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Microbiological diagnosis and antimicrobial resistance profile of bovine mastitis causing agents in the municipality of Placas, Pará, Brazil

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ABSTRACT. The study was carried out in the region of Placas, Pará. Objective: identify the main causing agents of mastitis, as well as evaluating the resistance profile of the isolated agents against antimicrobials. Methods: Ten properties were evaluated, where 161 animals were submitted to the California Mastitis Test (CMT) for the diagnosis of subclinical mastitis, and milk samples were collected from positive animals and led for culture and antibiogram. Results: In total, 31.06% of the animals were positive for CMT. The main agents identified were *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae* and *Streptococcus bovis*. The antibiogram indicated resistance to Penicillin, Ceftazidime and Cephalothin. Conclusion: The properties surveyed showed a high rate of subclinical mastitis, regarding antimicrobial resistance, in general, all agents showed high resistance.

Keywords: ampicillin; antibiotics; antibiogram; staphylococcus aureus; penicillin.

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Introduction

The milk production chain is one of the most important in the Brazilian agro-industrial complex, producing approximately 20 billion liters of milk per year, from the largest commercial herd in the world (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA], 2018). Pará has the fifth largest cattle herd in Brazil, with about 20 million animals, most of which are destined for meat production. However, there is a growing interest in the dairy products market, with a production of 1,200 liters of milk per day, being the 15th producer of milk in the country (Agência de Defesa Agropecuária do Estado do Pará [ADEPARÁ], 2017).

For the good performance of dairy farming, one of the essential factors to be observed and strictly controlled is the health of the herd. Animal health interferes with the evolution of herd productivity and, consequently, production on the property and profitability of the activity. Among the various pathologies that affect the dairy herd, mastitis stands out, as it causes great damages such as the disposal of milk, the drop in milk production, the expense of antibiotics and, eventually, the disposal of the animal (Rollin, Dhuyvetter, & Overton, 2015; Puerto et al., 2021).

In general, the infection presents itself in two ways: clinical and subclinical (Boonyayatra, Rin-Ut, & Punyapornwithaya, 2014). In clinical mastitis, the mammary gland presents an increase in volume, elevation of temperature, pain and stiffness. In subclinical mastitis, on the other hand, it is more difficult to establish a clinical diagnosis, since the bacterial infection when installed in the teat remains without the presence of classic clinical signs. Despite this absence of evident signs, there are losses to milk production, as well as reduced product quality and influence on animal welfare (Hogeveen & Van Der Voort, 2017; Lopes, Manzi, & Langoni, 2018).

Mastitis can be caused by irritant toxic agents or trauma, but in the vast majority of cases it is caused by an infectious agent that invades the udder, which multiplies in the glandular tissue, leading to tissue damage in different degrees, causing inflammatory processes (Ruegg, 2017; Taponen, Liski, Heikkilä, & Pyörälä, 2017).

More than 140 different types of microorganisms can cause mastitis (Motaung, Petrovski, Petzer, Thekisoe, & Tsilo, 2017; Ndahetuve et al., 2019), and although some fungi, yeasts and chlamydiae can be found among these agents, bacteria are the main etiologic agents of this disease, being isolated more frequently the bacteria of the genus Staphylococcus, mainly Staphylococcus aureus, as well as Streptococcus agalactiae (Lopes et al., 2018).

However, recently developed studies describe a shift in the agents that cause mastitis from major pathogens to minor pathogens such as coagulase-negative Staphylococcus and other bacilli (Ndahetuve et al., 2019; Mbindyo, Gitao, & Mulei, 2020).

The treatment of this disease is mainly based on antibiotic therapy protocols, but it is worth mentioning that the treatment has greater efficacy and safety, if stipulated from the result of the microbiological culture, together with the antimicrobial sensitivity test, also known as antibiogram (Barkema, Schukken, & Zadoks, 2006; Laven, Balcomb, Tulley, & Lawrence, 2014; Vakkamäki, Taponen, Heikkilä, & Pyörälä, 2017).

It is common in dairy farms to carry out a drug therapy soon after the identification of the infection, or even in a preventive way, as in the case of animals in the dry season. With a few exceptions, the choice of antimicrobial is empirically based, normally based on the availability of the drug in the region and the cost of the product, not considering the sensitivity of microorganisms to different antimicrobial agents.

Due to the cost or lack of access to carry out the culture, isolation and identification of infectious agents and antimicrobial sensitivity tests, many properties make indiscriminate use of antibiotics, which sometimes do not present satisfactory results, providing an increase in resistant bacteria and increasing the cost of production (Hogeveen & Van Der Voort, 2017; Pascu, Herman, Iancu, & Costinar, 2022).

Given the above, the objective of this paper was to identify and evaluate the antibiotic resistance profile of the most common microorganisms that cause mastitis in dairy farms in the region of Placas, Pará.

Material and methods

Study location

The study was carried out in the milk basin of Placas-PA, located in the North region Latitude: 03° 52' 04" S Longitude: 54° 13' 12" W Altitude: 95m Area, totaling 7194.1 Km^2.

Animals

A total of 161 animals were evaluated, in 10 properties in different branches of the municipality, previously selected after indicating the dairy receiving the raw material.

California Mastitis Test (CMT)

Lactating cows were initially submitted to the California Mastitis Test (CMT) test to detect subclinical mastitis, following the guidelines described by (Santos, 2013). After the CMT test, and with the confirmation of the presence of subclinical mastitis, the animals underwent cleaning and disinfection of the teats, after cleaning milk samples were collected from the teats affected by subclinical mastitis and placed in a sterile collection bottle, composing a single sample. per animal for microbiological evaluation.

Microbiological analysis

All samples were properly identified and stored in isothermal boxes under refrigeration, and transported to the microbiology laboratory of the Centro Universitario da Amazônia – UNAMA Santarém.

In the laboratory, the samples were heated in a water bath for approximately five minutes and then homogenized and streaked with the aid of a sterile swab in culture plates containing Muller-Hinton-Blood medium and incubated in a bacteriological oven at 37°C for 24 to 48 hours for bacterial isolation. After incubation for 24 hours, morphological characteristics of colonies such as size, type and color were analyzed. A small portion of the distinct colonies were collected and destined for Gram staining, and with the aid of a microscope the disposition of the cells and the staining characteristics to Gram (Barbalho, 2001).

For identification, the isolated colonies were submitted to Catalase and Coagulase tests. The catalase test was performed according to Brito and Brito (1999), to differentiate between staphylococci and streptococci. Bacteria identified as Staphylococcus were submitted to a coagulase test to differentiate between coagulase-positive Staphylococcus and coagulase-negative Staphylococcus. Colony samples that were positive for the catalase test and negative for the coagulase test were submitted to the Novobiocin sensitivity test to identify Staphylococcus epidermidis, and Staphylococcus saprophyticcus.

After the cultivation in Muller-Hinton-Blood, already isolated and identified, the colonies were submitted to the test of sensitivity and resistance to antimicrobials (antibiogram) following the method of diffusion by discs in Muller-Hinton Agar medium (Bauer, 1996), following the specifications of the Clinical and Laboratory Standards Institute - CLSI. For that, colonies with the same morphological and biochemical characteristics, corresponding to the same genus and/or species of bacteria, were transferred to eppendorf tubes containing peptoned water, forming a composite sample. Then, seeding was carried out with a sterile swab on a plate containing Mueller-Hinton Agar medium, arranged with discs impregnated with the main antibiotics and incubated in a bacteriological oven at 37°C.

The reading was performed after 24 and 48 hours of incubation, by measuring the inhibition halos, with the aid of a millimeter ruler and table for reading sensitivity to antimicrobials. The measurements of the diameters of the zones of inhibition were defined in three categories of strains: sensitive, intermediate, resistant.

Data analysis

For the analysis and discussion of the results, those strains that showed intermediate resistance and susceptibility to the antibiotic were considered resistant.

To obtain the association between classes of classificatory effects with classes greater than two levels, the contingency chi-square test was used. For situations in which classes had only two levels, Fisher's Exact (Fisher-Irwin Test) test was used, in all cases a significance level of 0.05 was used.

Results

Of the 161 animals submitted to the CMT test, 31.06% showed positive results in the test for subclinical mastitis (Table 1), and samples were collected for microbiological evaluation.

Table 1. Result of California Mastitis Test (CMT) carried out in the 10 properties evaluated in the milk basin in the municipality of Placas, Pará State.

Property	Herd by property	N# of tested animals (%)	N# of positive animals (%)
A	70	20 (28, 57)	5 (25,00)
B	25	15 (60,00)	5 (33,33)
C	31	18 (58,06)	7 (38,89)
D	45	19 (42,22)	5 (26,32)
E	32	16 (50,00)	4 (25,00)
F	40	15 (37,50)	6 (40,00)
G	12	12 (100,00)	4 (33,33)
H	16	8 (50, 00)	3 (37,50)
I	54	22 (40,74)	8 (36,36)
J	10	6 (60,00)	3 (50,00)
TOTAL: 10	335	161	50 (31,06)

X^2=3,01 (p-value=0,964).

Of the 50 (31.5%) positive samples collected for CMT, there was bacterial growth and colony formation in all samples, making it possible to isolate and identify the agents. The agents isolated from samples from each property are described in Table 2. It was found that the main agent causing mastitis in the region was Staphylococcus aureus, being isolated in all analyzed properties, followed by Staphylococcus epidermidis, isolated in five properties.

In the antimicrobial resistance test (antibiogram), it was found that Staphylococcus aureus, isolated in the 10 properties analyzed, showed greater sensitivity to Tetracycline and Sulfamethoxazole/Trimethoprim, having Gentamicin as an intermediate antibiotic, and being totally resistant to Penicillin G (Table 3).

In five of the properties, *Staphylococcus epidermidis* was isolated, this group of bacteria was mainly sensitive to Tobramycin and Amikacin, being resistant to Chloramphenicol and Ceftazidime (Table 4).

Streptococcus agalactiae, isolated in 4 of the properties, was sensitive to Amikacin, and to Sulfamethoxazole/Trimethoprim, and resistant to Penicillin G, Clindamycin, and Ceftazidime (Table 5).

Table 2. Microorganisms isolated from milk samples from cows with subclinical mastitis in herds from 10 properties in the dairy basin of the municipality of Placas, Pará State.

Property	Isolated bactéria
A	*Streptococcus agalactiae, Staphylococcus epidermidis, Staphylococcus aureus,* Gram positive bacilli
B	*Staphylococcus epidermidis, Staphylococcus aureus*
C	*Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus agalactiae,* Gram positive bacilli
D	*Staphylococcus aureus, Streptococcus agalactiae,* Gram positive bacilli
E	*Staphylococcus epidermidis, Staphylococcus aureus* Gram positive bacilli
F	*Staphylococcus aureus, Streptococcus agalactiae,* Gram positive bacilli
G	*Staphylococcus aureus,* Gram positive bacilli
H	Bacilos Gram positivos, *Staphylococcus aureus*
I	*Staphylococcus epidermidis*, *Streptococcus bovis, Staphylococcus aureus*
J	Gram positive bacilli *Staphylococcus aureus*

Table 3. Sensitivity test of Staphylococcus aureus isolated in the properties evaluated in the municipality of Placas-PA to antibacterials.

Antibiotics (group)	Properties										% total of resistance
	A	B	C	D	E	F	G	H	I	J	
Tetracycline (1)	S	S	S	S	S	S	S	S	S	S	0
Tobramycin (2)	S	R	S	S	S	S	S	S	S	S	10
Amikacin (2)	S	R	S	S	S	I	S	S	S	S	10
Ampicillin (2)	I	S	S	R	S	R	R	I	R	R	50
Ceftazidime (3)	R	R	R	R	I	S	R	R	R	R	80
Chloramphenicol (2)	S	I	S	R	I	S	S	S	R	I	20
Sulfamethoxazole/Trimethoprim (1)	S	S	S	S	S	S	S	S	S	S	0
Gentamicin (1)	S	I	S	S	S	S	S	S	I	I	0
Ciprofloxacin (2)	S	R	S	S	S	S	S	S	I	S	10
Clindamycin (3)	R	S	R	R	R	S	R	R	R	R	80
Erythromycin (2)	I	R	I	R	S	I	S	S	R	R	40
Cephalothin (3)	R	R	R	R	S	R	R	R	R	R	90
Oxacillin (3)	R	S	R	R	S	R	R	R	R	R	80
Penicillin (3)	R	R	R	R	R	R	R	R	R	R	100
Vancomycin (2)	S	S	S	S	S	S	S	S	R	S	10
Group (% of resistense)			Resistense (%)					Sensitivity (%)			Total (%)
1*			-					-			-
2 (< 50%)			15(13.76)					45(41.28)			60(55.05)
3 (≥ 50%)			43(39.45)					6(5.50)			49(44.95)
Total			58(53.21)					51(46.79)			109(100)

Fisher's Exact Test=0,00 (p-value < 0,0156). *Group 1 antibiotics were left out of the test, as they showed 0% resistance.

Table 4. Sensitivity test of *Staphylococcus epidermidis* isolated in the properties evaluated in the municipality of Placas-PA to antibacterials.

Antibiotic (group)	Properties					% total of resistance
	A	B	C	E	I	
Tetracycline (1)	S	I	S	S	S	0
Tobramycin (1)	S	S	S	S	S	0
Amikacin (1)	S	S	S	S	S	0
Ampicillin (2)	R	R	S	R	S	60
Ceftazidime (3)	R	R	I	R	R	80
Chloramphenicol (3)	R	R	R	R	R	100
Sulfamethoxazole/Trimethoprim (2)	S	R	S	S	S	20
Gentamicin (1)	S	I	I	I	I	0
Ciprofloxacin (2)	R	I	S	R	R	60
Clindamycin (3)	R	R	S	R	R	80
Erythromycin (3)	R	R	S	R	R	80
Cephalothin (3)	R	R	S	R	R	80
Oxacillin (3)	R	R	S	R	R	80
Penicillin (3)	R	R	S	R	R	80
Vancomycin (3)	R	R	S	R	R	80
Group (% of resistense)		Resistense (%)		Sensitivity (%)		Total (%)
1*		-		-		-
2 (≤ 60%)		7.00(13.21)		7.00(13.21)		14.00(26.42)
3 (> 60%)		33.00(61.26)		6.00(11.32)		39.00(73.58)
Total		40.00(75.47)		13.00(34.53)		53.00(100.00)

Fisher's Exact Test=7.00 (p-value < 0.0156). *Group 1 antibiotics were left out of the test, as they showed 0% resistance.

Table 5. Sensitivity test of *Streptococcus agalactiae* isolated in the properties evaluated in the municipality of Placas-PA to antibacterials.

Antibiotics (group)	Properties				% total of resistance
	A	C	D	F	
Tetracycline (1)	S	S	I	I	0
Tobramycin (1)	S	S	R	S	25
Amikacin (1)	S	S	S	S	0
Ampicillin (1)	S	I	R	S	25
Ceftazidime (2)	R	R	R	R	100
Chloramphenicol (2)	I	R	R	S	50
Sulfamethoxazole/Trimethoprim (1)	S	S	S	S	0
Gentamicin (1)	S	I	I	S	0
Ciprofloxacin (1)	S	S	I	S	0
Clindamycin (2)	R	R	R	R	100
Erythromycin (2)	I	R	R	S	50
Cephalothin (1)	S	S	R	S	25
Oxacillin (2)	S	S	R	R	50
Penicillin (2)	R	R	R	R	100
Vancomycin (1)	S	S	R	S	25
Group (% of resistense)	Resistense (%)		Sensitivity (%)		Total (%)
1 (< 50%)	4.00(7.69)		26.00(50.00)		30.00(57.69)
2 (≥ 50%)	18.00(34.62)		4.00(7.69)		22.00(42.31)
Total	22.00(43.31)		30.00(57.69)		52.00(100.00)

Fisher's Exact Test=4,00 (p-value < 0,0001).

Streptococcus bovis was isolated in only one of the properties, presenting in the antibiogram greater sensitivity to Amikacin, and to Sulfamethoxazole/Trimethoprim, intermediate sensitivity to Gentamicin and Ciprofloxacin, and resistant to Penicillin G.

Discussion

A 31.5% rate of involvement was observed for subclinical mastitis, a high percentage, since a maximum of 15% of subclinical mastitis in a herd is considered acceptable (Langoni et al., 2011), for mastitis Subclinical disease is considered the main form of this disease in dairy herds worldwide (Östensson, Lam, Sjögren, Wredle, 2013; León-Galván et al., 2015; Abebe, Hatiya, Abera, Megersa, & Asmare, 2016), which results in a greater number of somatic cells in the milk produced and, consequently, changes in its physical and chemical qualities (Reis, Barreiro, Mestieri, Porcionato, & dos Santos, 2013; Pérez et al., 2020).

During the collection of the samples, the handling conditions on the properties were observed, and it was verified that in 90% of them there was no coverage in the corrals (data not shown) and, in turn, due to the rainy season, a lot of mud was found in the place of the milking, practically no form of asepsis of the teats before or after milking, as well as some form of management related to the order of entry of these animals that have already presented clinical signs of mastitis, favoring, therefore, a cross-contamination.

Of the microorganisms isolated, four etiological agents were identified as the main agents responsible for subclinical mastitis *Streptococcus agalactiae, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus bovis*, in addition to Gram positive bacilli.

Gram-positive cocci are the main group of bacteria responsible for mastitis, with more than 90% of isolates (Abdi et al., 2021).

Within this group, Staphylococcus aureus and Streptococcus agalactiae are the most frequent species (Amer et al., 2018), and despite the large number of pathogens that cause mastitis in cattle, *S. aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis* and *Escherichia coli* account for about 80% of cases (Ranjan, Swarup, Patra, & Nandi, 2006; Zeryehum & Abera, 2017).

E. coli is characterized as the Gram-negative species that most causes the development of mastitis in dairy cows (Mpatswenumugabo et al., 2017; Gao et al., 2017).

In contrast, *S. aureus* has been the main species of microorganism found in intramammary infections of cattle on all continents, being the main agent causing losses in dairy farming (Rollin et al., 2015; Kromker & Leimbach, 2017; Liu et al., 2017; Bobbo et al., 2017; Sender, Pawlik, & Korwin-Kossakowska, 2017; Helmy, Deblais, Kassem, Kathayat, & Rajashekara, 2018; El-Sayed, Algammal, Abouel-Atta, Mabrok, & Emam, 2019; Forno-Bell et al., 2021).

Thus, *S. aureus* came to be considered the main causal agent of bovine mastitis (Abebe et al., 2016; Acosta, Silva, Medeiros, Pinheiro-Júnior, & Mota, 2016; Levison et al., 2016), with an isolation rate between herds ranging from 8. 3 to 49.23% (Helmy et al., 2018; Algammal, Wahdan, & Elhaig, 2019; Algammal, Enany, El-Tarabili, Ghobashy, & Helmy, 2020), with frequent cases in Brazilian territory (Tomazi et al., 2018), with an index of 100% in Minas Gerais (Teixeira, Silva, Fonseca, & Costa, 2014).

The results found in this study are in accordance with those observations available in the literature, since this agent was isolated in all properties surveyed. Results described by other authors indicate that S. aureus was isolated in up to 40% of mastitis cases in China (Wang et al., 2018) and in other countries (Piessens et al., 2011; Silveira-Filho et al., 2014; Condas et al., 2017).

Likewise, studies carried out on the European continent and in the Americas point to a high prevalence of S. aureus in dairy cows, reaching 41% in France, between 1995 and 2012 (Papadopoulos et al., 2018) and 47.2% in Italy (Poutrel, Bareille, Lequeux, & Leboeuf, 2018).

According to Sadashiv and Kaliwal (2014) and Song et al. (2020), Gram-positive bacilli, especially those belonging to the genus Bacillus spp, are widely distributed in the air, soil, water, feces and, therefore, in the milking environment. These are found as milk contaminants, the vast majority being saprophytes. Bacillus cereus and Bacillus subtilis can be isolated solely as the main agent in cultures or associated with other pathogens that cause mastitis, which is the most common form of isolation.

Gram-positive bacilli were isolated in eight properties; however, it was not possible to carry out tests for the classification of the genera of the bacilli. According to Christiansson and Te Giffel (2000) the taxonomic studies of these species do not clearly determine the morphological characteristics of the spores or in tests with their carbohydrates. In addition to these characteristics that make their classification ineffective, there is also the fact that genomic studies that use routine phenotypic characteristics commonly used to distinguish these species as a requirement no longer have much value, having little recognition in the scientific environment.

It should be noted that strains of S. aureus resistant to multiple antimicrobials have been identified in different countries (Wang et al.,2014; Salauddin et al., 2020), including Brazil (Fontana et al., 2010).

In recent decades it has been increasingly difficult to control and maintain the health of animals when it comes to infections by these microbial agents, especially *S. aureus* (Peréz et al., 2020). Because it is so frequent, this agent has developed resistance to the most frequently used antibiotics, especially those widespread in local therapy in a given region.

Studies indicate that *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis*, in addition to being the most frequently isolated in bovine mastitis in Brazil (Langoni et al., 2017), show an increasing pattern of resistance to antimicrobials (Lopes et al., 2018; Freu, Tomazi, Filho, Heinemann, & Santos, 2022).

In a study carried out with 237 crossbred dairy cows, belonging to nine properties located in the dairy basin of Rondon do Pará, the bacteria isolated with the highest incidence were Staphylococcus aureus, which, according to the antibiogram, were resistant mainly to Ampicillin and Penicillin and sensitive to cephalothin and cefoxitin (Oliveira et al., 2011).

These results corroborate the data obtained in the present study when reporting resistance to Ampicillin and Penicillin. However, cephalothin showed a higher resistance index in dairy herds in Placas, Pará State. This variation within the same state may be linked to the therapeutic measures adopted, since these also vary depending on the availability of medicines in the regional market.

Similar results were evidenced by Zhang and Buckling (2012) who showed a rate of 94.8% of S. *aureus* isolates resistant to ampicillin and penicillin. Similar to that described by Varela-Ortiz et al. (2018) in the state of Guanajuato, Mexico, who also identified resistance of *S. aureus* to ampicillin and penicillin.

Likewise León-Galván et al. (2015), signaled 60 to 90% resistance to β-lactam antibiotics. This resistance, according to them, is linked to the acquisition of mobile genetic components, such as, for example, plasmids, transposable genetic elements (insertion sequences and transposons).

Studies developed in Nordic countries, such as Sweden, Norway and Denmark, point out that penicillin is the most used drug in the treatment of mastitis caused by S. aureus, and indicate that penicillin resistance is considered low. This may be associated with the strict legislation adopted in these countries (Persson, Nyman, & Grönlund-Andersson, 2011; Chehabi, Nonnemann, Astrup, Farre, & Pedersen, 2019).

In a meta-analysis carried out with different articles, from different regions, such as Europe, Asia, America, Africa, North America and Oceania, it was possible to observe greater resistance of S. aureus against penicillin, and cephalothin showed a lower prevalence of resistance antimicrobial (Molineri et al., 2021).

Conclusion

The properties surveyed showed a high rate of subclinical mastitis. The main agents causing mastitis in the region were Staphylococcus aureus, Staphylococcus epidermidis and Streptococcus agalactiae, with concomitant presence of Gram-positive bacilli. Regarding antimicrobial resistance, in general, all agents showed high resistance, mainly to Penicillin, Cephalothin, Oxacycline, Clidamycin and Ceftazidime.

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