATGGTCAAGCCCAGACAGAG Reverse: AGTATTTTCAATGTTTAAAGCAA	211	Arciola *et al.* [20]

Table 2: Prevalence of *Staphylococcus aureus* isolated from Mastitic Milk of Sheep and Goats in Diyala, Iraq

Animal Species	Number of Samples	*S. aureus* Positive (n (%))	95% Confidence Interval for %	Other Bacteria (n (%))
Sheep	50	41 (82.0%)	68.6-91.4%	9 (18.0%)
Goats	50	28 (56.0%)	41.3-70.0%	22 (44.0%)
Total	100	69 (69.0%)	59.0-77.8%	31 (31.0%)

Statistical comparison, $\chi^2 = 6.73$, df = 1, p = 0.0094

Table 3: Tests Confirming the Phenotypic Characteristics of *Staphylococcus aureus* Isolates from Sheep and Goats

Animal Source	Number of Isolates	Catalase Positive (n (%))	Tube Coagulase Positive (n (%))	VITEK-2 Confirmation (n (%))
Sheep	41	41 (100%)	41 (100%)	41 (100%)
Goats	28	28 (100%)	28 (100%)	28 (100%)
Total	69	69 (100%)	69 (100%)	69 (100%)

95°C for 5 min for initial denaturation, followed by 35 cycles at 95°C for 30 sec for denaturation, 55°C for 30 sec for annealing and 72°C for 30 sec for extension and then a final extension for 7 min at 72°C. *S. aureus* ATCC 25923 (which is a known biofilm producer) was included as a positive control. A mixture of reactions which contained nuclease-free water as a substitute for the DNA template served as the no template control (NTC) for examining contaminants.

Agarose Gel Electrophoresis

Electrophoresis of the PCR amplicons was done over 1.5% (w/v) agarose gel (Vivantis Technologies, Malaysia) which was made with 1X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and stained with 5 µL of SafeView™ Classic nucleic acid stain (Applied Biological Materials Inc., Canada) along with 100 bp DNA Ladder (GeneDireX, Taiwan) as the standard. Electrophoresis was done at 100 V for 45 minutes using 1X TAE running buffer. Visualization and photography of the gel was done with Gel Doc™ XR+ system using Image Lab™ Software (Bio-Rad) under UV light.

Statistical Analysis

All the data was compiled in a Microsoft Excel spreadsheet and analyzed using IBM SPSS Statistics for Windows, Version 28.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were done for the prevalence data. Chi-square (χ^2) test of independence was done for comparison of prevalence of *S. aureus* in sheep and goats. When, the expected cell counts were less than 5, Fisher's exact test was done. The modified Wald method was used for the calculation of 95% Confidence Intervals (CI) for the proportions (prevalence and gene frequency). All the analyses were conducted at 95% confidence interval and significance level of $p<0.05$.

RESULTS

Prevalence and Phenotypic Characterization of *S. aureus*

About 69 out of the 100 cultured samples from mastitic milk were confirmed to be Staph. aureus. With regards to the analysis on host species, a notable difference emerged. Staph. aureus had a prevalence of 82% (41 out of 50) for the samples from sheep and 56% (28 out of 50) for those from goats. The analysis on host species showed a statistically significant difference ($\chi^2 = 6.73$, df = 1, p = 0.0094). The other 31 samples had growth of other, primarily coagulase-negative staphylococci (CoNS), streptococci and entero bacteria, which were outside the scope of this study. Further details on the prevalence can be found in Table 2.

All 69 *S. aureus* isolates displayed the same classic phenotypic characteristics on the selective media. When cultured on Sheep Blood Agar, *S. aureus* isolates produced colonies that were circular, smooth, convex and golden yellow, exhibiting a complete (β) zone of hemolysis. In the case of all isolates cultured on Mannitol Salt Agar, growth (indicating the ability to tolerate the salt) and a distinct yellow color change were both present in the colony and the medium surrounding the colony, an indication of mannitol fermentation. All isolates on Baird-Parker Agar produced colonies that were black, convex with a slight halo and opaque white, indicating lecithinase activity. All isolates were dark field Gram-positive cocci and were observed in typical grape-like clusters. All isolates exhibited immediate catalase activity (bubbling in the presence of 3% H_2O_2) and demonstrated clotting in the tube coagulase test in 4 hours. The identification of all 69 isolates as *Staphylococcus aureus* by the VITEK-2 Compact system was classified as "Excellent" or "Very Good" confidence and these test results are summarized in Table 3.

Molecular Detection of icaA and icaD Genes

PCR analysis of the 69 *S. aureus* isolates yielded amplifications of the target genes. Each isolate for the icaA gene had a clearly defined band of the expected size (151 bp). In the same manner, all isolates of the icaD gene had a band of 211 bp. No amplifications were observed on the no-template control lanes. Thus, the prevalence for icaA and icaD genes was 100% (69/69). The proportion for this prevalence at a 95% confidence interval was between 94.8 and 100.0%. No statistically significant difference was observed regarding the prevalence of these genes

Table 4: Prevalence of Biofilm-Associated icaA and icaD Genes in *Staphylococcus aureus* Isolates

Group (Host)	Total Isolates	*icaA* Positive, n (%) [95% CI]	*icaD* Positive, n (%) [95% CI]
Sheep	41	41 (100%) [91.4-100%]	41 (100%) [91.4-100%]
Goats	28	28 (100%) [87.7-100%]	28 (100%) [87.7-100%]
Total	69	69 (100%) [94.8-100%]	69 (100%) [94.8-100%]

between isolates emerging from sheep and those from goats, as both groups had 100% positivity. Molecular results are detailed in Table 4.

Staphylococcus aureus mastitis in small ruminants in the Diyala Governorate of Iraq was examined as part of this study. The isolation from clinical mastitis *S. aureus* averages 69%, which suggests that this pathogen is a parasite of significant clinical importance and is a major detriment to the health and productivity of the herd. The high level of isolation from mastitis is more significant than the averages of 20-40% that have been reported from multiple studies in European countries [17,18]. In a recently published study from Karbala, Iraq, the average was 21.33% [21]. However, this is a common finding in studies in the Middle East and North Africa regions [22]. The reported prevalence of *S. aureus* is often surrounded by controversy and it may be a result of variations in the definitions of clinical and subclinical mastitis, herd management and biosecurity practices and levels of prior antimicrobial use.

One of the most notable results was a clear difference in the occurrence rate of *S. aureus* in sheep (82%) and in goats (56%). This difference in occurrence rate seems to be species specific which is consistent with previous studies [23]. This species-specific difference can be explained by a number of reasons. Some anatomical considerations include a shorter and wider sheep teat canal, which can be more facilitative of bacterial entry [24]. Other possible reasons can also be the differences in the innate immunity of the milk, such as differences in concentrations of immune modulatory proteins like lysozyme and lactoferrin and/or in the expression of pattern recognition receptors in mammary epithelial cells [25]. This observed difference can also be a result of specific management factors which include differences in the milking technique, bedding materials and the overall hygiene of the flock.

The most remarkable and important finding of this study is the universal carriage (100%) of both icaA and icaD biofilm-forming genes in all *S. aureus* isolates, regardless of the host species of origin. The almost clonal biofilm genetic potential is remarkable and no other study has come close. For instance, in Turkey, 15.9 and 43.2% of mastitis staphylococci (icaA and icaD, respectively) were reported to have these genes [26]. The icaA and icaD genes have been reported to be present in 40 to 70% of the isolates responsible for mastitis in cattle in studies conducted in Brazil and China [27]. A meta-analysis conducted in 2022 estimated the global prevalence of icaA in *S. aureus* strains of animal origin to be 58.5% [28]. The 100% prevalence in our study is only comparable to a small number of studies; one study presents this data in Basra, Iraq [29] and another study includes human clinical isolates [30]. This suggests that the region has a unique epidemiological situation.

The global occurrence of the icaAD genes is indicative of strong selection pressure within the biosphere studied that favors the survival and dominance of biofilm producing *S. aureus* strains. Possible causes are: (1) Anecdotal and/or widespread antimicrobial usage: In many agricultural systems, antibiotics are utilized prophylactically or metaphyletically, without prior sensitivity testing. Biofilm formation is an established tolerance mechanism against a wide variety of antibiotics. Strains that are able to form biofilms would thus have a survival advantage and, consequently, become enriched in the population, (2) Chronic untreated infections: The inability to access veterinary care or perform diagnostics may lead to lasting subclinical or mild clinical infections. Biofilms allow bacteria to enter a dormant or slowly growing state within the mammary gland, evading immune clearance and short courses of antibiotics, thus fostering chronic infection and (3) Persistent environmental reservoirs: Biofilms can form on milking equipment, water troughs or animal housing, serving as an enduring source of infection. Strains that are adapted to life in biofilms in the environment are more likely to establish infection in the host.

This finding has important practical implications. As shown, the genetic potential for biofilm formation, due to elements icaA and icaD, is a prerequisite for robust biofilm production that is PIA dependent. Although the phenotypic expression may be clonal and there may be additional control mechanisms (e.g., repressor icaR, alternative sigma factor sigB and environmental conditions such as anaerobiosis or low sub-inhibitory concentrations of antibiotics) the universal genetic background shows that there is a high risk for biofilm-associated chronic mastitis in the area. Biofilms are a direct cause of the treatment failure, high somatic cell counts and recurrent bacterial shedding, as well as the potential zoonotic transmission of the pathogen via contaminated milk [31,32].

The absence of additional phenotypic biofilm assays (i.e., microtiter plates, crystal violet and the Congo red agar test) that would allow the authors to correlate the genotype of the biofilm-forming organisms to their actual phenotypic biofilm-forming capacity is a limitation of this study. Future research should include such phenotypic characterizations [33-35]. In addition, the combination of some molecular typing methods such as Multi-Locus Sequence Typing (MLST) and /or spa- typing methods, along with antimicrobial susceptibility testing will be important to establish whether the high prevalence and universal gene carriage is due to the dissemination of a single, successful clonal lineage or is reflecting the presence of multiple co-existing competing lineages [36,37].

CONCLUSIONS

There is a considerable presence of clinical mastitis caused by *Staphylococcus aureus* cases in small ruminants in the Diyala region of Iraq. There is a larger burden in sheep than in goats. The most significant finding is that all isolates in the region of study have a presence of biofilm-positive icaA

and icaD genes. This means that all local *S. aureus* populations have the ability to form biofilms; this is a potentially important virulence factor. Biofilms are a significant obstacle in the management of mastitis because they are extremely difficult to eradicate.

In this region, mastitis management strategies will need to move away from conventional antibiotic treatments. A multifaceted approach is warranted. This may include, (1) Adherence to milking hygiene practices (disinfection of teats and cleaning and maintenance of milking apparatuses and gear), (2) The use of antiseptic and antibiofilm agents, (3) Responsible, evidence-based use of antibiotics in order to minimize the control of the infectious agent and (4) removing infected animals that do not respond to treatment in order to reduce the infectious cycle. The maintenance of these strategies will enable the identification of antimicrobial resistance and virulence factors.

Institutional Review Board Statement

The study protocol was reviewed and approved by the Research Ethics Committee of the College of Veterinary Medicine, University of Diyala (Reference No. CVM-REC/2022-15). Verbal informed consent was obtained from all animal owners prior to sample collection.

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Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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