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Mastitis-Associated Bacterial Etiologies and Antimicrobial Susceptibility Profiles

Etiologias Bacterianas Associadas à Mastite e Perfis de Suscetibilidade a Antimicrobianos

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Abstract

Cattle mastitis is common in dairy farms, affecting animal welfare and the profitability of production. Mastitis contributes to antimicrobial agent consumption in animal and agriculture sectors, which might impact the occurrence and dissemination of multidrug-resistant microorganisms. To overcome this scenario, it is important to identify the etiology and treat it correctly, which does not occur in the majority of farms in Brazil. This study identified the etiology, by MALDI-TOF MS, and analyzed the antimicrobial susceptibility profile of bacterial isolates recovered from milk samples of 122 animals, with clinical or subclinical mastitis. Sampling occurred during June and July 2021, in farms located in the cities of Lima Duarte, Pedro Teixeira, and Bias Fortes, mesoregion Zona da Mata of Minas Gerais, the major milk producer state of Brazil. The prevalent species was *Staphylococcus aureus* (27.7%), followed by *Streptococcus uberis* (13.1%) and *Streptococcus agalactiae* (8%). *Staphylococcus* and *Streptococcus* species showed resistance to several antimicrobials, however, they were susceptible to the recommended veterinary drugs ceftiofur and penicillin-novobiocin. *Escherichia coli* was recovered in 3.6% and showed resistance to ceftiofur and other drugs. Multidrug resistance has varied from 2.6% in *S. aureus* to 60% in *E. coli* isolates. Results show that all classical mastitis pathogens continue to cause infection in this region, despite improved control programs. The spread of bacterial isolates resistant to veterinary and human antimicrobials, besides contributing to treatment failure, represents a major concern under the view of the one health approach.

Keywords: Cattle Mastitis. Bacterial Pathogens. MALDI-TOF MS. Antimicrobial Resistance.

Resumo

A mastite bovina é comum nas fazendas leiteiras, afetando o bem-estar animal e a lucratividade da produção. A mastite contribui para o consumo de antimicrobianos nos setores animal e agrícola, o que pode impactar na ocorrência e disseminação de microrganismos multirresistentes. Para superar esse cenário, é importante identificar a etiologia e tratá-la corretamente, o que não ocorre na maioria das fazendas do Brasil. Este estudo identificou a etiologia, por MALDI-TOF MS, e analisou o perfil de suscetibilidade antimicrobiana de bactérias isoladas de amostras de leite de 122 animais, com mastite clínica ou subclínica. As coletas ocorreram entre junho e julho de 2021, em fazendas localizadas nos municípios de Lima Duarte, Pedro Teixeira e Bias Fortes, mesorregião Zona da Mata de Minas Gerais, o maior estado produtor de leite do Brasil. A espécie prevalente foi Staphylococcus aureus (27,7%), seguida de Streptococcus uberis (13,1%) e Streptococcus agalactiae (8%). Espécies de Staphylococcus e Streptococcus mostraram resistência a vários antimicrobianos, entretanto, foram suscetíveis a ceftiofur e penicilina-novobiocina, drogas recomendadas na veterinária. Escherichia coli foi isolada em 3,6% e apresentou resistência ao ceftiofur e outras drogas. A resistência a múltiplas drogas variou de 2,6% em S. aureus a 60% em E. coli. Os resultados mostram que todos os patógenos clássicos da mastite continuam a causar infecção nessa região, apesar dos programas de controle aprimorados. A disseminação de amostras bacterianas resistentes a antimicrobianos veterinários e humanos, além de contribuir para falhas de tratamento, representa uma grande preocupação sob a ótica da abordagem de saúde única.

Palavras-chave*: Mastite Bovina. Patógenos Bacterianos. MALDI-TOF MS. Resistência antimicrobiana.*

1 Introduction

Cattle mastitis is a common disease with a relevant impact on animal welfare and the profitability of dairy farms. Mastitis strongly impacts milk quality, which has motivated herds' development of control programs. Standards in milk quality have been established and when these are achieved, the industry financially rewards the producer. This policy establishes a virtuous circle culminating in practices that result in milk production with better sanitary quality. Strict hygiene measures during the milking process and control of mastitis incidence are mandatory to achieve the legally required milk quality standards. Well-known actions include pre-milking teat disinfection, drying off with individual towels, usage of milking gloves, sanitation of teat cups, and effective post-milking teat dipping (Oliveira Cruz *et al*., 2022; Ruegg, 2017).

Despite the adoption of such good milking practices, some animals develop clinical or subclinical mastitis. Identification of such animals should be done promptly, using methodologies such as the strip cup test, California mastitis test (CMT), and somatic cell count (SCC). Bacteria are the most important etiological agents, especially species of *Streptococcus* and *Staphylococcus* genera. Mastitis accounts for the majority of antimicrobials used by dairy cows, with beta-lactams, lincosamides, and tetracyclines being largely used for prevention and therapy (Oliver; Murinda, 2012). Antimicrobial usage is a constant concern for public health since it is the main factor associated with the selection of resistant microorganisms (Ashraf; Imran, 2020). To control the evolution of antimicrobial resistance, it is essential to use antimicrobials rationally. For this, the therapeutic choices should be based on the identification of the infectious agents as well as on antimicrobial susceptibility tests. Unfortunately, this is not the scenario on most dairy farms in Brazil, where cases of mastitis are treated empirically.

Brazil is one of the major milk producers in the world and the state of Minas Gerais is the major producer, with almost one-third of the national production (IBGE, 2023). The cities of Lima Duarte, Bias Fortes and Pedro Teixeira belong to Zona da Mata mesoregion. Despite this region not being the major producer of the state, Lima Duarte is one of the major milk producers of Zona da Mata, with production occurring mainly in small and medium properties (IBGE, 2023).

Considering the economic impact of milk production, as well as the importance of mastitis management in such farms, studies that identify the main infectious agents and their antimicrobial susceptibility profiles are essential to help veterinarians and producers make better therapeutic choices. Therefore, the present study identified the etiology, by MALDI-TOF MS, and analyzed the antimicrobial susceptibility profile of bacterial isolates recovered from milk samples of 122 animals, with clinical or subclinical mastitis.

2 Material and Methods

2.1 Study design

Bacterial isolates analyzed in this study were primarily recovered during a quality control program of one dairy industry, located in the city of Lima Duarte, Minas Gerais (Brazil). Between June and July 2021, milk aliquots of 122 animals were obtained, after teat disinfection, and deposited in sterile flasks. Selected animals were from farms located in the cities of Lima Duarte, Pedro Teixeira, and Bias Fortes with a previous history of elevated SCC, positive CMT, or positive strip cup test. All properties have a milk cooling tank and mechanical milking. Collected milk samples were transported in a thermal box, and inoculated into SmartColor 2® (OnFarm Tecnologia, Piracicaba, SP, Brazil), a commercial system of non-selective chromogenic culture media that allows a preliminary bacterial identification. Plates were incubated under conventional conditions (35°C for 24 to 48h).

2.2 Species identification

Colonies with distinct characteristics in the original chromogenic media were cultured on blood agar medium. Suggestive colonies of major mastitis pathogens were submitted to presumptive species identification by classical phenotypic tests (growth in salt mannitol agar and coagulase test for staphylococci isolates; growth in blood agar and hippurate hydrolysis test for streptococci isolates). All isolates were also submitted to species identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis (Bruker Daltonik, Bremen, Germany). Briefly, isolates were cultured overnight under conventional conditions. On the surface of a metal plate, one colony of each isolate was mixed with one drop of the MALDI-TOF matrix (2,5 dyhydroxybenzoic acid) in duplicate and submitted for analysis. The time of flight of the ionized housekeeping and ribosomal proteins was compared with the database, and a few minutes later, the software released the best score of species identification for each isolate.

2.3 Antimicrobial susceptibility testing (AST)

Staphylococcus and *Streptococcus* species, as well as *Escherichia coli* isolates, were submitted to AST by agar diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018; 2020). Macrolide resistance phenotypes were detected according to CLSI (2020). Other species were not submitted to AST because no standardization was available in CLSI, BrCAST, nor EUCAST documents specific for veterinary pathogens.

3 Results and Discussion

The primary chromogenic culture media used to inoculate milk samples of 122 animals yielded 155 distinct colonies. All of them were submitted to MALDI-TOF MS analysis, yielding identification in three categories of reliability: probable genus (PG) n=30 (19.35%), identified genus (IG) n=70 (45.16%) and identified species (IS) n=37 (23.87%). Eighteen (11.61%) isolates were not identified by this methodology.

Considering 137 isolates identified, it was possible to observe that both contagious (*S. aureus* and *S. agalactiae*) and environmental (non-*agalactiae Streptococcus* and *E. coli*) mastitis-associated etiologies were predominant. The most frequent species were: *Staphylococcus aureus* (38, 27.7%; IG=26, IS=12); *Streptococcus uberis* (18, 13.1%; PG=3, IG=9, IS=6); *Streptococcus agalactiae* (11, 8%; IG=8, IS=3); *Streptococcus dysgalactiae* (6, 4.4%; PG=1, IG=2, IS=3); *Escherichia coli* (3.6%; IS=5) and *Lactococcus lactis* (4, 2.9%; IG=3, IS=1). Other species associated with mastitis were coagulase-negative staphylococci (CNS, 16, 11.7%), with the predominance of *Staphylococcus chromogenes* (6). *Corynebacterium bovis* (9, 6.6%), and *Aerococcus viridans* (7, 5.1%) were also detected.

S. aureus has accounted for almost 30% of identified species, while CNS species accounted for less than one-third of staphylococci detected. Some authors have reported the emergence of CNS, especially *S. chromogenes* and *S. simulans* prevailing in both clinical and subclinical mastitis in several regions (Pyorala; Tuponent, 2009; Chung *et al*., 2021). Here, *S. chromogenes* was the most detected CNS.

S. agalactiae, the classical mastitis pathogen, accounted for 8% of isolates, although its recovery has become scarce or null in recent studies from other countries (Chung *et al*., 2021; Maalaoui *et al*., 2021; Crespi *et al*., 2022). Our findings, together with another previously reported in the same state (Oliveira *et al*., 2015) or other regions of the country (Oliveira *et al*., 2022), may indicate that, despite advances in mastitis control worldwide, in Brazil, this classical pathogen is still responsible for a great amount of cattle mastitis.

MALDI-TOF MS identification of streptococci species whose score was at PG or IG categories was confirmed by classical phenotypic tests. Staphylococci species identified by MALDI-TOF MS at PG or IG categories were also confirmed by traditional tests, except for two isolates. One isolate was identified as *S. aureus*, but the coagulase test was negative. Another isolate, identified as *S. chromogenes,* yielded a positive coagulase test. In general, MALDI-TOF MS allowed the identification of almost 90% of isolates. This fast and reliable methodology has been used for bacterial identification in the last two decades. Its application, although more robust in the clinical setting, has been extrapolated to environment and food analysis, including the dairy industry (Jang; Kim, 2018).

Regarding AST results, it is noteworthy that both staphylococci and streptococci species were susceptible to ceftiofur and penicillin-novobiocin, antimicrobials recommended by CLSI to be tested against isolates recovered for cattle mastitis (CLSI, 2018). Resistance to antimicrobials recommended for human infections has been detected, as shown in Table 1.

Table 1 - Antimicrobial susceptibility profile of *Staphylococcus* species recovered from cattle mastitis

Species (n)	Antimicrobial susceptibility (%)										
	CTF	PNM	TET	OFX	CLI	ERI	CFO	GEN	SUT	LNZ	PEN
S. aureus (38)	100	100	86,8	100	94,7	92,1	100	97,3	44,7	100	100
S. chromogenes (6)	100	100	100	100	100	100	100	100	66,6	100	100
S. equorum (1)	100	100	100	100	100	100	100	100	100	100	100
S. haemolyticus (2)	100	100	50	100	100	100	100	100	50	100	100
S. lugdunensis (1)	100	100	0	100	100	0	100	100	100	100	100
S. saprophyticus (1)	100	100	100	100	100	100	100	100	100	100	100
S. sciuri (1)	100	100	100	100	100	0	100	100	100	100	100
S. warneri (1)	100	100	100	100	100	100	100	100	100	100	100
S. xylosus (3)	100	100	33,3	0	100	100	100	100	66,6	100	100

n: number of isolates; CTF: Ceftiofur (30µg); PNM: Penicillin + Novobiocin (40 µg); TET: Tetracycline (30 µg); OFX: Ofloxacin (5 µg); CLI: Clindamycin (2 µg); ERI: Erythromycin (15 µg); CFO: Cefoxitin (30 µg); GEN: Gentamicin (10 µg); SUT: Trimethoprim-sulfamethoxazole (25 µg); LNZ: Linezolide (30 µg); PEN: Penicillin (10UI).
Source: research data.

Staphylococci isolates were susceptible to beta-lactams and linezolid; therefore, no methicillin-resistant *Staphylococcus* isolate was detected. Such a finding is noteworthy, considering the massive usage of beta-lactams in the field and the resistant trend observed elsewhere (Chung *et al*., 2021; Maaloui *et al*., 2021; Crespi *et al*., 2022). Except for *S. warneri, S. equorum,* and *S. saprophyticus,* susceptible to all agents, variable rates of resistance to erythromycin, clindamycin, gentamicin, trimethoprim-sulfamethoxazole, and more prominently, to tetracycline, were observed among staphylococci isolates.

Streptococci isolates were susceptible to beta-lactams and ofloxacin. However, all species but *S. pluranimalium* presented resistance to at least one antimicrobial, as shown in Table 2. Tetracycline resistance calls attention, since it has been largely used in veterinary medicine practice (Oliver; Murinda, 2012). Resistance to erythromycin was also observed, and the most common macrolide resistance phenotypes, cMLS, iMLS, and M, were detected among three erythromycin-resistant *S. uberis* isolates. Three *S. agalactiae* isolates also resistant to erythromycin presented the cMLS phenotype. These findings show that lincosamides should be used with care since isolates with cMLS or iMLS phenotypes are also resistant to these antimicrobials (Barros, 2021).

Table 2 - Antimicrobial susceptibility profiles of *Streptococcus* species recovered from cattle mastitis

Species (n)	**Antimicrobial susceptibility (%)**					
	CTF	**PNM**	**TET**	**OFX**	**CLI**	**ERI**
S. agalactiae (11)	100	100	27,3	100	72.7	72.7
S. dysgalactiae (6)	100	100	50	100	100	100
S. pluranimalium (1)	100	100	100	100	100	100
S. uberis (18)	100	100	94,4	100	94.4	83.3

n: number of isolates; CTF: Ceftiofur (30µg); PNM: Penicillin + Novobiocin (40 µg); TET: Tetracycline (30 µg); OFX: Ofloxacin (5 µg); CLI: Clindamycin (2 µg); ERI: Erythromycin (15 µg).
Source: research data.

Resistance to tetracycline and to erythromycin and clindamycin has been detected in similar studies worldwide (Chung *et al*., 2021; Crespi *et al*., 2022). In Brazil, resistance to these antimicrobials is relatively common among *S. agalactiae* recovered from humans, although tetracycline has not been recommended to treat human streptococcal infections (Barros, 2021).

Escherichia coli isolates (5 isolates) were susceptible to piperacillin-tazobactam, meropenem, and amikacin. Isolates were also susceptible to gentamycin (80%), levofloxacin and ceftazidime (60%), cefepime, ceftiofur, aztreonam, and sulfamethoxazole-trimethoprim (40%), and tetracycline (20%).

Multidrug resistance, defined as resistance to three or more antibiotic classes, was detected in *S. aureus* (1 isolate, 2,6%), *S. uberis* (1 isolate, 5.5%), *S. agalactiae* (3 isolates, 27.3%) and *E. coli* (3 isolates, 60%). Despite the limited sample, it is noteworthy the finding of multidrug resistance, especially among *S. agalactiae* and *E. coli* isolates. This last showed resistance to several antimicrobial agents, including ceftiofur, the unique recommendation of CLSI for testing *E. coli* recovered from cattle mastitis (CLSI, 2018). In addition, mastitis pathogens showing resistance to antimicrobials that are used in both humans and animals should be viewed with concern. According to the One Health approach, the finding and/or dissemination of antimicrobial-resistant microorganisms in different settings represents an enormous risk for human and animal health, as well as to the global environment (Mcewen; Collingnon, 2018). Therefore, according to the data presented here, a practical approach would be the implementation of microbiological diagnostics in an affordable manner for milk producers, to identify mastitis pathogens and their antibiotic resistance profile. Such knowledge would lead to better therapy strategies and would contribute to minimizing the burden of bacterial antimicrobial resistance, not only among animals but also among humans and the environment.

4 Conclusion

This study confirms the use of MALDI-TOF MS as a reliable tool for the identification of bacterial isolates recovered from milk. To our knowledge, this is the first report using this technique to identify microorganisms recovered from farms in the state of Minas Gerais, Brazil's largest milk producer. Additionally, the prevalence of classical pathogens such as *S. aureus*, *S. uberis*, and *S. agalactiae* is noteworthy. It draws attention for the multidrug resistance observed to antimicrobial agents recommended for treating bovine mastitis according to international guidelines, as well as to drugs used by humans, particularly among *E. coli* isolates. These findings underscore the importance of continuous surveillance on dairy farms, not only for animal welfare and farm profitability but also for monitoring the spread of antimicrobial resistance.

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