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Antimicrobial resistance of bacterial agents of bovine mastitis from dairy properties in the metropolitan region of São Luís – MA

Resistência antimicrobiana de agentes bacterianos da mastite bovina de propriedades de leiteiras da região metropolitana de São Luís – MA

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ABSTRACT

The objective of this study was to evaluate the “in vitro” antimicrobial susceptibility profiles of *Staphylococcus* spp. and *Streptococcus* spp. isolated from the milk of cows with infectious mastitis. This study was conducted on 14 dairy farms located in four municipalities on the Island of São Luís, Maranhão, Brazil. The isolates were obtained by bacteriological cultivation of milk samples, followed by morphological and biochemical characterization. Antimicrobial susceptibility tests were performed using the disk diffusion method. The results showed that none of the principles were 100% effective against the isolates. Penicillin, ampicillin, and amoxicillin showed higher rates of resistance in isolates of Coagulase-positive *Staphylococcus* (80%, 77.2%, and 77.2%, respectively), Coagulase-negative *Staphylococcus* (80%, 75.4%, and 75.4%, respectively), and *S. aureus* (94.3%, 88.6%, and 88.6%, respectively). Isolates of *Streptococcus* showed a higher frequency of resistance to streptomycin (94%), tetracycline (86%), and lincomycin (76%). Isolates from the Coagulase-negative *Staphylococcus* group had the highest multidrug resistance profile, with 32.65% of the strains being simultaneously resistant to more than eight antibiotics. A high frequency of isolates of agents that cause bovine mastitis with multidrug-resistant phenotypes was determined, making it necessary to base the treatment of animals on the diagnosis of the causative pathogen and patterns of sensitivity to antimicrobials.

Keywords: milk, *Streptococcus*, *Staphylococcus*, beta-lactamics, and tetracyclines.

RESUMO

Objetivou-se avaliar o perfil de suscetibilidade antimicrobiana “in vitro” de isolados do *Staphylococcus* spp. e de *Streptococcus* spp. originados do leite de vacas com mastite

infeciosa. O estudo foi realizado em 14 propriedades leiteiras situadas em quatro municípios da Ilha de São Luís, Maranhão. Os isolados foram obtidos do cultivo bacteriológico das amostras de leite seguido de caracterização morfológica e bioquímica. Realizaram-se os testes de suscetibilidade antimicrobiana pela técnica de difusão em discos para 14 princípios antimicrobianos. Os resultados mostraram que nenhum dos princípios apresentou 100% de eficácia contra os isolados. A penicilina, ampicilina e amoxicilina apresentam maiores índices de resistência nos isolados de *S. coagulase* positiva (80%, 77,2% e 77,2%), *S. coagulase* negativa (80%, 75,4%, 75,4%) e *S. aureus* (94,3%, 88,6% e 88,6%), respectivamente. Os isolados do gênero *Streptococcus* apresentaram maior frequência de resistência a estreptomicina (94%), tetraciclina (86%) e lincomicina (76%). Os isolados do grupo *S. coagulase* negativa o com maior perfil de multirresistência, com 32,65% das cepas resistentes a mais de oito antibióticos, simultaneamente. Concluiu-se que há alta frequência de isolados de agentes causadores de mastite bovina com fenótipos de multirresistência, sendo necessário embasar o tratamento dos animais no diagnóstico do patógeno causador e nos padrões de sensibilidade a antimicrobianos.

Palavras-chave: leite, *Streptococcus*, *Staphylococcus*, beta-lactâmicos, tetraciclínas.

INTRODUCTION

Bovine mastitis frequently occurs in dairy farms worldwide and is considered one of the main causes of the indiscriminate use of antimicrobials in herds (CENITI et al., 2017). This has led to serious animal and public health issues. through a consistent selection process for infectious agents with multiple antibiotic resistances (WHO, 2019).

Many bacterial species have been identified as the causative agents of intramammary infections responsible for bovine mastitis. *Staphylococcus aureus* and *Streptococcus agalactiae* are the main bacterial species responsible for contagious mastitis, which is transmitted between cows during milking (CHEN & HAN, 2020). These pathogens are highly prevalent in cases of clinical and subclinical mastitis and often make the mammary gland chronically infected and act as a reservoir of infectious agents (RABELLO et al., 2023).

The main strategy for treating infectious mastitis is the administration of antimicrobials via intramammary infusion and intramuscular injections

(HOSSAIN et al., 2017). When conducted effectively, this tool reduces the number of pathogenic microorganisms in milk, increases the number of healthy quarters, and improves animal welfare by reducing the number of infected cows on dairy farms (SANTOS & FONSECA, 2007). However, the massive use of antimicrobials in animal production without professional supervision has contributed to the progressive increase in bacteria with a multidrug resistance (MDR) profile, limiting animal treatment and affecting productivity (RIBEIRO et al., 2023). The failure of antibiotic therapy for mastitis due to antimicrobial resistance has implications for public health as it increases the possibility of chemical residues in milk in the environment, in addition to the transmission of resistant bacteria through the consumption of dairy products (RABELLO et al., 2023). The spread of bacteria with MDR profiles has had a major impact on global health, increasing the risk of death by 50% in patients undergoing treatment (WHO, 2018). Thus, surveillance and the impact of antimicrobial resistance have become

high priorities for global political bodies involved in One Health, generating guidance on the prudent use of critically important antimicrobials, including those used in animal therapy. (WOAH, 2021; WHO, 2019).

One of the reasons for mastitis treatment failure is the use of antimicrobial agents before or without the results of antimicrobial susceptibility testing for pathogens (CENITI et al., 2017). These are useful for monitoring the development of pathogen resistance and assisting veterinarians in choosing the appropriate medication for mastitis therapies. Thus, this study aimed to evaluate the “in vitro” antimicrobial sensitivity profiles of *Staphylococcus* spp. and *Streptococcus* spp. isolated from cows with clinical and subclinical mastitis on dairy farms in the Metropolitan Region of São Luís.

MATERIAL AND METHODS

A total of 135 isolates of *Staphylococcus* spp. and 50 isolates of *Streptococcus* spp. originated from dairy cow milk, 27 from clinical cases, and 215 from subclinical cases, were analyzed. Isolates were obtained from 40 dairy farms located in São Luís (n=10), Paço do Lumiar (n=1), Raposa (n=1), and São José de Ribamar (n=2) in the state of Maranhão.

The sampled herd consisted of primiparous and multiparous Holstein cows that were crossed with zebu, raised in a semi-intensive system under manual milking, and not subjected to antibiotic therapy for up to 1 week before harvest. Information was obtained on the antimicrobials used in previous cases of clinical mastitis in the properties visited, with the principles of gentamicin (7/14), streptomycin associated with penicillin (3/14), and tetracycline (10/14) being reported as those used.

The isolates were obtained from 0.01 mL (10 µL) aliquots of the milk sample sown on Blood Agar base plates, containing 5% defibrinated sheep blood, and incubated in a bacteriological oven at 37 °C, carrying out be read after 24, 48, and 72 h. The morphological characteristics of the colonies (appearance, size, color, and type of hemolysis) and the morphological characteristics of the microbial cells (shape, arrangement, and color) were observed. The bacterial genera *Staphylococcus* and *Streptococcus* were identified and classified (APHA, 2015). Colonies of *Staphylococcus* spp. were subjected to biochemical tests for producing free coagulase, DNase, acetoin, glucose fermentation (anaerobiosis), and mannitol and were classified into 65 coagulase-negative *Staphylococcus* (SCN), 35 coagulase-positive *Staphylococcus* (SCP), and 35 strains of *Staphylococcus aureus* (SA) (APHA, 2015).

After isolation and identification, the *Staphylococcus* and *Streptococcus* strains were subjected to “in vitro” sensitivity tests for antimicrobials using the disc diffusion method, following the recommendations of the Clinical and Laboratory Standards Institute (2013). Bacterial strains were cultured in 2 mL of brain heart broth (BHI) and incubated at 37 °C until turbidity (corresponding to tube 0.5 on the MacFarland scale). Then, seeding was carried out on Müeller Hinton Agar and then the discs impregnated with antimicrobials were deposited: gentamicin (10 µg), streptomycin (10 µg), penicillin (10 IU), ampicillin (10 µg), amoxicillin (10 µg), oxacillin (1 µg), vancomycin (30 µg), erythromycin (15 µg), lincomycin (2 µg), tetracycline (30 µg), norfloxacin (10 µg), enrofloxacin (5 µg), bacitracin (10 I.U.), and sulfazotrim (sulfa 25 mcg + trimethoprim 5 mcg). Incubation was

carried out at 37 °C for 24 h and then the diameters of the inhibition zones were measured. A standard strain of *Staphylococcus aureus* ATCC 25923 was used for the quality control. Phenotypes with MDR were considered, isolates with simultaneous resistance to two or more classes of antimicrobials, as defined by the Agência Nacional de Vigilância Sanitária do Brasil (BRASIL, 2021).

RESULTADOS E DISCUSSÃO

The susceptibility profiles of *Staphylococcus* isolates showed that none of the active ingredients evaluated were 100% effective (Table 1). Bacitracin, sulfazotrim, and gentamicin were the antimicrobials with the highest sensitivity rates against isolates of *Staphylococcus*.

Table 1. Relative frequency (%) of sensitivity (S), intermediate sensitivity (I), and resistance (R) “in vitro” to antimicrobials of *Staphylococcus* isolated from cows with mastitis

| Antimicrobial | Coagulase-positive (n = 35) | | | Coagulase-negative (n = 65) | | | <i>S. aureus</i> (n = 35) | | |
|------------------------------|--------------------------------|------|------|--------------------------------|------|------|------------------------------|------|------|
| | S | I | R | S | I | R | S | I | R |
| Penicillins Class | | | | | | | | | |
| Ampicillin | 22,8 | 0 | 77,2 | 24,6 | 0 | 75,4 | 11,4 | 0 | 88,6 |
| Amoxicillin | 22,8 | 0 | 77,2 | 24,6 | 0 | 75,4 | 11,4 | 0 | 88,6 |
| Penicillin | 20,0 | 0 | 80,0 | 20,0 | 0 | 80,0 | 5,7 | 0 | 94,3 |
| Oxacillin | 68,6 | 0 | 31,4 | 63,0 | 0 | 37,0 | 77,1 | 2,8 | 20 |
| Polypeptide Class | | | | | | | | | |
| Bacitracin | 91,4 | 0 | 8,6 | 89,2 | 7,8 | 3,0 | 91,4 | 0 | 8,6 |
| Macrolide Class | | | | | | | | | |
| Erythromycin | 51,4 | 17,4 | 31,4 | 63,0 | 23,2 | 13,8 | 54,3 | 25,7 | 20 |
| Fluorquinolones Class | | | | | | | | | |
| Enrofloxacin | 68,5 | 0 | 31,4 | 76,9 | 3,1 | 20,0 | 88,6 | 5,7 | 5,7 |
| Norfloxacin | 77,2 | 0 | 22,9 | 75,4 | 13,9 | 10,7 | 88,6 | 5,7 | 5,7 |
| Aminoglycosides Class | | | | | | | | | |
| Streptomycin | 60,0 | 0 | 40,0 | 40,0 | 0 | 60,0 | 77,1 | 0 | 22,9 |
| Gentamicin | 81,5 | 0 | 18,5 | 83 | 0 | 17,0 | 91,4 | 0 | 8,6 |
| Lincosamide class | | | | | | | | | |
| Lincomycin | 17,1 | 31,2 | 51,4 | 18,4 | 29,3 | 52,3 | 11,4 | 45,8 | 42,8 |
| Folate Inhibitors | | | | | | | | | |
| Sulfazotrim | 92,0 | 0 | 8,0 | 81,5 | 0 | 18,5 | 94,2 | 0 | 5,8 |
| Tetracyclines Class | | | | | | | | | |
| Tetracycline | 57,1 | 0 | 42,9 | 61,2 | 0 | 38,8 | 54,3 | 0 | 45,7 |

The efficacy of bacitracin and sulfazotrim against the *Staphylococcus* groups may be related to the lack of use of these principles in the treatment of bovine mastitis on the properties visited, allowing the presence of bacteria sensitive to these antimicrobials.

Bacitracin is a drug that acts by inhibiting the formation of peptidoglycans in the cell wall of gram-positive bacteria; its use on the skin is recommended, and cases of bacterial resistance to this drug are rare (MACEDO et al., 2023).

Sulfazotrim has a synergistic effect between sulfa and trimethoprim, presenting a broad spectrum of bactericidal actions, which can hinder bacterial resistance (MACEDO et al., 2023). Similar results were reported by Mesquita et al. (2019) in antibiogram tests with *S. aureus* isolated from dairy farms in Minas Gerais, which is considered to be the principle with the greatest efficacy (98%) against mastitis. Samples of *S. aureus* (92.9%), SCP (71.4%), and SCN (72.0%) showed high sensitivity to gentamicin, an antimicrobial used to treat clinical mastitis, at 7 of the 14 properties analyzed. These results are similar to the high sensitivity rates of *Staphylococcus* spp. to gentamicin reported in studies on dairy herds in Brazil (OLIVEIRA et al., 2011; SOUZA et al., 2016; BENITES et al., 2021). Gentamicin is considered one of the most effective antimicrobial agents for treating staphylococcal mastitis, showing good correlation “in vitro” and “in vivo,” even with its wide use in mastitis treatment (ZAFALON et al., 2007).

Despite belonging to the same group as the aminoglycosides, the efficacy of streptomycin against strains SCP (60%), SCN (40.8%), and *S. aureus* (78.6%) was lower than that of gentamicin. Streptomycin is a first-generation broad-spectrum aminoglycoside that also acts on gram-negative bacteria and is often associated with penicillin. In this study, this antibiotic was used in association with penicillin to treat diseases in 3 of the 14 properties visited. Chromosomal resistance to streptomycin, but not to

other aminoglycosides, is very common and generally develops during antibiotic therapy (REYGAERT, 2018).

Evaluation of the antimicrobial resistance profile revealed that most SCP, SCN, and *S. aureus* strains showed a high percentage of resistance to beta-lactam penicillin, amoxicillin, and ampicillin. These beta-lactams are considered penicillase-resistant penicillins and are the most widespread antimicrobials used to treat animal diseases (RABELO et al., 2020).

However, *Staphylococci* isolated from mastitis cases almost always show high levels of resistance to PLSP beta-lactams, which restrict their use in the treatment of mastitis (RABELO et al., 2020). Penicillin was used in five properties visited, which may have contributed to the selection of resistant *Staphylococcus* strains present in intramammary infections. Susceptibility assessment research on *Staphylococcus* isolated from bovine mastitis in several Brazilian regions exhibits a phenotypic pattern of high resistance of this pathogen to penicillins (FREITAS et al., 2018; MESQUITA et al., 2019; BENITES et al., 2021; FREU et al., 2022; GONÇALVES et al., 2023).

In the beta-lactam class, oxacillin was the only ingredient that showed good efficacy, represented by the lowest resistance rates compared with the SCP (31.4%), SCN (37%), and *S. aureus* (20%) groups. Strains of *Staphylococcus* resistant to this principle have a phenotype of resistance to all available β -lactam antibiotics, being used to identify methicillin resistant *Staphylococcus aureus* (MRSA) (CLSI, 2018). Although high rates of resistance to oxacillin are infrequent in specimens isolated from mammary glands, it is noteworthy that the occurrence of *Staphylococcus* isolates with MRSA phenotypes is worrying, as they cause

hospital-acquired infections, and there is a risk that these bacteria are transmitted to humans. human health owing to the consumption of contaminated dairy products (SILVA et al., 2018).

The high frequency of antimicrobial-resistant bacterial strains found in the herd in the properties of the São Luís Island dairy basin may be related to the indiscriminate use of these medications without professional guidance. In almost all the properties evaluated, producers used broad-spectrum antimicrobials, such as penicillin and tetracycline, based solely on the availability of the product in the local market. This may have exerted selective pressure on the bacterial population that caused contagious mastitis in cattle herds.

Streptococcus spp. showed higher levels of sensitivity to the antimicrobials tested (Table 2), with bacitracin (98%) and the β -lactam group showing the greatest “in vitro” efficacy. Streptomycin, tetracycline, and lincomycin showed the highest antimicrobial resistance rates (94%, 86%, and 76%, respectively).

In *Streptococcus*, the high sensitivity to penicillin and resistance to aminoglycosides (streptomycin and tetracyclines) in bovine mastitis isolates has been a phenotypic pattern described in several studies in Brazil (SILVA et al., 2017; MIRANDA et al., 2018 ; MESQUITA et al., 2019; COSTA et al., 2021). These results reflect the extensive use of veterinary antimicrobials in the Brazilian market for treating intramammary infections.

Table 2. Susceptibility profile of 50 *Streptococcus* spp. isolates. of bovine mastitis against antimicrobials

| Antimicrobiano | Perfil de suscetibilidade (%) | | |
|------------------------------|-------------------------------|---------------|-------------|
| | Sensibilidade | Intermediária | Resistência |
| Penicillins Class | | | |
| Ampicillin | 90 | 0 | 10 |
| Amoxicillin | 92 | 0 | 8 |
| Penicillin | 82 | 0 | 18 |
| Oxacillin | 92 | 0 | 8 |
| Polypeptide Class | | | |
| Bacitracin | 98 | 0 | 4 |
| Macrolide Class | | | |
| Erythromycin | 28 | 22 | 50 |
| Fluorquinolones Class | | | |
| Enrofloxacin | 82 | 14 | 4 |
| Norfloxacin | 84 | 14 | 2 |
| Aminoglycosides Class | | | |
| Streptomycin | 6 | 0 | 94 |
| Gentamicin | 68 | 2 | 30 |
| Lincosamide class | | | |
| Lincomycin | 24 | 0 | 76 |
| Folate Inhibitors | | | |

| | | | |
|---------------------|----|---|----|
| Sulfazotrim | 68 | 0 | 32 |
| <hr/> | | | |
| Tetracyclines Class | | | |
| Tetracycline | 12 | 2 | 86 |

Notably, tetracycline was reported as the main antimicrobial agent used in the treatment of herd diseases in 10 of the 14 properties. Based on the time of manifestation of the clinical signs of the disease, the dosage of the medication and the duration of treatment with antimicrobials were incorrect. This may have contributed to high levels of resistance. Resistance to tetracycline is mediated by a plasmid that confers the capacity to produce the Tet protein, which is responsible for transporting this antibiotic out of the bacterial cells. This plasmid is highly transferable to other bacteria and is capable of conferring resistance to other antimicrobials (SILVA et al., 2017).

In the evaluation of multiple antimicrobial resistance profiles of the bacterial strains (Figure 1), majority of SCP (59%), SCN (46%), SA (70%), and *Streptococcus* (56%) isolates were found to have a simultaneous resistance profile for three to seven antimicrobials. The Coagulase-negative *Staphylococcus* group had the highest MDR, with 32% of the isolates being simultaneously

resistant to more than eight antimicrobials.

MDR of the *Staphylococcus* strains isolated from bovine mastitis has been reported by several studies (RABELO et al., 2020; MESQUITA et al., 2021; FREU et al., 2022; GONÇALVES et al., 2023). There is strong evidence that resistance genes can be transferred and disseminated among different bacterial populations, including among different groups of the same genus (XU et al., 2022). Therefore, the bovine mastitis pathogens that presented a high frequency of MDR profile in this study may function as a reservoir of resistance genes for other pathogenic or commensal bacterial species and genera (GOLÇALVES et al., 2023). Given the vision of One Health, which is based on the interrelationships between humans, animals, and the environment, the surveillance of pathogens with an MDR profile originating from livestock farming has increased, as the presence of MDR mastitis agents is directly related to the spread of microorganisms resistant to dairy products and other foods (PAHO/WHO, 2022).

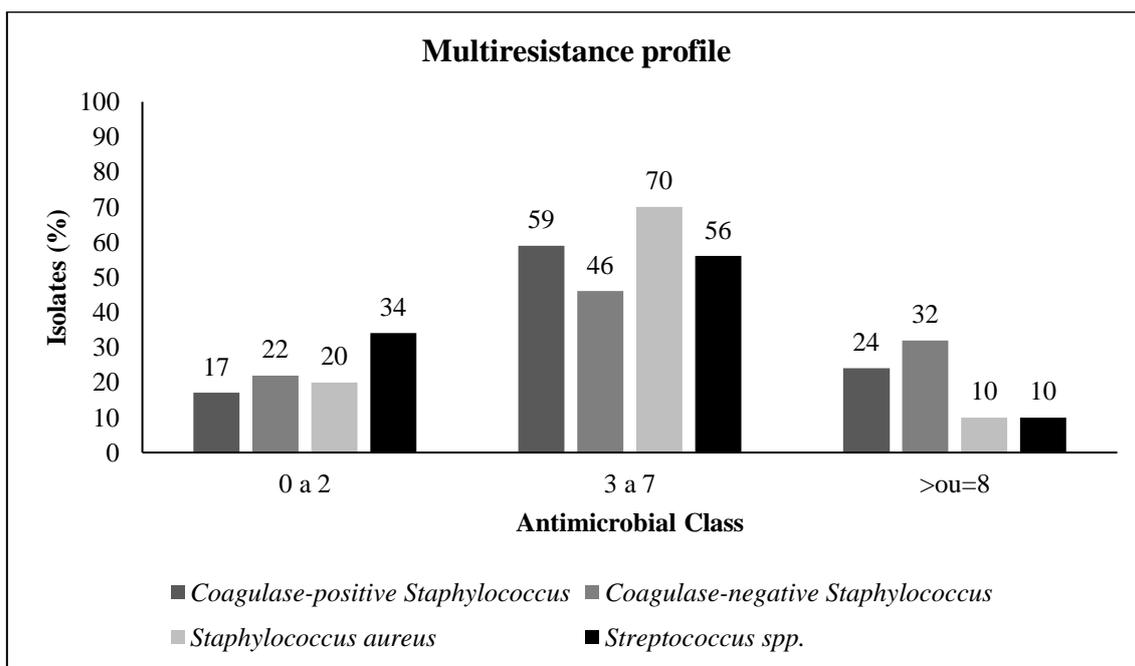


Figure 1. Antimicrobial multiresistance profile of groups of *Staphylococcus* and *Streptococcus* spp. isolated from cows with mastitis, São Luís-MA

The high bacterial resistance to several antimicrobials is worrisome, as it restricts the treatment options for bovine mastitis in dairy herds. Furthermore, the results of the high frequency of resistance to the classes of penicillins, tetracyclines, and antimicrobials are considered critically important for veterinary medicine according to the recommendations of the World Organization for Animal Health (WOAH, 2021), and under priority for evaluation of the risk of resistance. To reduce the selective pressure that favors the emergence of multiresistant isolates, the importance of carefully choosing the antimicrobial used in the therapeutic protocol for bovine mastitis is emphasized under professional supervision, based on microbiological tests to isolate the etiological agent and antimicrobial sensitivity tests (PAHO/WHO, 2022).

CONCLUSION

Staphylococcus isolates. and *Streptococcus* spp. of bovine mastitis from dairy herds in the Metropolitan Region of São Luís, Maranhão, showed a high frequency of resistance to the penicillin and tetracycline classes of antimicrobials; thus, there was a high occurrence of phenotypes with multiple resistance to antimicrobials. The coagulase-negative *Staphylococcus* group presented itself as an emerging pathogen with high multiresistance to the antimicrobials tested and could be a risk factor for spreading resistant bacteria to milk and dairy products offered to consumers.

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