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Phenotype detection of biofilm from *Serratia* and *Staphylococcus aureus* isolated from clinical and subclinical mastitis of bovine in Diyala governorate

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Persistent intramammary infections caused by biofilm-producing bacterial pathogens represent a critical challenge for dairy cattle health and veterinary management. This research aimed to isolate *Staphylococcus aureus* and *Serratia marcescens* from clinical and subclinical bovine mastitis cases in Baqubah city within the Diyala governorate, evaluate their phenotypic and genotypic biofilm-forming profiles, and determine their susceptibility to *Capsicum frutescens* crude ethanolic extract. Out of 65 milk samples collected from lactating dairy cows exhibiting evident symptoms of mastitis, bacterial pathogens were successfully recovered from 58 samples, representing a 89.23 % isolation success rate, whereas 7 samples exhibited no pathogen development. Based on standard morphological characterization, conventional biochemical profiling, and definitive verification via the automated VITEK 2 identification system, 10 isolates were classified as *Staphylococcus aureus*, constituting 15.38 % of the total specimens, and 4 isolates were identified as *Serratia marcescens*, accounting for 6.15 % of the evaluated sample pool. Phenotypic quantification of biofilm production using the microtiter plate assay method revealed that among the 10 staphylococcal strains, 3 isolates (30 %) developed weak biofilms, 3 isolates (30 %) generated moderate biofilms, and 4 isolates (40 %) established strong, highly adherent biofilm architectures. For the 4 *Serratia marcescens* environmental-opportunistic strains, 1 isolate (25 %) exhibited moderate biofilm layers, while the remaining 3 isolates (75 %) demonstrated robust, strong biofilm matrix development. Molecular characterization via the Polymerase Chain Reaction verified the presence of the multidrug efflux pump gene *norA* at 391 bp in ciprofloxacin-resistant staphylococcal strains and the biofilm-associated genetic marker *bsmA* at 298 bp in *Serratia marcescens* strains. Evaluation of the alternative antibacterial efficacy of the crude *Capsicum frutescens* extract using the agar well diffusion assay at targeted concentrations of 500 and 1000 µg/mL demonstrated profound, dose-dependent inhibitory activity against both persistent bacterial pathogens, with the measured diameters of the inhibition zones expanding proportionally in direct correlation to the rising concentration of the agent. These findings confirm that biofilm matrix production directly accelerates chronic disease development and long-term bovine mastitis persistence within the region, establishing the crucial practical utility of implementing alternative biofilm-targeted control programs and natural phytotherapeutic alternatives in dairy herds.

Keywords: *Staphylococcus aureus*, *Serratia marcescens*, bovine mastitis, biofilm formation, *Capsicum frutescens*.

Фенотипове визначення біоплівок *Serratia* та *Staphylococcus aureus*, виділених від великої рогатої худоби з клінічним та субклінічним маститом у провінції Діяла

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Хронічні внутрішньовименні інфекції, спричинені бактеріальними патогенами, що утворюють біоплівки, становлять серйозну проблему для здоров'я великої рогатої худоби та ветеринарного менеджменту. Це дослідження було спрямоване на ізоляцію штамів *Staphylococcus aureus* та *Serratia marcescens* у випадках клінічного та субклінічного маститу корів у місті Бакуба в провінції Діяла, оцінку фенотипових та генотипових профілів утворення біоплівок, а також визначення їхньої чутливості до неочищеного етанольного екстракту *Capsicum frutescens*. Із 65 зразків молока, відібраних від лактуючих молочних корів із очевидними симптомами маститу, бактеріальні патогени були успішно виділені з 58 зразків, що становить 89,23 %, тоді як 7 зразків не продемонстрували росту патогенів. На основі стандартної морфологічної характеристики, загальноприйнятого біохімічного профілювання та остаточного підтвердження за допомогою автоматизованої системи ідентифікації VITEK 2 10 ізолятів було класифіковано як *Staphylococcus aureus*, що становить 15,38 % від загальної кількості зразків, а 4 ізоляти було ідентифіковано як *Serratia marcescens*, що становить 6,15 % досліджуваних зразків. Фенотипове кількісне визначення утворення біоплівок за допомогою методу мікротитраційного планшета показало, що серед 10 стафілококових штамів 3 ізоляти (30 %) утворювали слабкі біоплівки, 3 ізоляти (30 %) продемонстрували помірне утворення біоплівок, і 4 ізоляти (40 %) сформували сильні, високоадгезивні архітектури біоплівок. Серед 4 умовно-патогенних штамів *Serratia marcescens* 1 ізолят (25 %) виявив помірне утворення біоплівок, тоді як інші 3 ізоляти (75 %) продемонстрували інтенсивний розвиток матриксу біоплівки. Молекулярна характеристика за допомогою полімеразної ланцюгової реакції підтвердила наявність гена мультирезистентного ефлюксного насоса *norA* розміром 391 пар нуклеотидів у ципрофлоксацинорезистентних стафілококових штамів та генетичного маркера біоплівок *bsmA* розміром 298 пар нуклеотидів у штамів *Serratia marcescens*. Оцінка антибактеріальної ефективності неочищеного екстракту *Capsicum frutescens* методом дифузії в агарі у цільових концентраціях 500 та 1000 мкг/мл продемонструвала виражену дозозалежну інгібуючу активність проти обох хронічних бактеріальних патогенів, при цьому виміряні діаметри зон пригнічення росту розширювалися пропорційно до збільшення концентрації екстракту. Отримані результати підтверджують, що утворення матриксу біоплівки безпосередньо сприяє розвитку хронічного захворювання та тривалому перебігу маститу великої рогатої худоби в регіоні, що вказує на важливу практичну доцільність впровадження альтернативних програм контролю, спрямованих на біоплівки, а також на застосування природних фітотерапевтичних засобів у молочних господарствах.

Ключові слова: *Staphylococcus aureus*, *Serratia marcescens*, мастит великої рогатої худоби, утворення біоплівок, *Capsicum frutescens*.

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Introduction

Bacterial pathogens exist throughout the dairy cow environment which creates a danger for mammary gland health [1]. The collection of mastitis-positive cow milk samples revealed more than 130 distinct microorganisms which consisted mainly of bacterial species. The primary pathogens consist of contagious bacteria which include *Staphylococcus aureus* and *Streptococcus agalactiae* and environmental bacteria which consist of coliforms and various streptococcal species found in natural surroundings [2].

The mastitis condition develops because of three particular types of *Serratia* bacteria which include *S. marcescens*, *S. rubidaea* and *S. liquefaciens*. The dry period stands as the main stage which leads to infection, secondly the lactation phase. The source of the outbreak remained unidentified during the *Serratia* mastitis outbreak which probably resulted from bacterial growth inside teat dip containers and bedding materials. The occurrence of mastitis becomes more likely when cows experience unclean environments and when their teat ends sustain damage. The environmental microorganism *Serratia* becomes transmitted through the milking machine according to reference [3].

Mastitis is a multifactorial and clinically complex inflammatory condition affecting the mammary gland parenchyma. The disease develops from multiple damaging factors which include pathogenic microorganisms as the main cause and physical injuries and chemical exposures that trigger the disease. The teat and udder tissue must first experience damage before microbes can enter the gland to cause an infection.

Staphylococcus aureus represents one of the main pathogens because it infects numerous animal species while producing various infections throughout different body regions. The bacteria can colonize various surfaces because it possesses multiple virulence factors which include specific adhesion molecules and biofilm production capabilities on living and non-living surfaces [4].

Environmental pollutants now serve as the main factor which causes bovine mastitis through their impact on *Serratia* species that consist of *Serratia marcescens* and *Serratia liquefaciens* members. Scientists have proven that these organisms exist throughout every environmental space because they exist in water and soil and they can be found on plants and inside insects which distribute them [5].

The presence of *Serratia* spp. in dairy operations becomes evident through their detection in bedding materials and milking equipment and bovine fecal matter while bulk tank milk surveys show their existence in 1.3 % to 2 % of cases [6]. The bacteria form biofilms on production equipment which creates a major danger to milk quality because their enzymes persist through pasteurization and lead to milk spoilage during both processing and storage operations [7].

Bovine mastitis serves as the leading economic disease which threatens the global dairy sector because it triggers mammary gland inflammation in cows. The financial burden stems primarily from depressed milk yields, deterioration in milk composition, and elevated expenditures on veterinary interventions [8].

Veterinary clinicians diagnose the disease through two primary clinical patterns which it presents. Mastitis in its clinical form produces milk alterations which include color changes and floating clumps and solid masses, while the udder becomes swollen and red and develops touch sensitivity. The glandular tissue develops fibrotic tissue through a process of remodeling which occurs in severe or long-lasting cases [7, 9].

The clinical presentation of subclinical mastitis remains hidden because the disease does not show any visible symptoms, which makes it necessary to use diagnostic tools such as the California Mastitis Test (CMT) for detection. During mastitis, the inflammatory response leads to an influx of somatic cells into the milk, increasing its pH and often causing a change in color, which can range from yellowish to watery or even reddish in severe cases, indicating the extent of inflammation and tissue damage [9].

The aim of the study

The objectives of this study were to identify the prevalence of *Staphylococcus aureus* and *Serratia marcescens* in clinical and subclinical mastitis cases in Diyala governorate and to assess their biofilm-forming capabilities, which contribute significantly to the persistence and recalcitrance of these infections.

Materials and methods

Milk sample collection

Veterinary experts examined the cow's udder together with its teat to identify any clinical mastitis symptoms. The researchers conducted visual inspections and manual udders palpation of their study cows to detect clinical mastitis symptoms. The examination process focused on five essential inflammation signs which include redness and swelling and pain and hotness and functional loss, and also evaluated udder quarter dimensions and their texture. The milk underwent inspection to identify clinical mastitis indicators through its color and its texture and its clot formation characteristics.

Milk specimens were collected from sixty-five dairy cattle which showed clinical signs of mastitis throughout Diyala governorate while researchers used strict aseptic procedures to prevent any outside contamination during the sample collection process [3].

The California Mastitis Test (CMT) functioned as the first diagnostic tool which detected subclinical diseases while providing the benefit of instant testing at the cow's location. The assay is performed by admixing equivalent aliquots of milk from individual quarters with the supplied reagent within a compartmentalized four-well paddle. The reagent functions through detergent-based cell destruction which breaks down somatic cells to release their nuclear DNA while developing into a gel-like structure; bromocresol purple serves as the standard pH-based indicator to help users identify color differences [9].

The resultant degree of gelation provides a semi-quantitative approximation of the somatic cell count, thereby reflecting the severity of underlying udder

inflammation. Conversely, milk from unaffected glands – possessing a physiological pH near 6.6 – yields a mixture that typically persists in a liquid state with a characteristic purple-to-blue-gray coloration. However, in mastitic milk, the increased pH due to inflammation causes the indicator to turn a deeper purple, while a yellow color, though less common, can indicate an acidic shift often associated with acute coliform mastitis. The visual assessment of both gelling and color change allows for a semi-quantitative scoring of mastitis severity, enabling differentiation between healthy, subclinical, and clinical cases [9].

Researchers acquired 65 milk samples from cows which developed clinical mastitis symptoms during their lactation period in Baqubah city and its surrounding areas. The research team took milk samples from animal quarters which showed infection, but the animals had not received any form of antibiotic treatment in the past. The collection procedure adhered to strict aseptic protocols: the udder surface was cleansed with water, after which teats and teat orifices were swabbed with 70 % ethyl alcohol-soaked cotton wool and allowed to dry using fresh sterile gauze prior to milking out the sample.

Researchers collected 5–6 milliliters of milk from the infected quarter through sterile bottle collection after they removed the initial foremilk for bacteriological testing and they properly labeled the samples. The samples reached the Veterinary laboratory microbiology facility through quick transportation after staff members put them in ice containers which contained frozen ice packs. The samples which did not receive immediate processing were preserved in a 4°C refrigerator until scientists could begin their work (up to 48 hours).

Bacterial isolation and identification

Scientists used various media types to isolate different bacterial species from their samples. The research team used MacConkey agar and Mannitol salt agar as their standard media throughout their study. The laboratory analysis media were prepared using the standard procedures which recommended for this purpose [10].

Laboratory personnel used standard morphological and biochemical testing methods to identify bacteria during their isolation process according to the *Manual of Clinical Microbiology* [11]. In addition, VITEK 2 system was used to confirm each isolate.

Biofilm assay

The quantitative assessment of biofilm production was performed according to a modified microtiter plate protocol [12], with the following sequential steps:

1. Preparation of inoculum: Laboratory personnel extracted individual colonies from fresh agar plates to create a suspension which they combined with 5 mL brain heart infusion broth containing 2 % sucrose before incubating it at 37°C under aerobic conditions for 24 hours [13].

2. Plate inoculation: Laboratory personnel used 20 µL of bacterial suspension from each isolate which they adjusted to 0.5 McFarland opacity to fill individual wells in a 96-well microplate that contained 180 µL of BHI broth. The plate received an incubation period of 24 hours at 37°C through static incubation after it was covered following inoculation.

3. Washing: Following incubation, planktonic cells were eliminated by three successive rinses with physiological saline (0.9 % NaCl).

4. Fixation: Surface-attached biofilms were fixed by adding 200 µL of absolute methanol per well for 15 minutes, then air-dried for 30 minutes.

5. Staining: Biofilm biomass was visualized by adding 200 µL of 1 % crystal violet solution to each well for 15 minutes.

6. Destaining and quantification: Unbound dye was aspirated, wells were gently washed with sterile distilled water, and captured stain was eluted with 96 % ethanol. The automated microplate reader monitored optical density at 630 nm while following the classification standards listed in ***Table 1***.

Table 1

Classification criteria for bacterial biofilm adherence based on optical density values at 630 nm

Optical density	Biofilm-forming capacity
OD ≤ ODc	Non-adherent (Non-producer)
ODc < OD ≤ 2ODc	Weak producer
2ODc < OD ≤ 4ODc	Moderate producer
OD > 4ODc	Strong producer

Plant collection and extraction

Red hot peppers (*Capsicum frutescens*) which originated from Iraq were purchased at Baghdad markets before laboratory staff members performed two consecutive water rinses to eliminate dirt and foreign substances from the peppers. The process of drying fruits required the whole fruits to be cut into small pieces which then dried at room temperature until they reached stable weight. The dried pepper segments were subsequently pulverized into a homogeneous powder, which was then placed in a 500 mL borosilicate beaker.

The extraction process required 100 g of dried powder which was combined with 300 mL of absolute ethanol before being stirred with a magnetic stirrer at room temperature for seven days. The suspension underwent vacuum filtration at the end of extraction time before the clear filtrate was concentrated through room temperature evaporation to produce a thick brown substance which scientists saved for upcoming research [14].

Evaluation of antibacterial activity

The antibacterial activity of the prepared extract samples was evaluated using the agar well diffusion assay, with the following procedural steps:

1. Each bacterial isolate received 18 to 24 hours of growth in nutrient broth at 37°C before reaching the exponential growth phase.

2. Standardization: Laboratory personnel combined one microbial colony with 5 mL of sterile saline which they then adjusted to produce a 0.5 McFarland standard equivalent to ~1.5×10^8 CFU/mL.

3. Plate inoculation: The adjusted bacterial suspension was streaked across the entire surface of Mueller-Hinton agar using a sterile cotton swab; plates were allowed to rest undisturbed for ten minutes prior to well formation.

4. Sample loading: Laboratory personnel created 5 mm diameter wells in the agar, then they used a micropipette to distribute 0.1 mL of each extract sample into distinct wells.

5. The plates which had been loaded underwent an 18-hour incubation at 37°C before laboratory personnel measured and recorded the sizes of the inhibition zones which appeared.

Molecular detection of biofilm-associated genes

The existence of biofilm genes (*norA*) for *Staphylococcus aureus* and (*bsmA*) for *Serratia marcescens* were examined in two isolates for each bacteria. DNA extraction was carried out following a G-spin™ extraction kit (iNtRON, Korea). The primers employed for determining genes are documented in ***Table 2***.

Table 2

Oligonucleotide primer sequences employed for the detection of *norA* and *bsmA* biofilm-associated genes

Target gene	Primer	Sequence (5′ → 3′)	Size of amplicon (bp)
norA	F	TTTCATATGATCAATCCCCTTT	391
	R	ACCACCAAACGGCGATATAA	
bsmA	F	CGTTTGTCCGCACACTCAT	298
	R	TACAGGATCGCCTGGGAATA	

A total volume of 20 µl was used to establish the reaction mixtures. Specifically, 2 µl of DNA extracted from the isolates was added to 8 µl of the master mix (Bioneer, USA), 0.5 µl of magnesium chloride ($MgCl_2$), and 7.5 µl of deionized water (ddH_2O). After that, 2 µl of both primers was added to the resultant mixture and then placed in the thermocycler (Biometra, Germany) for 5 minutes at 94°C. PCR programs for detecting respective genes are represented in ***Table 3***.

Table 3

Optimized thermocycling protocols and conditions for targeted amplification of biofilm-associated determinants

Step	Temperature (°C)	Time	Cycles
Initial denaturation	94 °C	5 min	1
Denaturation	94 °C	30 sec	35
Annealing	55 °C	30 sec	35
Extension	72 °C	30 sec	35
Final extension	72 °C	5 min	1

The PCR products were stained with RedSafe™ (iNtRON Biotechnology, Korea), 1 % agarose gel (Merck, Darmstadt, Germany) electrophoresis, and photographed under UV light. Images were digitally captured using the Gel Doc apparatus.

Results and discussion

Field manifestations and California Mastitis Test profile

Clinical examination of the dairy cows revealed distinct pathological changes indicating active infections. Affected quarters exhibited notable local inflammation and structural swelling of the udder parenchyma, which signified typical clinical manifestations associated with bovine mammary gland disease (***Fig. 1***).

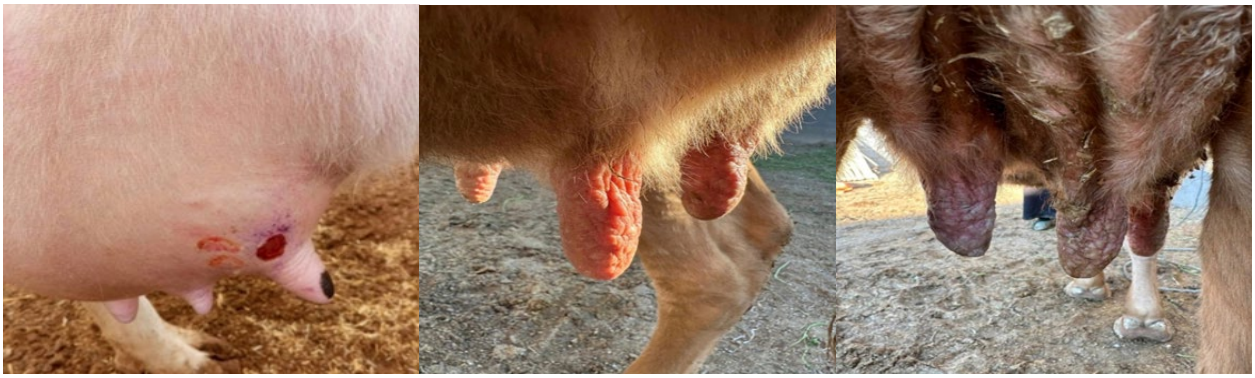


Fig. 1. Clinical bovine mastitis presenting with udder inflammation and swelling

Following the initial field visual evaluation, the California Mastitis Test was implemented for deeper diagnostic profiling. The CMT showed positive reactions with varying degrees of gel formation and precipitate in milk samples from the affected quarters, indicating significantly elevated somatic cell counts associated with both clinical and subclinical mastitis cases.

Isolation and characterization of bacteria

Isolation of *S. aureus*

A total of 65 samples were collected from cows which showed clinical symptoms of mastitis. The period from August to December 2025 saw the collection and testing of these samples. The research findings demonstrated that 58 isolates which represented 89.23 % of the samples showed pathogen development from cows with clinical mastitis, but 7 samples which accounted for 10.76 % exhibited no pathogen development.

The study obtained growth of *Staphylococcus aureus* in 10 samples which represented 15.38 % of the total samples. The growth patterns of *S. aureus* become evident through various media including Blood agar and Mannitol salt agar, and the process of microscopic analysis which involved Gram staining all 10 isolates. The cells appeared as Gram-positive cocci, mostly arranged in grape-like irregular clusters. The first step of identification involved using biochemical tests to identify all isolates through Catalase and Coagulase and DNAse testing. All these tests were positive for *S. aureus*.

Isolation of *Serratia marcescens*

Bacterial isolation was performed on 65 milk samples, yielding four isolates identified as *Serratia marcescens*, constituting 6.15 % of the total specimens examined. Upon cultivation under standard conditions (37 °C for 24 hours), distinct morphological phenotypes were observed across the various culture media employed. Through MacConkey agar testing, the bacteria showed their delayed or weak ability to ferment lactose, producing prominent red colonies primarily due to the unique feature where *S. marcescens* naturally produces its own pigment; these findings match the information which previous studies have already presented [15].

The light microscope revealed that the isolated bacteria showed rod-shaped structures while maintaining their Gram-negative cell wall characteristics. The complete biochemical analysis showed that *Serratia marcescens* exhibits the following characteristics: Gram-negative staining and rod-shaped structure and positive catalase test and negative oxidase test and lactose non-fermentation and positive motility and negative indole production and positive citrate test and yellow slant and yellow butt (A/A) in Triple Sugar Iron (TSI) medium and positive DNase test and negative urease test according to standard criteria [16].

VITEK-2 compact system analysis

For definitive verification, the isolates were subjected to the VITEK 2 automated identification system, which revealed perfect (100 %) congruence at the species level, supported by a probability index of 99 %. The analytical method produced confirmed taxonomic results which scientists could depend on with their highest degree of accuracy and trustworthiness.

Biofilm formation assay

Bacterial biofilms create persistent infections which veterinary professionals find difficult to manage. The research divides bacteria into two groups which include biofilm-producing bacteria and non-biofilm-producing bacteria by using the microtiter method for analysis. The method functions as the primary technique which detects biofilm formation through its frequent application in research.

Biofilm production in *S. aureus*

The ten *Staphylococcus* isolates underwent biofilm formation evaluation which resulted in their classification into three groups based on their biofilm production levels: weak, moderate, and strong. The distribution of biofilm phenotypes was as follows: three isolates (30 %) were classified as weak producers, three isolates (30 %) demonstrated moderate biofilm formation, and four isolates (40 %) exhibited strong biofilm-producing capability, as summarized in ***Table 4***.

The experimental variations in biomass density and the distinct gradients of crystal violet staining reflecting weak, moderate, and strong biofilm categories among the tested staphylococcal strains are visually illustrated in ***Figure 2***.

Table 4

Distribution and intensity of phenotypic biofilm production among individual *Staphylococcus aureus* bovine mastitis isolates

Isolate designation	Weak biofilm	Moderate biofilm	Strong biofilm
S. aureus Staph 1	+	–	–
S. aureus Staph 2	–	–	+
S. aureus Staph 3	–	–	+
S. aureus Staph 4	–	–	+
S. aureus Staph 5	–	+	–
S. aureus Staph 6	–	–	+
S. aureus Staph 7	+	–	–
S. aureus Staph 8	+	–	–
S. aureus Staph 9	–	+	–
S. aureus Staph 10	–	+	–

Note: «+» indicates the presence of a specific biofilm-forming phenotype; «–» indicates the absence of the corresponding biofilm-forming intensity.

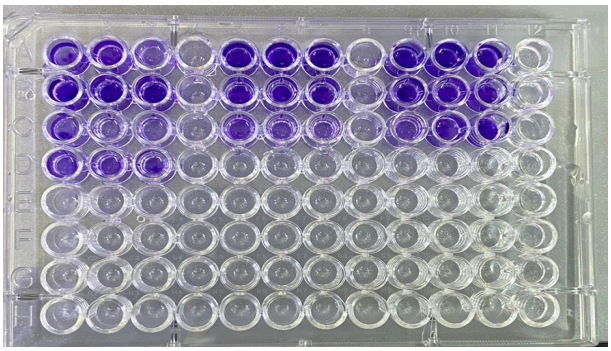


Fig. 2. Microtiter plate quantitative assay for *Staphylococcus aureus* isolates displaying distinct gradients of biofilm biomass production intensity

The observed variations in staining intensity visually reflect the total accumulated mass of the extracellular polymeric matrix. Well-developed, strong biofilm architectures retained a higher concentration of the crystal violet dye, resulting in deeper optical density values during subsequent spectrophotometric quantification.

Biofilm production in *S. marcescens*

Serratia marcescens has recently become a source of rising concern due to its tendency to form biofilms. The research study identified four *S. marcescens* isolates which demonstrated biofilm formation, as summarized in ***Table 5***.

Table 5

Distribution and intensity of phenotypic biofilm production among individual *Serratia marcescens* bovine mastitis isolates

Isolate designation	Weak biofilm	Moderate biofilm	Strong biofilm
S. marcescens Se.1	–	+	–
S. marcescens Se.2	–	–	+
S. marcescens Se.3	–	–	+
S. marcescens Se.4	–	–	+

Note: «+» indicates the presence of a specific biofilm-forming phenotype; «–» indicates the absence of the corresponding biofilm-forming intensity.

Biofilms exist as surface-attached microbial communities which produce an extracellular matrix that contains polysaccharides and proteins and nucleic acids and lipids and various other macromolecular substances and chemical compounds. The matrix depends on extracellular polysaccharides which perform multiple essential roles including surface and cellular adhesion and biofilm development and maintenance and cell defense against environmental threats and predators which include antimicrobial substances and immune system components. The experimental manifestations of these macro-structures across different intensities are illustrated in ***Figure 3***.

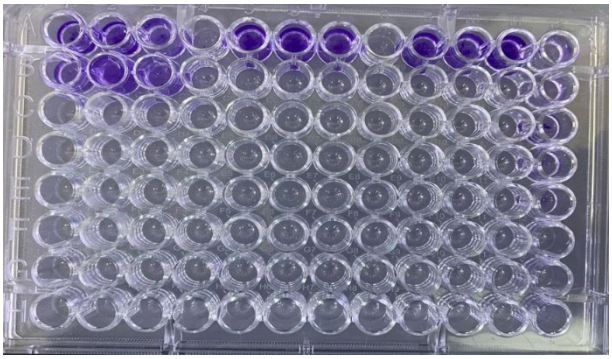


Fig. 3. Microtiter plate quantitative assay for *Serratia marcescens* isolates displaying distinct gradients of biofilm biomass production intensity

The documented phenotypic shifts in staining intensity among the environmental-opportunistic strains visually reflect the accumulated volume of the extracellular polymeric substance (EPS) matrix. The dominant portion of the isolates exhibited a strong biofilm layer, which secures the embedded cells and enhances structural stability during colonization.

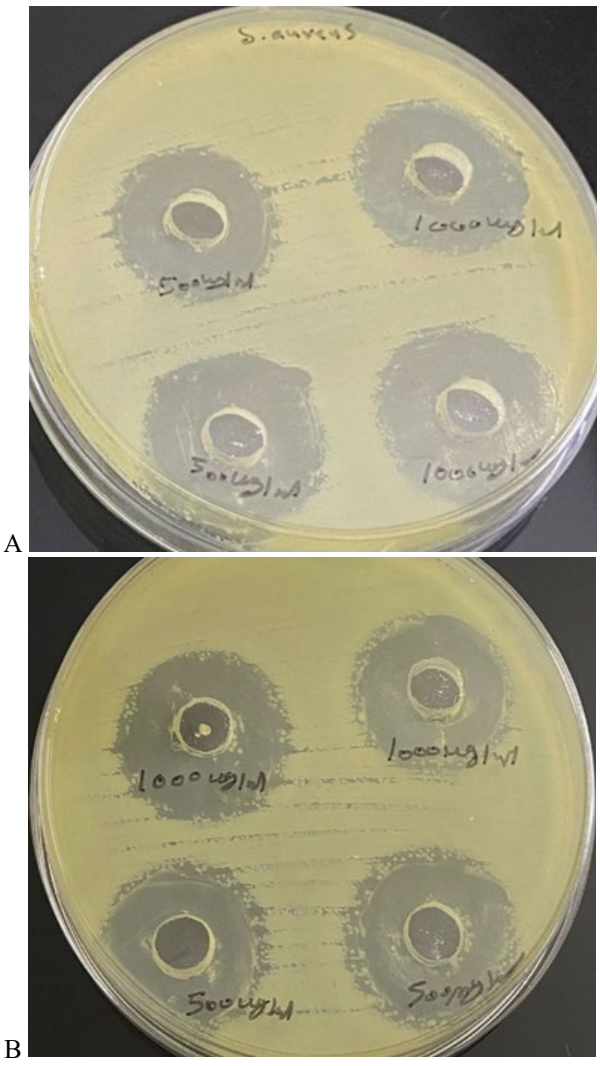
Antimicrobial activity of C. frutescens

The research investigated the antibacterial effectiveness of *Capsicum frutescens* against two bacterial strains which included *Staphylococcus aureus* and *Serratia marcescens*. The agar well diffusion assay was used to test different amounts of *C. frutescens* through two concentration levels (500 and 1000 µg/ml) which produced different sizes of inhibition zones against the isolated pathogens.

The diameter of the zones of inhibition demonstrated a clear concentration-dependent relationship with respect to *C. frutescens*; progressively higher concentrations of the extract yielded a concomitant and proportional increase in the extent of the inhibitory halos. This extract had an effective impact in inhibiting these types of bacteria, as illustrated in ***Figure 4***.

The recorded diameters of the inhibitory halos visually reflect the diffusion kinetics of the bioactive phytochemical components through the agar matrix. The maximum concentration of 1000 µg/ml consistently achieved a more expansive zone of clearance compared to the lower dose, confirming the potent dose-dependent bactericidal action of the extract against both Gram-positive and Gram-negative mastitis-associated strains.

From the 65 milk samples collected from cows with clinical mastitis, bacterial isolation identified *Staphylococcus aureus* in 10 (15.38 %) samples and *Serratia marcescens* in 4 (6.15 %) samples. The biofilm formation ability of *S. aureus* isolates became evident through phenotypic testing which showed 3 isolates (30 %) produced weak biofilms and 3 isolates (30 %) made moderate biofilms and 4 isolates (40 %) produced strong biofilms. For the *S. marcescens* isolates, 1 strain created a moderate biofilm layout while the other 3 isolates formed robust biofilm structures.



A

B

Fig. 4. Antibacterial activity of *Capsicum frutescens* extract against (A) *Staphylococcus aureus* and (B) *Serratia marcescens*

The milk samples from clinical mastitis cases showed various visible changes which included yellowish to watery discoloration and flaky or clotted milk that indicated an inflammatory process. The California Mastitis Test (CMT) showed different levels of milk gel formation and color changes which indicated increased somatic cell counts and modified pH values that occurred during clinical and subclinical mastitis cases [9].

Molecular detection of biofilm-associated genes

The Polymerase Chain Reaction (PCR) method served to screen the local isolates for specific genetic determinants associated with active efflux pump platforms and matrix production. Genotypic analysis utilizing primers targeting the *norA* gene revealed positive amplification products in 2 of the evaluated *Staphylococcus aureus* strains (***Fig. 5***).

Electrophoretic separation in the first lane on the left contains the DNA marker (Ladder), which serves as a molecular ruler to determine the size of the amplified PCR products. The two distinct bands visualized in lanes 2 and 3 confirm that the target DNA sequence underwent successful amplification, migrating precisely at the expected size of 391 base pairs, which matches the target *norA* gene fragment. Consequently, these findings confirm that the evaluated staphylococcal isolates demonstrate positive genotypic profiles for the *norA* determinant.

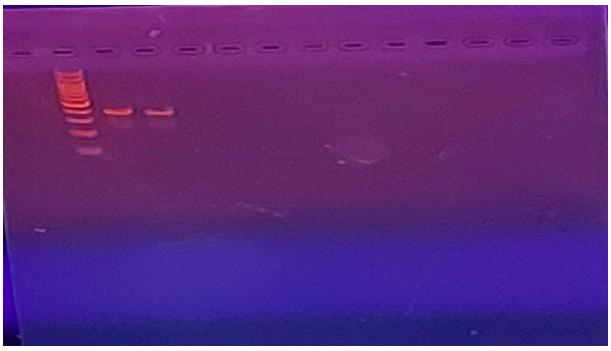


Fig. 5. Agarose gel electrophoresis (1 %) showing PCR amplification products of the *norA* gene from *Staphylococcus aureus* milk isolates: Lane 1 – DNA ladder; Lanes 2 and 3 – positive *norA* amplicons (391 bp)

Concurrently, the results of Agarose Gel Electrophoresis for the molecular detection of the *bsmA* gene in *Serratia marcescens* were evaluated to verify the genotypic potential for biofilm maturation. The left lane contains the DNA marker (Ladder), serving as a reference scale that enables users to measure the size of PCR products through base pair measurement.

The successful targeted amplification of the *bsmA* virulence factor becomes evident through the clear, consistent bands appearing in lanes 2 and 3 (***Fig. 6***). The migration of these targeted fragments at 298 bp confirms that the evaluated environmental-opportunistic *S. marcescens* isolates harbor this essential biofilm-mediating determinant.

In conclusion, the successful genotypic screening of the selected isolates highlights the solid molecular foundation underpinning their virulence profiles. The identification of the multidrug efflux pump gene *norA* in *Staphylococcus aureus* strains, coupled with the ubiquitous detection of the *bsmA* biofilm-mediating determinant in *Serratia marcescens* strains, confirms that these mastitis-associated pathogens possess the necessary genetic machinery to sustain long-term persistence within the mammary gland tissue. These molecular findings provide crucial baseline data that directly support the phenotypic resistance and robust biofilm traits observed during the initial laboratory assays.

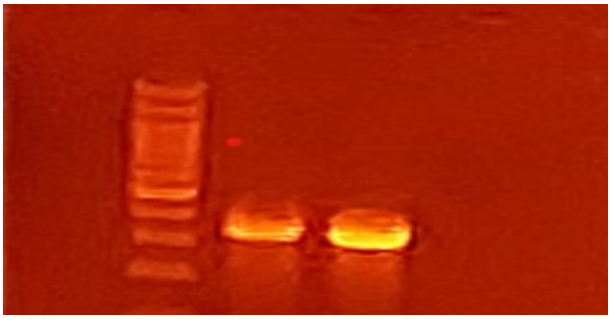


Fig. 6. Agarose gel electrophoresis (1 %) for the molecular identification of the *bsmA* gene in *Serratia marcescens* isolates: Lane 1 – DNA ladder; Lanes 2 and 3 – positive *bsmA* amplicons (298 bp)

Laboratory personnel collected and identified pathogenic bacteria which included *Staphylococcus aureus* to prove that microbial agents trigger mastitis infections in this particular region. The study revealed that *S. aureus* represented the most common pathogen among coagulase-positive *Staphylococcus* species with a frequency of 15.38 % within the evaluated cohort. These surveillance findings match the results from [17], but are lower than the findings from [18], who discovered a 30.0 % isolation rate during their historical screenings. The primary reason for the persistent isolation of *S. aureus* is its wide ecological distribution inside the bovine mammary gland and on the surrounding udder skin. Furthermore, the widespread practice of manual hand milking, combined with incorrect or unverified drug administration to combat mastitis infections, seems to support the horizontal spread of these contagious pathogens through direct milker hand contact during routine milk handling operations [19].

The bacterial analysis of the 65 milk samples also detected *Serratia marcescens* in 4 samples, which equals 6.15 % of the total milk samples examined. In comparison, previous epidemiological screening [20] found that 4 (3.33 %) of 120 monitored dairy cows developed *Serratia marcescens* mastitis, which contrasts with the slightly higher prevalence rate noted in the current research findings. The milk samples which underwent culturing developed various bacterial growth patterns on distinct media types after laboratory personnel incubated them under aerobic conditions at 37°C for 24 hours. On MacConkey agar, the growing colonies exhibited a delayed or weak ability to ferment lactose, appearing as characteristic red colonies primarily due to the inherent biochemical capability of *S. marcescens* to synthesize its own endogenous pigment. These morphological and pigment-producing results are in complete agreement with the diagnostic baselines established by [3]. The bacteria which were isolated showed up as classic Gram-negative rods when viewed through a standard light microscope.

The VITEK 2 Compact automated system successfully confirmed the definitive diagnosis of the recovered milk isolates through its improved biochemical testing cards, which leads to significantly enhanced and reliable diagnostic results. The automated VITEK 2 system produces better results than traditional manual tube methods by generating rapid diagnostic solutions which deliver precise species identification results. This automated platform employs a standardized method which enables fast pathogen identification while maintaining accurate diagnostic results [21].

Previous studies have shown that *Staphylococcus aureus* strains exhibit different levels of biofilm formation ability depending on their origin and environmental triggers. For instance, a prior study evaluated 31 clinical *S. aureus* strains, showing that nine strains developed strong biofilms while twelve strains produced moderate biofilms and ten strains had minimal biofilm formation [22]. Concurrently, separate research conducted on 100 clinical isolates showed a different distribution pattern, which found that twenty strains did not produce biofilms while thirty-two strains formed moderate biofilms and forty-eight strains developed strong, highly adherent biofilms [23].

The current study discovered that 100 % of the *S. aureus* isolates which researchers tested developed biofilms during the microtiter assays. These research findings closely match the structural results from [24], who found that all 58 *S. aureus* strains obtained from various clinical samples tested positive for biofilm formation, with eight strains showing weak production, twenty-eight strains showing moderate production, and twenty-two strains showing strong matrix production.

Biofilm formation serves as the main virulence factor which allows *S. aureus* to survive within the host environment, because the multi-layered extracellular matrix effectively shields the embedded bacteria from both antimicrobial medication attacks and host immune system responses [25]. These sessile biofilm communities serve as essential factors which drive the development and worsening of multiple severe chronic diseases in dairy cattle. The research suggests that *Staphylococcus aureus* strains develop different biofilm-forming capacities because their genetic expression patterns and regulatory networks show significant variations [26]. Equally significant is the profound influence of ambient environmental parameters on the initiation, cellular attachment, and subsequent maturation of these complex biofilm architectures [27].

Research demonstrates that *Serratia marcescens* bacteria produce robust biofilms. This study follows the research guidelines which previous investigators [28, 29] used to identify the phenotypic biofilm profiles of *S. marcescens* strains obtained from clinical samples. Research has proven that *S. marcescens* clinical isolates create biofilms which protect them from standard medical antibiotics. Biofilm formation by clinical bacterial pathogens leads to device-associated chronic infections, which represent the main cause of these persistent conditions [28].

Bacterial biofilms create an antimicrobial resistance system which significantly exceeds the protective level found in free-floating planktonic cells. *S. marcescens* has developed an adaptive method which shows how bacteria buried in extracellular polymeric matrices can build resistance to environmental forces that help them survive in nature and fight for ecological resources. Biofilm production creates two major problems which affect both animal health and industrial bio-processing facilities. Laboratory personnel have not fully discovered the exact molecular process which *S. marcescens* uses to stick to living and non-living surfaces, even though they know about its broad ability to attach to many different surfaces [30].

Preparations derived from *Capsicum frutescens* exhibited substantial antimicrobial efficacy against the complete spectrum of bacterial strains subjected to testing, thereby confirming the potent bactericidal potential of this plant material. The observed therapeutic activity is attributable to pharmacologically active constituents present within the extract matrix. The bioactive substances seem to be secondary metabolites, which include capsaicin and dihydrocapsaicin according to the study by [31], who conducted a complete phytochemical investigation of *Capsicum* species and found these substances to be the main factors which produced the antibacterial effects in the extracted samples.

The research outcomes from this study match the results which previous literature [32] published about *Capsicum frutescens* extracts that showed strong antibacterial effects against all bacterial strains tested. The current research findings duplicate the results from [33], who discovered that *Capsicum* species develop strong antibacterial properties which stop the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* species, and *Bacillus subtilis*.

The study authors confirmed that *Capsicum* plants produce their own natural antimicrobial properties through their research findings. The unprocessed crude *C. frutescens* extract component showed better antibacterial properties than the highly processed version during the evaluation of crude extracts. The intensive downstream filtration process seems to remove the essential active components which exist in the extract. The research findings from [34] showed similar results in their evaluation of botanical spices and condiments.

The research found *Staphylococcus aureus* and *Serratia marcescens* at rates of 15.38 % and 6.15 % respectively in mastitic milk samples from Diyala governorate, which confirms their established position as major mastitis pathogens according to previous studies [8, 9]. The study reveals an important discovery about the extensive presence of biofilm-producing bacteria which create robust biofilms in *S. aureus* and *S. marcescens* bacteria. Biofilms create a defensive shield which prevents immune system attacks and blocks antibiotic access, thus leading to ongoing infections that become resistant to treatment, which explains why mastitis continues to affect animals [35, 36].

Clinical mastitis causes milk to change from its normal white appearance into yellowish or watery form while it develops flakes and clots because of the inflammatory process, which enables blood elements and

white blood cells to penetrate milk through increased blood vessel leakage [9]. The California Mastitis Test (CMT) detects milk changes through somatic cell count elevation and pH modification to identify inflammation at its earliest stage, which includes cases without any visible milk alterations. The CMT reagent contains a pH indicator which changes to a stronger purple shade when milk pH levels rise because of inflammation. The progression of severe cases results in permanent damage to the udder, which includes fibrosis, because the tissue transforms into connective tissue that creates a hardened udder which no longer produces milk [7, 9].

The chromosomal structure of *Staphylococcus aureus* contains various genetic elements which produce virulence factors, antimicrobial resistance systems, and multi-drug efflux systems that create its ability to cause disease and adapt to new environments. The organism develops antimicrobial agent resistance through various independent mechanisms which operate differently from each other. The efflux pump system functions as an essential mechanism which removes antimicrobial substances from inside cells to reduce their internal levels and decrease their effectiveness as treatments.

Research has shown that *S. aureus* clinical isolates which contain these efflux systems will develop ciprofloxacin resistance [37]. The transporters operate with an extensive range of substrate recognition which extends to antibiotics, antiseptic agents, chromogenic dyes, and detergent compounds. The genes which control efflux pumps exist on bacterial chromosomes to maintain their presence in bacteria and to enable their transfer to following bacterial populations. These systems function as a fundamental defensive strategy, enabling *S. aureus* to withstand antimicrobial pressure through active compound expulsion [38].

Based on phylogenetic and amino acid sequence homology, bacterial efflux transporters are taxonomically classified into five principal superfamilies: (1) the Major Facilitator Superfamily (MFS), (2) the ATP-Binding Cassette (ABC) family, (3) the Resistance-Nodulation-Division (RND) family, (4) the Small Multidrug Resistance (SMR) family, and (5) the Multidrug and Toxic Compound Extrusion (MATE) family [39]. Notably, the *norA* gene product represents a constituent of the MFS efflux pump superfamily. Moreover, *norA* was present in ciprofloxacin-resistant MRSA strains as reported by studies conducted by [40–42].

Serratia marcescens depends on the quorum-sensing system to create biofilms by going through a series of unique steps that produce a highly porous, filamentous biofilm composed of clusters, filaments, and chains of cells. An increase in population density activates a gene regulatory system called quorum sensing. *S. marcescens* coordinates quorum sensing via acyl homoserine lactone (AHL), which controls the formation of biofilms, swarming motility, and prodigiosin production [43].

It is common knowledge that quorum sensing (QS) contributes to bacterial pathogenicity, antibiotic resistance, and biofilm formation. Thus, QS inhibition may lower the chance of microbial pathogenicity in both local and systemic infections [43]. Previous genotypic characterization has confirmed that the evaluated isolates harbored the *bsmA* gene [44], establishing this determinant as a key genetic marker for biofilm validation in environmental-opportunistic lineages.

Conclusions

This study confirms the significant prevalence of biofilm-producing *Staphylococcus aureus* (15.38 %) and *Serratia marcescens* (6.15 %) in 65 examined bovine clinical and subclinical mastitis milk samples within Diyala governorate, establishing that 100% of these isolated pathogens possess phenotypic biofilm-forming capacities (ranging from weak to strong adherence) with verified molecular expression of the multidrug efflux pump gene *norA* (391 bp) and the matrix-mediating determinant *bsmA* (298 bp) which directly underpins the chronicity, therapeutic recalcitrance, and mitigation difficulties of chronic intramammary infections. The California Mastitis Test (CMT) proved to be an invaluable and highly sensitive field diagnostic tool for the early-stage detection of both clinical and subclinical configurations before irreversible parenchymal tissue remodeling occurs, using pathognomonic milk color variations and specific gel formation profiles as critical inflammation indicators. Furthermore, crude ethanolic extract of *Capsicum frutescens* at targeted concentrations of 500 and 1000 µg/mL demonstrated profound, concentration-dependent antibacterial and anti-biofilm potential by generating prominent zones of inhibition against both persistent strains, proving that understanding these genotypic matrix mechanisms alongside the practical field utility of the CMT is paramount for engineering effective targeted phytotherapeutic prevention protocols to mitigate the severe economic impact of bovine mastitis in dairy herds.

DECLARATIONS

Ethical Statement

The authors confirm that the studies were conducted in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), national bioethical norms, and international standards for the humane treatment of animals. The study protocol was reviewed and approved by the Ethics Committee of the College of Veterinary Medicine, University of Diyala, Iraq (Approval No. SR 1233 - 1/6/2025). The *Serratia marcescens* and *Staphylococcus aureus* samples investigated in this research were recovered from clinical veterinary cases during routine diagnostic and sanitary screening.

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Conflict of Interest

The authors state that there is no conflict of interest.

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Declaration of AI and AI-assisted technologies

The authors declare that no generative AI or AI-assisted technologies were used during the preparation, writing, translation, or language polishing of this manuscript. The final content was fully authored and reviewed by the researchers, who take joint responsibility for the work.

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