Iosr Journal Of Agriculture And Veterinary Science (Iosr-Javs)
E-Issn: 2319-2380, P-Issn: 2319-2372. Volume 18, Issue 4 Ser. 1 (April. 2025), Pp 24-28
www.Iosrjournals.Org

Assessment Of Biofilm Production By *Staphylococcus Aureus* Isolates From Subclinical Mastitis On Congo Red Agar Supplemented With NaCL

Agueda Maria De França Tavares[1], Leonardo Ferreira Oliveira[1], Adriana Froes Do Nascimento Souto[1], Ellem Cristina Gomes Damascena[1], Maria Júlia Ribeiro Magalhães[1], Cintya Neves De Souza[1], Anna Christina De Almeida[1], Demerson Arruda Sanglard[2], Eliane Macedo Sobrinho Santos[3], Hércules Otacílio Santos[4], Carolina Magalhães Caires Carvalho[5], Franciane Gabrielle Dos Santos[6], Ageu Emerson Braz Do Carmo[7], Joao Bosco De Souza Júnior[8]

[1](*Animal Health Laboratory - CPCA, Federal University Of Minas Gerais, Brazil)*
[2](*Biotechnology Laboratory - CPCA, Federal University Of Minas Gerais, Brazil)*
[3](*Department Of Agricultural And Environmental Engineering, Federal Institute Of Northern Minas Gerais, Brazil)*
[4](*Production Core, Federal Institute Of Northern Minas Gerais, Brazil)*
[5](*CPCA, Federal University Of Minas Gerais, Brazil)*
[6](*Phd Student In Plant Production, Federal University Of Minas Gerais, Brazil)*
[7](*Microbial Ecology Laboratory - CPCA, Federal University Of Minas Gerais, Brazil)*
[8](*Bachelor Of Chemical Engineering, Faculty Of Science And Technology Of Montes Claros, Brazil)*

Abstract:
Background*: Phenotypic methods are essential for identifying biofilms, allowing for direct observation of their formation and characteristics. Techniques such as microscopy and staining, particularly the use of Congo Red Agar (CRA), are widely employed to study the structure and behavior of biofilms, as well as to evaluate the efficacy of antimicrobial treatments. This study investigates the influence of sodium chloride (NaCl) concentration on biofilm production in Staphylococcus aureus strains.*
Materials and Methods*: A total of six S. aureus strains were tested, including three biofilm-producing isolates, two non-producing isolates, and one reference strain (ATCC 25923). The CRA medium was prepared with varying NaCl concentrations (0%, 1%, 2.5%, 5%, and 7%), and the strains were incubated at 35°C ± 2°C for 24 to 48 hours. Biofilm production was assessed based on the morphology and coloration of the colonies, where black colonies indicated biofilm production and red or pink colonies indicated non-producers. Each test was performed in triplicate to ensure reproducibility.*
Results*: The biofilm-producing strains exhibited intense formation at NaCl concentrations of 0%, 1%, and 2.5%, displaying characteristic black coloration. In contrast, at NaCl concentrations of 5% and 7%, although bacterial growth was observed, there was no black coloration, indicating inhibition of biofilm production. The non-producing strains maintained red coloration at all tested concentrations.*
Conclusion: *Biofilm production by S. aureus is favored in media with up to 2.5% NaCl, while it is inhibited at higher concentrations (5% and 7%). These findings demonstrate that elevated levels of NaCl compromise the expression of the biofilm-producing phenotype, highlighting the impact of salt stress on this important virulence characteristic of the bacterium.*
Key Word*: Biofilms, S. aureus, NaCl, Phenotypic.*

Date of Submission: 15-04-2025 Date of Acceptance: 25-04-2025

I. Introduction

Phenotypic methods play a crucial role in identifying biofilms, as they allow for direct observation of their formation and characteristics. Techniques such as microscopy, staining, and culture-based assays help evaluate the structure and behavior of biofilms, providing important insights into their development. These

methods are widely used to study interactions within bacterial communities and to test the efficacy of antimicrobial treatments[1,2].

However, phenotypic methods face challenges in terms of sensitivity and specificity compared to molecular biology techniques. Identifying biofilms in clinical samples can be difficult due to their heterogeneous nature and the presence of the extracellular matrix, which can hinder visualization. Molecular methods, such as PCR (Polymerase Chain Reaction) and sequencing, offer greater sensitivity in detecting specific genetic markers of biofilm-forming bacteria. Nevertheless, combining phenotypic and molecular approaches can provide a more comprehensive view, as phenotypic methods reveal information about the structure and activity of biofilms that molecular techniques alone may not capture[3,4].

Congo Red Agar (CRA) is a widely used phenotypic method for detecting biofilm formation in bacteria. This technique relies on culturing microorganisms in a medium supplemented with the Congo Red dye, which interacts with the polysaccharides in the biofilm matrix. Biofilm-producing strains form black colonies with a dry, crystalline appearance, while non-producing strains exhibit red or pink colonies. As a simple and cost-effective approach, CRA is a viable option for initial screening of biofilm formation capacity[5,6,7,8].

However, the sensitivity and specificity of the method can vary. Some studies indicate that CRA may have low sensitivity, failing to detect all biofilm-forming strains[9,10]. For instance, one study demonstrated that CRA sensitivity was only 0.9%, while specificity reached 97.4% in identifying biofilms in Staphylococcus isolates. These results suggest that while CRA is useful for initial screening, it should be complemented with other tests to ensure more accurate identification of biofilm production[11,12].

The concentration of sodium chloride (NaCl) in the growth medium can significantly influence the expression of genes related to antibiotic resistance and biofilm formation in bacteria[13,14]. Therefore, this study aims to analyze the influence of NaCl on biofilm production in *S. aureus* strains.

II. Material And Methods

Strain Selection and Identification

A total of six *S. aureus* strains were tested, comprising three biofilm-producing isolates, two non-producing isolates, and one reference strain ATCC 25923, which is a biofilm producer. All isolates were previously identified as S. aureus through MALDI-TOF methods, as part of previous research conducted at the Animal Health Laboratory of the Institute of Agricultural Sciences (ICA) at the Federal University of Minas Gerais (UFMG).

Preparation of Congo Red Agar (CRA) Medium

The CRA medium was prepared according to Freeman *et al*.[15], with modifications to incorporate varying concentrations of NaCl. The base composition of CRA included BHI broth (37 g/L), base agar (10 g/L), sucrose (50 g/L), and Congo Red dye (0.8 g/L). NaCl was added to the medium at final concentrations of 0%, 1%, 2.5%, 5%, and 7% (w/v) to assess the impact of salinity on biofilm detection.

Inoculation and Incubation Conditions

Each strain was inoculated onto CRA plates with different NaCl concentrations and incubated aerobically at 35°C ± 2°C for 24 and 48 hours. Biofilm production was evaluated based on the morphology and color of the colonies. Black, dry, and crystalline colonies were interpreted as positive for biofilm production, while red or pink colonies indicated non-producers.

Reproducibility and Data Analysis

Each strain was tested in triplicate at each NaCl concentration to ensure reproducibility. Data analysis involved observing consistency in biofilm production patterns across the NaCl gradient and comparing the morphologies of biofilm-producing and non-producing strains to evaluate the effect of NaCl on the phenotypic expression of biofilms.

III. Result

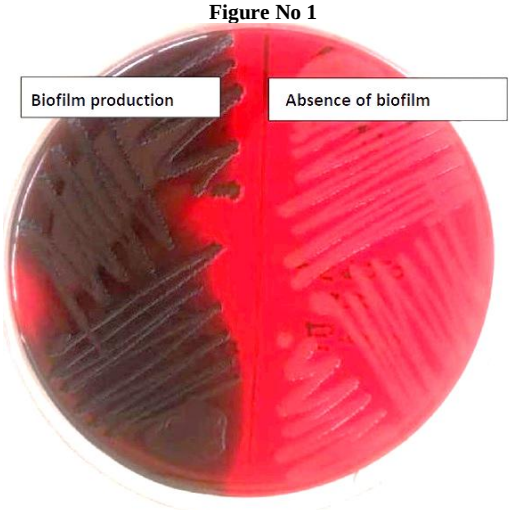
The assessment of biofilm production by *S. aureus* strains isolated from subclinical mastitis, using CRA supplemented with different NaCl concentrations, revealed that biofilm-producing strains, including the standard strain ATCC 25923, exhibited intense biofilm formation at NaCl concentrations of 0%, 1%, and 2.5%. In these media, the colonies displayed characteristic black coloration, indicating the production of the typical exopolysaccharide associated with biofilms. No visible variation in the intensity of biofilm production was observed among the different tested concentrations (Figure no 1, 2A, 2B). The results are detailed in Table no 1.

Table no 1 - Biofilm Production by S. aureus Isolates in CRA Medium Supplemented with Different NaCl Concentrations

NaCl Concentrations	0%	1%	2,5%	5%	7%
A15	-	-	-	-	-
LM261	-	-	-	-	-
A6	+	+	+	-	-
R9	+	+	+	-	-
R13	+	+	+	-	-
ATCC 25923	+	+	+	-	-

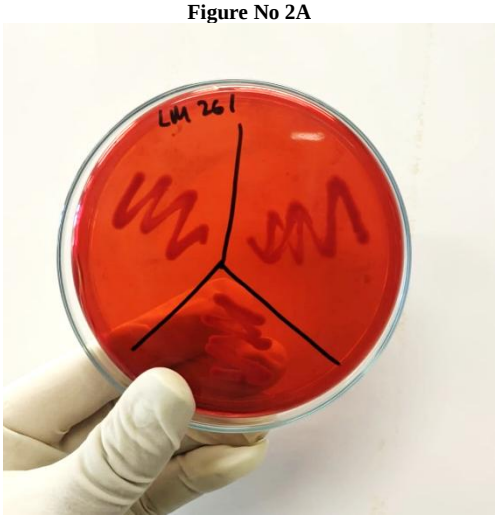
In contrast, at NaCl concentrations of 5% and 7%, although bacterial growth was noted, there was an absence of black coloration, indicating that under these conditions, the expression of the biofilm-producing phenotype was inhibited. The non-producing strains showed no changes in expected behavior, displaying red colonies at all tested NaCl concentrations, confirming the absence of exopolysaccharide and indicating an inability to produce biofilm.

Figure No 1



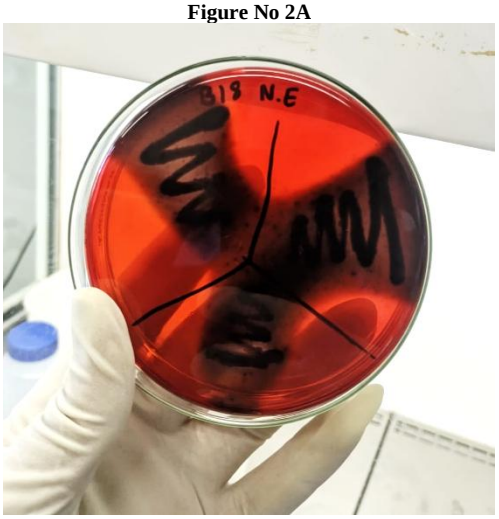
Source: Adapted By The Author

Figure No 2A



Source: Adapted By The Author

Figure No 2A



Source: Adapted By The Author

IV. Discussion

Bacterial biofilms are organized communities of microorganisms encased in a protective matrix that they produce themselves. These structures adhere to surfaces and confer greater resistance to antibiotics and immune system attacks. Among the species well-known for their ability to form biofilms is *S. aureus*, a pathogen responsible for persistent and difficult-to-treat infections[16,17].

The *Staphylococcus* genus is recognized for its resistance to high concentrations of NaCl, which allows survival in high-salinity environments. This capability is linked to adaptations that adjust gene expression in response to osmotic stress caused by NaCl. Research shows that the presence of NaCl can stimulate biofilm

formation in some *S. aureus* strains, particularly methicillin-sensitive strains (MSSA). This occurs through the activation of the *ica*ADBC operon, responsible for producing a substance called PIA (Polysaccharide Intercellular Adhesin), which is fundamental to the biofilm structure[18,19].

Conversely, in methicillin-resistant strains (MRSA), biofilm formation under the influence of NaCl may occur through different mechanisms involving adhesion proteins regulated by the SarA protein. Additionally, the stress caused by NaCl activates genes such as *sig*B, which coordinates a general stress response and enhances bacterial resistance under adverse conditions. These adjustments in gene functioning demonstrate how *S. aureus* can rapidly adapt, ensuring its survival and increasing its infectious potential in high-salinity environments[20].

In *Staphylococcus* species, both NaCl and other substances, such as glucose, can stimulate or inhibit biofilm formation. Studies have identified that biofilm-forming strains include, in addition to *S. aureus*, at least nine different species of coagulase-negative *Staphylococcus*. Furthermore, the presence of the *ica*A gene has been detected in many of these strains and correlated with a higher capacity for biofilm formation[21].

Kaiser *et al*.[22] tested different formulations of the CRA medium, evaluating separately and in combination various concentrations of NaCl (1.5%, 3%, 4%, 5%, and 7%) and glucose (1%, 2%, and 3%) to establish an optimized formulation for biofilm detection. The best results were obtained with the addition of 1.5% NaCl, 2% glucose, and vancomycin at a concentration of 0.5 mg/mL, allowing for efficient identification of biofilm-producing *S. epidermidis* strains.

In *Salmonella enterica* (serovar Dublin), the presence of NaCl is essential for biofilm development, but concentrations above 1.0% may inhibit this process. This suggests that there is an optimal level of salt for biofilm formation, an important factor for resistance to antibiotics such as ampicillin[13]. Iliadis *et al*.[23] evaluated the effect of different NaCl concentrations (0.5% to 8.5%) on biofilm formation by two *S. enterica* strains (serovars Enteritidis and Typhimurium). The results showed that for both strains, increasing NaCl concentration restricted sessile growth. Conversely, under low salinity conditions (NaCl < 4%), biofilm formation was favored.

Another example comes from *Piscirickettsia salmonis*, a pathogenic bacterium affecting fish. Increased NaCl concentration in the culture medium significantly stimulates biofilm production, making the bacterium more resistant and virulent. This indicates that NaCl not only regulates biofilm formation but can also influence a microorganism's ability to cause infections[24]. These examples highlight the importance of NaCl concentration in regulating gene expression, directly affecting characteristics such as antibiotic resistance and biofilm formation, which can have implications in clinical microbiology, the food industry, and aquaculture.

V. Conclusion

In conclusion, biofilm production by *S. aureus* is favored in media supplemented with up to 2.5% NaCl, while it is inhibited at higher concentrations (5% and 7%). This finding indicates that elevated levels of NaCl may compromise the expression of the biofilm-producing phenotype, underscoring the influence of salt stress on this important virulence characteristic of the bacterium.

References

[1]. Rodrigues RL, Lima JLDC, Sena KXDFR, Maciel MAV. Phenotypic And Genotypic Analysis Of Biofilm Production By Pseudomonas Aeruginosa Isolates From Infection And Colonization Samples. Rev Soc Bras Med Trop. 2020; 53:E20200399.
[2]. Yan J, Bassler BL. Surviving As A Community: Antibiotic Tolerance And Persistence In Bacterial Biofilms. Cell Host Microbe. 2019; 26(1): 15-21.
[3]. Barros EM, Lemos M, Souto-Padrón T, Giambiagi-Demarval M. Phenotypic And Genotypic Characterization Of Biofilm Formation In Staphylococcus Haemolyticus. Curr Microbiol. 2015; 70(6): 829-834.
[4]. Aniba R, Dihmane A, Raqraq H, Ressmi A, Nayme K, Timinouni M, Barguigua A. Molecular And Phenotypic Characterization Of Biofilm Formation And Antimicrobial Resistance Patterns Of Uropathogenic Staphylococcus Haemolyticus Isolates In Casablanca, Morocco. Diagn Microbiol Infect Dis. 2024; 110(4): 116483.
[5]. Harika K, Shenoy VP, Narasimhaswamy N, Chawla K. Detection Of Biofilm Production And Its Impact On Antibiotic Resistance Profile Of Bacterial Isolates From Chronic Wound Infections. J Glob Infect Dis. 2020; 12(3): 129-134.
[6]. Ürer EK, Aslantaş Ö, Tek E, Yılmaz MA, Ergün Y. Antimicrobial Susceptibility And Biofilm Forming Ability Of Staphylococci From Subclinical Buffalo Mastitis. J Dairy Res. 2023; 90(1): 1-4.
[7]. Nguyen JM, Moore RE, Spicer SK, Gaddy JA, Townsend SD. Synthetic Phosphoethanolamine Cellobiose Promotes Escherichia Coli Biofilm Formation And Congo Red Binding. Chembiochem. 2021; 22(15): 2540-2545.
[8]. Akbar MU, Haque A, Liaquat S, Schierack P, Ali A. Biofilm Formation By Staphylococcus Epidermidis And Its Inhibition Using Carvacrol, 2-Aminobenzemidazole, And 3-Indole Acetonitrile. ACS Omega. 2022; 8(1): 682-687. Doi: 10.1021/Acsomega.2c05893.
[9]. Parai D, Banerjee M, Dey P, Mukherjee SK. Reserpine Attenuates Biofilm Formation And Virulence Of Staphylococcus Aureus. Microb Pathog. 2020; 138: 103790.
[10]. Ballah FM, Islam MS, Rana ML, Ferdous FB, Ahmed R, Pramanik PK, Karmoker J, Ievy S, Sobur MA, Siddique MP, Khatun MM, Rahman M, Rahman MT. Phenotypic And Genotypic Detection Of Biofilm-Forming Staphylococcus Aureus From Different Food Sources In Bangladesh. Biology (Basel). 2022; 11(7): 949.
[11]. Abdel Halim RM, Kassem NN, Mahmoud BS. Detection Of Biofilm Producing Staphylococci Among Different Clinical Isolates And Its Relation To Methicillin Susceptibility. Open Access Maced J Med Sci. 2018; 6(8): 1335-1341.
[12]. El-Nagdy AH, Abdel-Fattah GM, Emarah Z. Detection And Control Of Biofilm Formation By Staphylococcus Aureus From Febrile Neutropenic Patient. Infect Drug Resist. 2020; 13: 3091-3101.
[13]. Ju X, Li J, Zhu M, Lu Z, Lv F, Zhu X, Bie X. Effect Of The Luxs Gene On Biofilm Formation And Antibiotic Resistance By Salmonella Serovar Dublin. Food Res Int. 2018; 107: 385-393.

[14]. Kotian A, Aditya V, Sheikh J, Saikrishnan S, Rai P, Chakraborty A, Karunasagar I, Deekshit VK. Effect Of Nacl, High Iron, Iron Chelator And Antibiotics On Growth, Virulence Gene Expression And Drug Susceptibility In Non-Typhoidal Salmonella: An In Vitro Fitness Study. Arch Microbiol. 2022; 204(11): 667. Doi: 10.1007/S00203-022-03278-X.
[15]. Freeman DJ, Falkiner FR, Keane CT. New Method For Detecting Slime Production By Coagulase Negative Staphylococci. J Clin Pathol. 1989; 42: 872-874.
[16]. Peng Q, Tang X, Dong W, Sun N, Yuan W. A Review Of Biofilm Formation Of Staphylococcus Aureus And Its Regulation Mechanism. Antibiotics (Basel). 2022; 12(1): 12.
[17]. Bhattacharya M, Berends ETM, Chan R, Schwab E, Roy S, Sen CK, Torres VJ, Wozniak DJ. Staphylococcus Aureus Biofilms Release Leukocidins To Elicit Extracellular Trap Formation And Evade Neutrophil-Mediated Killing. Proc Natl Acad Sci U S A. 2018; 115(28): 7416-7421.
[18]. O'Neill E, Pozzi C, Houston P, Smyth D, Humphreys H, Robinson DA, O'Gara JP. Association Between Methicillin Susceptibility And Biofilm Regulation In Staphylococcus Aureus Isolates From Device-Related Infections. J Clin Microbiol. 2007; 45(5): 1379-1388.
[19]. Jyoti K, Soni K, Chandra R. Optimization Of The Production Of Exopolysaccharide (EPS) From Biofilm-Forming Bacterial Consortium Using Different Parameters. The Microbe. 2024; 4: 100117.
[20]. Kubistova L, Dvoracek L, Tkadlec J, Melter O, Licha I. Environmental Stress Affects The Formation Of Staphylococcus Aureus Persisters Tolerant To Antibiotics. Microb Drug Resist. 2018; 24(5): 547-555.
[21]. Møretrø T, Hermansen L, Holck AL, Sidhu MS, Rudi K, Langsrud S. Biofilm Formation And The Presence Of The Intercellular Adhesion Locus Ica Among Staphylococci From Food And Food Processing Environments. Appl Environ Microbiol. 2003; 69(9): 5648-5655.
[22]. Kaiser TD, Pereira EM, Dos Santos KR, Maciel EL, Schuenck RP, Nunes AP. Modification Of The Congo Red Agar Method To Detect Biofilm Production By Staphylococcus Epidermidis. Diagn Microbiol Infect Dis. 2013; 75(3): 235-239.
[23]. Iliadis I, Daskalopoulou A, Simões M, Giaouris E. Integrated Combined Effects Of Temperature, Ph And Sodium Chloride Concentration On Biofilm Formation By Salmonella Enterica Ser. Enteritidis And Typhimurium Under Low Nutrient Food-Related Conditions. Food Res Int. 2018; 107: 10-18.
[24]. Santibañez N, Vega M, Pérez T, Yáñez A, González-Stegmaier R, Figueroa J, Enríquez R, Oliver C, Romero A. Biofilm Produced In Vitro By Piscirickettsia Salmonis Generates Differential Cytotoxicity Levels And Expression Patterns Of Immune Genes In The Atlantic Salmon Cell Line SHK-1. Microorganisms. 2020; 8(10): 1609.