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Multidrug Resistance And Phenotypic Characterization Of Biofilm Formation By Staphylococcus Aureus: Contributions To Public Health

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Abstract:
Background*: In humans, the increase in bacterial resistance in samples isolated from infectious processes seems to be caused not only by the inappropriate and intensive use of antimicrobials, but also by transmission through food of animal origin or direct contact with farm animals. Microbial biofilms have effects on public health and industrial considerations regarding their influence on the economy, energy use, deterioration of equipment and the occurrence of infections. Thus, biofilm production can significantly impact disease progression and treatment outcomes, and may favor antimicrobial resistance. Studies that evaluate the relationship between biofilm production and antimicrobial resistance can help to elucidate mechanisms that may influence the efficacy of antimicrobial therapy against microorganisms. This study aimed to evaluate the profile of multidrug resistance and phenotypic characterization of biofilm formation by Staphylococcus aureus isolated from cases of infectious bovine mastitis on dairy farms in northern Minas Gerais, Brazil.* (10)
Materials and Methods*: We analyzed 23 S. aureus isolates (A - Z) from subclinical mastitis, previously identified by the MALDI-TOF MS technique. Sensitivity to antimicrobials was obtained using the disk diffusion method. Biofilm production was determined using Congo Red Agar and the plate adherence test. Descriptive statistics were used to distribute relative and absolute frequencies for the microbiological findings*. (10)
Results*: Most of the isolates were resistant to the antibiotics ASB (96%), OXA (92%) and ME1 (83%). Only isolate B proved resistant to all the antibiotics used. Using the Congo Red Agar test, 96.66% of the samples showed the ability to form biofilms, which were associated with resistance to the antibiotics ASB and IPM. However, only 16.66% of the isolates were positive for biofilm formation in the plaque adhesion test.* (10)
Conclusion: *The presence of multi-resistant, biofilm-forming bacteria represents a health risk. Rapid detection methods are essential to control these pathogens in the food industry*. (10)
Keyword*: Public health; Bovine mastitis; Plate adhesion tes;, Congo red agar; Sensitivity.*

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I. Introduction

Bovine mastitis is a disease that causes major losses in dairy production, and *Staphylococcus aureus* is one of the main causes of this infection. Traditionally, antimicrobials from various classes are used to control this disease (Campos *et al*.,2022). However, the treatment of bovine mastitis with antimicrobials is not always effective due to the development of multi-resistance characteristics in microorganisms, leading to public health problems (Zanela *et al*, 2022). In humans, the increase in bacterial resistance in samples isolated from infectious processes seems to be caused not only by the inappropriate and intensive use of antimicrobials, but also by transmission through food of animal origin or direct contact with farm animals (Tang *et al*., 2017).

Multiple resistance to antimicrobials represents a challenge, especially when the bacteria have additional defense mechanisms. One of these mechanisms is the formation of biofilms. The ability to form biofilms is associated with their ability to remain in the bovine udder, resisting both the animal's immune response and the action of antimicrobials, which become less effective against these structures (Chagas, 2015). Biofilms have effects on public health and industrial considerations regarding their influence on the economy, energy use, deterioration of equipment and the occurrence of infections (Sharma *et al*., 2023). Thus, biofilm production can significantly impact disease progression and treatment outcomes, and may favor antimicrobial resistance (Proctor *et al*., 2014).

S. aureus isolated from bovine mastitis have a scientifically proven ability to form biofilms (Bissong; Ateba, 2020). In addition, this pathogen is recognized for developing resistance to multiple antimicrobial classes, including β-lactams, tetracyclines, aminoglycosides, amphenicols, macrolides, trimethoprim, lipopeptides and lincosamides (Naranjo; Lucena; Slowey, 2023).

Studies that evaluate the relationship between biofilm production and antimicrobial resistance can help to elucidate mechanisms that may influence the efficacy of antimicrobial therapy against *S. aureus* in dairy herds. Therefore, this study aimed to evaluate multidrug resistance and phenotypic characterization of biofilm formation by *S. aureus* isolated from cases of infectious bovine mastitis on dairy farms in northern Minas Gerais, Brazil.

II. Material And Methods

Bacterial isolates

We used 23 isolates of *S. aureus*, obtained from the Animal Health Laboratory of the Institute of Agricultural Sciences at UFMG, previously identified using the MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) technique. These isolates were collected from milk samples of cows diagnosed with subclinical mastitis in previous studies.

The cryopreserved bacterial cultures were reactivated in BHI (Brain Heart Infusion) broth and then transferred to Plate Count Agar (PCA) plates using the streaking technique. The plates were incubated at 37°C for 24 hours to isolate the colonies.

Antimicrobial susceptibility testing

The 23 isolates were tested to determine their sensitivity to antimicrobials using the disk diffusion method following the protocol described by the Clinical and Laboratory Standards Institute (CLSI, 2018). The antimicrobials tested were Imipenem (IPM - 10 µg), Cefoxitin (CFO - 30 µg), Oxacillin (OXA - 1µg), Ampicillin + Sulbactam (ASB - 20µg) and Meropenem (ME1 - 10µg). A standard strain of *S. aureus* ATCC 25923 was used as a positive control.

Analysis of biofilm production using Congo Red Agar

The Congo Red Agar (CRA) medium was prepared according to a protocol adapted from Freeman *et al*. (1989), containing BHI broth (0.37%), sucrose (0.50%), agar (0.15%) and a 0.08% Congo Red dye solution. Using a sterile inoculation loop, a colony of each isolate was transferred to plates containing 0.08% CRA medium and incubated at 37°C for 24 to 48 hours. The test was carried out in triplicate. *S. aureus* ATCC 25923 and S. epidermidis ATCC 12228 were used as positive and negative controls, respectively.

Analysis of biofilm production using the plate adhesion test

Plate adherence was assessed according to the procedure described by Guimarães *et al*. (2012). 23 isolates of *S. aureus* were used and grown in TSB (Tryptone Soya Broth) medium at 37°C for 24 hours. After this period, an aliquot of each culture was diluted 1:40 in TSB medium supplemented with 0.25% glucose. The standardized samples, together with the controls, were distributed in 96-well U-bottom hemagglutination plates, in triplicate, with 200µL per well. After incubation (37°C for 24 hours), the plates were washed three times with distilled water and left to dry at room temperature.

Next, 200µL of crystal violet was added per well and incubated for 2 to 3 minutes at room temperature. Each well was then washed three times with distilled water and filled with an alcohol-acetone solution (80:20). The crystal violet-stained plates were subjected to spectrophotometry at 490 nm to measure the absorbance of each well. Isolates with an optical density equal to or less than 0.111 were classified as non-adherent, while those with values greater than 0.111 were classified as adherent.

Statistical analysis

The data obtained from the antimicrobial susceptibility and biofilm formation experiments was tabulated in Microsoft Excel spreadsheets®. Descriptive statistics were used to distribute relative and absolute frequencies for the microbiological findings of biofilm formation.

The Cytoscape platform was used to create an interaction network between bacterial isolates and their respective resistance to antimicrobials. In addition, a platform plugin was used to generate an Euler diagram showing the number of bacterial isolates resistant to each antibiotic, as well as the intersection between them.

To calculate the agreement between the resistance profile and biofilm formation capacity, Cohen's Kappa index was used, which is a measure of the agreement between the two tests, factoring in agreement due to chance. This was done using the Real Statistics Resource Pack computer program, which provides a tool for analyzing reliability data between the two tests.

The chi-square test was used to assess the association between the qualitative variables using the PAST software.

III. Result And Discussion

Of the 23 *S. aureus* isolates studied, the majority were resistant to ampicillin + sulbactam (96%), followed by oxacillin (92%) and meropenem (83%) (Figure 1). Ampicillin and oxacillin belong to the penicillin subgroup and can have disadvantages such as rapid elimination from the body and, above all, being vulnerable to the action of beta-lactamases (Arruda *et al.*, 2019). Cruz *et al.* (2020) found that 100% of 23 *S. aureus* isolates were resistant to ampicillin + sulbactam, while 73.26% of these isolates were resistant to oxacillin.

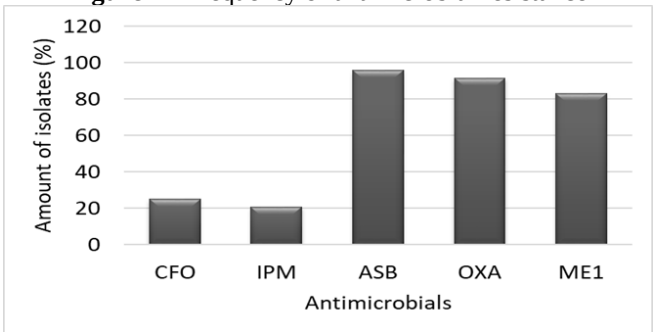
Although meropenem is a second-generation carbapenem that is resistant to hydrolysis by beta-lactamases and is an important therapeutic option against gram-positive bacteria and gram-negative bacilli (Pantalião; Ribeiro, 2023), its indiscriminate use has contributed to the increase in resistant bacteria. This antimicrobial can stimulate enzymes called carbapenemases in microorganisms, which are capable of degrading most beta-lactam antibiotics, which may explain the high percentage of meropenem-resistant strains (83%). *In* addition, *in vitro* studies have shown that meropenem acts more effectively against gram-negative bacteria (Edwards; Betts, 2000; Gales *et al.*, 1997; Hikida *et al.*, 1992).

Few isolates showed resistance to Cefoxitin and Imipenem (Figure 1). Cefoxitin belongs to the cephamycin subgroup, which is similar in structure to second-generation cephalosporins and acts against gram-negative bacilli, gram-positive and anaerobic bacteria (Arruda *et al.*, 2019; Tamma *et al*., 2019). Imipenem, which belongs to the carbapenem group, is effective against gram-positive, gram-negative, including non-glucose fermenting, and anaerobic bacteria (Zhanel *et al*, 2007). Although Meropenem is considered to be an evolution in the carbapenem class, studies have shown that Imipenem exhibited a slightly higher activity than Meropenem against gram-positive bacteria (Edwards; Betts, 2000; Gales *et al.*, 1997; Hikida *et al.*, 1992), substantiating the better action of Imipenem against *S. aureus* in the present study.

The differences observed in the resistance of *S. aureus* to different antibiotics can be attributed to factors linked to the microorganism and to the intrinsic characteristics of each class of antimicrobial. Microbial agents can mutate their genes and/or acquire resistance genes from other bacteria of the same species, or possibly from other species, giving microorganisms resistance to one or more antimicrobials depending on the gene involved in the resistance phenotype (Alós, 2015; Rodríguez-Noriega *et al*., 2013). Antibiotics, in turn, have different chemical structures which are responsible for their characteristics such as affinity for the receptor, spectrum of action, and others. The different subgroups of beta-lactams, such as Penicillins, Carbapenems and Cephamycins, differ according to the radical that binds to the beta-lactam ring, giving each subgroup specific activities (Arruda *et al*., 2019).

When assessing the sensitivity of *S. aureus* detected in bovine mastitis, using the same antibiotics as this study, Souza *et al*. (2020) found that the isolates did not show resistance to ampicillin + sulbactam and imipenem. In addition, the oxacillin-resistant isolates were also resistant to cefoxitin and were considered methicillin-resistant (MRSA). These findings show that differences between studies can occur from one herd to another and are related to factors such as the acquisition and transfer of certain resistance genes, selection and proliferation of a microbial type, carrying or transmission of microorganisms, as mentioned above by the author and collaborators.

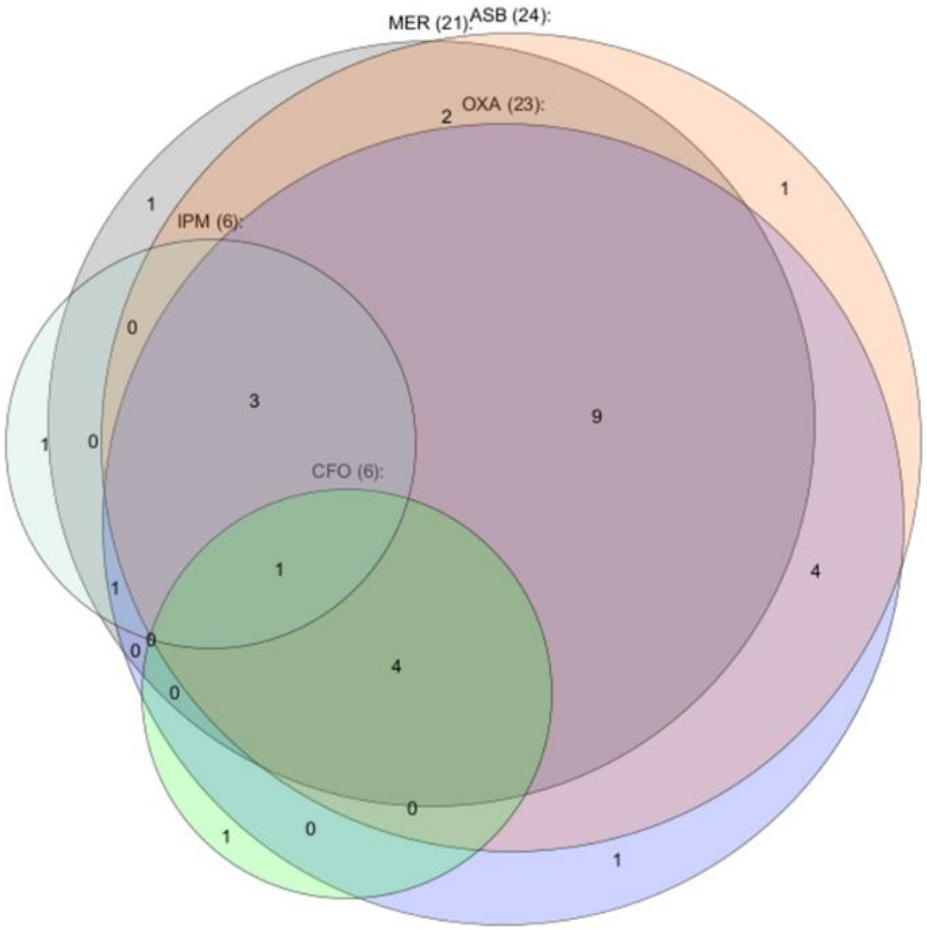
Figure 1 - Frequency of antimicrobial resistance



CFO = Cefoxitin. IPM - Imipenem. ASB = Ampicillin + Sulbactam. OXA = Oxacillin. ME1 = Meropenem.
Source: Prepared by the authors (2024)

Figure 2 shows the number of isolates resistant to each of the antimicrobials, as well as the number of isolates resistant to several antimicrobials at the same time (multidrug resistance), which can be seen at the intersection between antimicrobials. All the *S. aureus* isolates analyzed were resistant to at least two different antimicrobials. The majority of isolates (43%) were resistant to three antimicrobials. And 4% of the isolates were resistant to all the antimicrobials used. Microbial isolates resistant to two and four antibiotics, respectively, appeared at a frequency of 26%. Studies in the literature point to this characteristic of multi-resistance in *S. aureus* strains (Cruz *et al.*, 2020). *S. aureus* has several virulence mechanisms, including biofilm formation and multi-resistance to antibiotics (Kumar *et al.*, 2020).

Figure 2 - Euler diagram showing the number of bacterial isolates resistant to each antibiotic and the intersection between them



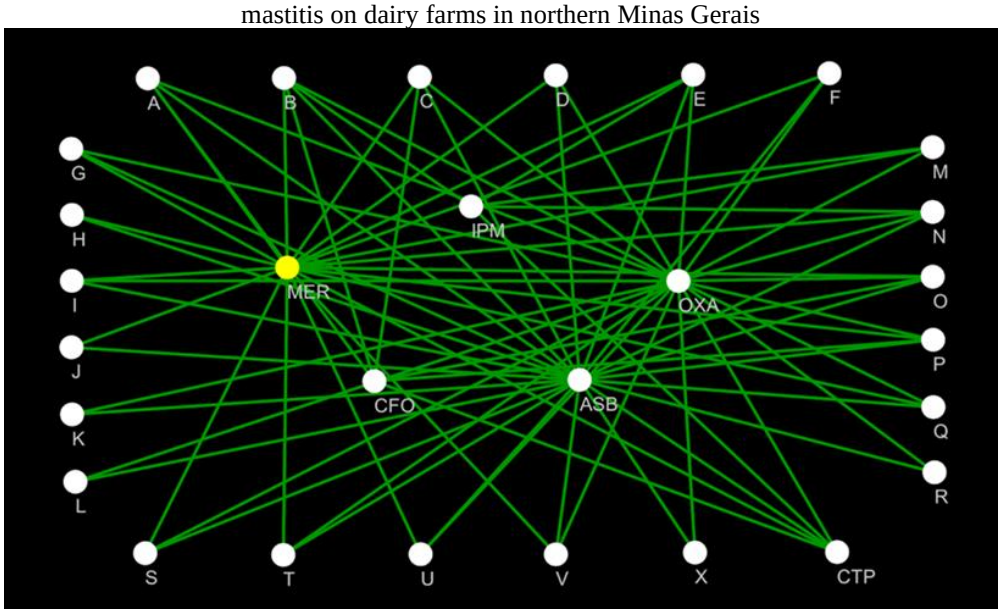
Source: Prepared by the authors (2024)

Only isolate B proved resistant to all the antibiotics used. On the other hand, isolates H, J, K, L, R, X were resistant to only 2 of the antimicrobials. The other isolates were resistant to 3 or 4 antibiotics. The ATCC AUREUS 25923 strain, used as a positive control, was resistant to all 5 antimicrobials (Figure 3).

Studies on the resistance profiles of *S. aureus* isolated from intramammary infections in cattle, conducted in other countries, indicate a trend towards high levels of resistance to beta-lactams, similar to the results of this study. In Ethiopia, *S. aureus* isolates from raw milk showed resistance to penicillin and other antibiotics, including cephalothin and oxacillin. In addition, research revealed that *S. aureus* strains continued to be resistant to multiple drugs, posing a challenge for the management of intramammary infections and leading to considerable economic losses for dairy farmers (Deddefo *et al.*, 2021).

In Africa, it has been observed that *S. aureus* resistance to antibiotics is a growing problem on dairy farms, with resistance rates similar to those observed in other countries. Furthermore, it has been revealed that while resistance to oxacillin and other beta-lactam antibiotics is high, susceptibility to new antibiotics such as vancomycin remains (Mphahlele *et al.*, 2020). The authors associate this resistance with the excessive use of antimicrobials in dairy production, which suggests the urgent need for more effective control strategies and prudent use of antibiotics to mitigate the spread of resistant strains.

Figure 3 - Multidrug resistance profile of *Staphylococcus aureus* isolated from cases of infectious bovine mastitis on dairy farms in northern Minas Gerais



A - Z: *S. aureus* isolated from cases of infectious bovine mastitis on dairy farms in northern Minas Gerais. CPT: ATCC strain of *S. aureus*. MER:(10 µg); IPM:(10 µg) OXA: (1µg) ASB (20µg). CFO:(30 µg).
Source: Prepared by the authors (2024)

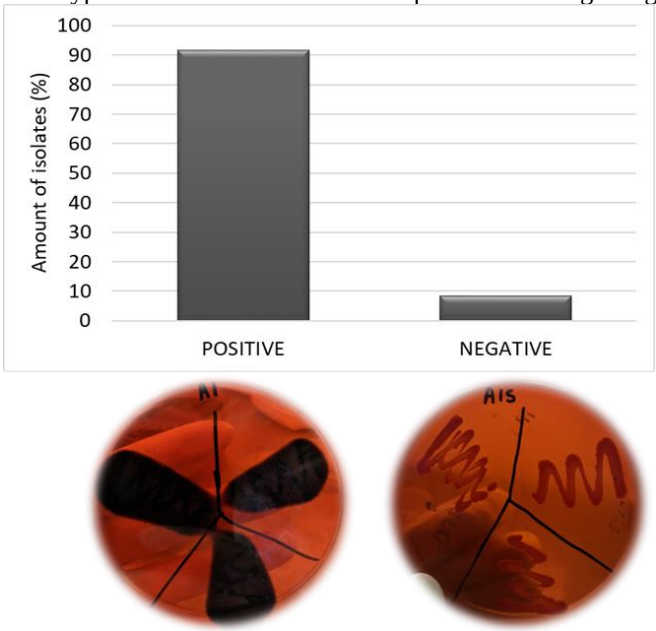
Biofilm formation is among the factors that favor the resistance of microorganisms to antimicrobials. Biofilms are more resistant than cells in their planktonic state and can survive sanitization processes and high concentrations of antimicrobials (Boari *et al*., 2009; Van Houdt; Michiels, 2010). It is therefore essential to use quick and simple methods to detect biofilm-producing bacteria, allowing more effective control and prophylaxis measures to be implemented. Culture on Congo red agar and the microplate adhesion test are used to detect bacterial biofilm production; the former verifies phenotypic expression by staining colonies, while the latter measures the amount of biofilm matrix (Sharma; Tyagi; Shrivastava; 2023). Methods for quantifying extracellular proteins and polysaccharides help to characterize the composition of the biofilm matrix, with variations in protein and polysaccharide concentrations being one of the factors that contribute to assessing the virulence of each strain (Chagas *et al*., 2015).

In the biofilm test, using the Congo Red Agar test, 96.66% of the samples showed the ability to form biofilms. However, in the Plate Adherence test, only 16.66% of the isolates were positive for biofilm formation (Figure 4, Figure 5), showing discrepancies between the two techniques for determining biofilm production by *S. aureus* strains. Corroborating these results, Cruz *et al.* (2021), when evaluating multidrug-resistant *S. aureus* isolated from the milk of cows with mastitis, found no association between the two techniques, which may be explained by the fact that the strains have varying polysaccharide concentrations and each compound acts differently in the formation and pathogenicity of the biofilm, as also reported in studies by Chagas *et al*., 2015.

Achek *et al.* (2020) analyzed biofilm formation by *S. aureus* isolated from different sources in Algeria and found that of 23 isolates from sheep's milk, only 17.9% tested positive in the Plate Adhesion test. On the other hand, only 3% tested positive for biofilm formation in the Congo Red test. Ahmed *et al* (2022) in a study carried out in Pakistan observed that of the 159 *S aureus* isolates obtained from bovine milk, 35.85% were positive for biofilm based on Congo red agar and 30.09% on the microplate test.

Congo Red is a secondary diazo dye that varies in color from blue to red depending on pH (Donadu *et al.*, 2022). This dye is widely used in methodologies aimed at identifying this production, because it detects the production of the polymeric extracellular matrix, also known as slime, which is an important factor for detecting biofilm (Rabha *et al*., 2021). In this case, samples that produce this matrix, i.e. are positive for biofilm production, acquire a blackish-red color in the medium in which it has been added, and if they are not producers, i.e. are negative for biofilm production, they acquire a brownish color (Figure 4). However, there are reports that the Congo Red methodology, despite being cheap and easy to perform, has low sensitivity and accuracy, which makes its results unreliable for determining biofilm (Sharlee; Sumangala, 2020; Shakya *et al*., 2022).

Figure 4 - Phenotypic characterization of biofilm production using Congo Red Agar



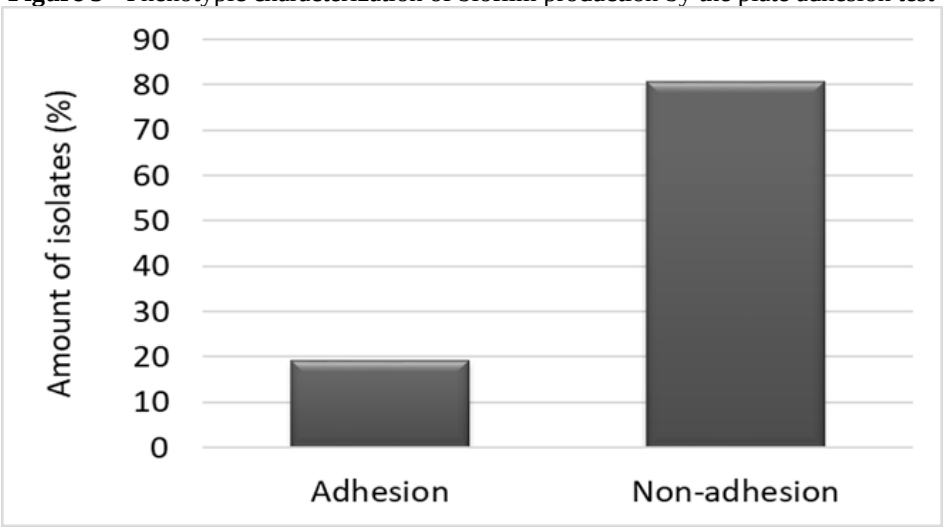
Blackish-red color = *S. aureus* colony positive for biofilm production. Brownish color = *S. aureus* colony negative for biofilm production.
Source: Prepared by the authors (2024)

The plate adherence test is used to quantify the formation of biofilms produced by *Staphylococcus sp.* It can also serve as an indicator of the pathogenicity of microorganisms. Strains that produce slime in the microplate test indicate a possible ability to adhere to mammary gland tissue. The presence of this mucopolysaccharide makes it difficult to treat mastitis with antibiotics and affects the host's innate immune response (Melo *et al*., 2012).

The low percentage of positives in the plate adhesion test (Figure 5) can be explained by the growth conditions used, the lack of ability of some biofilms to adhere to polystyrene plates due to different adhesion mechanisms, the presence of imperfections or grease. In addition, divergences in the detection of biofilm formation may be associated with the phase of variation in biofilm production (Melo *et al.*, 2012).

It should also be noted that the methods applied only measured the phenotypic expression of biofilm production, either by the morphology of the colonies or by the staining of the extracellular substance with crystal violet. Hoch (2022), analyzing bacterial adhesion and proliferation on stainless steel surfaces, proposes that further research, including a wider variety of surface patterns and bacterial strains, is necessary for the conclusions to be more definitive, since real biofilms are usually composed of several species, cohabiting or competing with each other.

Figure 5 - Phenotypic characterization of biofilm production by the plate adhesion test



Source: Prepared by the authors (2024)

Previous studies have shown that there is a relationship between the resistance profile of microorganisms to antibiotics and biofilm production (Cruz *et al*., 2021; Marques, 2016; Cunha., *et al.*, 2019;). In the present study, a Kappa index of 0.78 was obtained for agreement between resistance to the antibiotic ASB and biofilm formation determined by the Congo Red test (Table 1), showing excellent agreement between the parameters. Values greater than 0.75 represent excellent agreement. Values below 0.40 represent poor agreement and values between 0.40 and 0.75 represent average agreement (Landis; Koch, 1977).

Subjecting the data obtained to the Chi-square test, there was an association between *S. aureus* resistance to the antibiotics IPM and ASB and biofilm production as determined by the Congo Red test, but there was no association between antibiotic resistance and biofilm formation as determined by the plaque adherence test (Table 2). These results disagree with Cruz *et al.* (2021) who observed a relationship between biofilm formation obtained by the plaque adherence test and resistance to beta-lactam antimicrobials, but found no association when using the Congo Red test.

It has been reported in the literature that the relationship between resistance to beta-lactam class antimicrobials and biofilm formation in isolates can be explained by the extracellular matrix of the biofilm affecting the effectiveness of the antimicrobial due to diffusion limitation, with biofilm-producing microorganisms being more tolerant to these drugs (Blair; Richmond; Piddock, 2015).

Table 1 - Concordance between antibiotic resistance and biofilm formation obtained by the Congo Red and Adherence tests

Antibiotic	Cohen's Kappa	Standard error	95% confidence interval	
			lower	upper
Congo red test				
CFO	0.08	0.05	-0.02	0.17
IPM	-0.15	0.12	-0.38	0.08
ASB	0.78	0.21	0.37	1.19
OXA	-0.13	0.05	-0.23	-0.03
ME1	-0.17	0.07	-0.30	-0.03
Adhesion test				
CFO	-0.06	0.19	-0.43	0.32
IPM	0.24	0.23	-0.21	0.69
ASB	0.02	0.02	-0.02	0.05
OXA	-0.07	0.09	-0.24	0.11
ME1	0.10	0.06	-0.02	0.22

Source: Prepared by the authors (2024)

Table 2 - Association between antimicrobial resistance profile and biofilm formation obtained by Congo Red and Adherence tests

Antibiotic	Chi^2	p Value
Congo red test		
CFO	1.02	0.31
IPM	4.91	0.02
ASB	16.61	0.00
OXA	0.44	0.50
ME1	0.81	0.37
Adhesion test		
CFO	0.08	0.77
IPM	1.41	0.23
ASB	0.21	0.65
OXA	1.12	0.29
ME1	1.26	0.26

Source: Prepared by the authors (2024)

Antimicrobial resistance in *S. aureus* has been widely documented, especially in strains associated with persistent infections and the ability to form biofilms. Several studies have highlighted that this ability to form biofilms significantly increases the resistance of bacteria to antimicrobials, creating a cycle of bacterial persistence and ineffectiveness of conventional treatments. In the study by Bohrz *et al.* (2021), it was observed that the resistance profile of *S. aureus* strains is indicative of biofilm production using Congo Red Agar and the adherence test. These results indicated that antibiotic resistance and biofilm formation are interlinked adaptive strategies that facilitate the persistence of *S. aureus* in production environments, even after cleaning procedures.

The use of phenotypic methods to detect biofilms formed by multidrug-resistant *S aureus* is crucial for safety in the food industry. These biofilms, once formed on contact surfaces in food processing, represent a persistent reservoir of antibiotic-resistant microorganisms, making complete removal by conventional sanitization methods difficult. The resistance of *S. aureus* to various antimicrobial agents increases the risk of contamination

of food products, since the biofilm acts as a barrier that protects the bacteria, allowing them to survive and proliferate (Chagas *et al*., 2015).

In dairy production, the presence of biofilm-forming multidrug-resistant *S. aureus* is associated with high rates of subclinical bovine mastitis, an infection that often has no clear symptoms in the animals, making it difficult to control and facilitating the spread among the herd (Chagas *et al.,* 2015; Bohrz *et al.,* 2021). The use of phenotypic methods to characterize these biofilms allows the identification of resistance to conventional cleaning treatments, making it possible to optimize hygiene protocols and reduce the spread of multidrug-resistant pathogens in order to maintain microbiological quality and reduce public health risks.

IV. Conclusion

The *S. aureus* isolates from bovine milk showed resistance to various antimicrobial bases, with the majority showing the ability to form biofilms using the Congo red technique. On the other hand, for the plate adhesion analysis, the number of isolates was lower, indicating that not all phenotypic tests for detecting biofilm formation give the same result, thus highlighting the need to carry out other combined tests, such as gene detection and evaluation of their ability to adhere to stainless steel or polypropylene coupons, for a more accurate analysis of the biofilm formation capacity of these multi-resistant isolates.

The association between the resistance profile to the antimicrobials IPM and ASB and the ability to form biofilms by *S. aureus* isolated from bovine mastitis was also observed, demonstrating the greater ability of these isolates to develop resistance mechanisms under adverse conditions.

Studies of antimicrobial multidrug resistance, together with their ability to produce biofilms, are important tools for guiding well-planned and satisfactory antibiotic therapy. In addition, they are important from the point of view of food safety, as they can adhere to and resist disinfection on production surfaces, transferring resistance genes and representing a danger to public health. Rapid detection methods are essential for controlling these pathogens in the food industry.

Acknowledgments

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