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Microbiological Evaluation of Milk Quality and Antibiogram in Dairy Cows Managed on Pasture

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Abstract

Mastitis is a disease in dairy cattle that damage the milk chain. It was aimed identify the major causative agents of bovine mastitis in small dairy farms by producing an antibiogram and analysis of milk quality. Methods: During the summer, 280 dairy cows were examined on a farm located in Camapuã, MS, Brazil. The farm had a concrete milking facility and used cleaning and disinfection of the udder before and after milking to control mastitis. For assess microbial resistance, two samples of milk per cow were collected biweekly between January and March. The antibiotics sulfazotrim (25 μg), penicillin (10 μg), streptomycin (10 μg), vancomycin (30 μg), chloramphenicol (30 μg), tetracycline (30 μg), amoxicillin (10 μg), and gentamycin (10 μg) were used. Results: A total of 17.14% of the animals were positive for the California Mastitis Test (CMT). The identification of the pathogens revealed that *Staphylococcus aureus accounted* for 41.65% of the isolated organisms and *Escherichia coli* for 37.5%. Our results showed only sulfazotrim and chloramphenicol had effective results for both Gram-negative and Gram-positive bacteria. The isolated strains presented high resistance to the other tested antibiotics. Conclusions: sulfazotrim (25 μg) and chloramphenicol (30 μg) can be used to disinfect the udder of dairy cows.

Keywords: Antibiotics, bovine mastitis, milk quality

1. Introduction

Mastitis is a common and costly disease in dairy cattle that affects the profitability of the milk chain due to a reduction in milk yield, high amounts of discarded milk, treatment costs, and other factors. It can be present in both clinical and subclinical forms. The subclinical, the most frequent form, is a non-symptomatic intramammary inflammation and can affect up to 50% of cows in some herds (Forsbäck et al., 2009; Villa-Arcila et al., 2017). Most of the occurrences of mastitis are caused by bacteria, which are generally classified as either environmental or contagious (Gonçalves et al., 2018).

Among the contagious pathogens, *Staphylococcus aureus*, S*treptococcus agalactiae*,

Mycoplasma spp., and *Corynebacterium bovis* are noteworthy (Radostits et al., 2007) whereas the environmental pathogens correspond to *E. coli, Klebsiella* spp., *Streptococcus dysgalactiae,* and *Streptococcus uberis* (Harmon, 1994). *Staphylococcus aureus* is one of the most contagious bacteria that causes mastitis (Tollersrud et al., 2000); it is difficult to control and can rapidly invade all types of mammary gland cells. Clinical or subclinical mastitis caused by *S. aureus* tends to become chronic and has a low response to conventional antibiotics due to its intracellular localization in epithelial cells of the mammary gland (Dego et al., 2002; Leitner et al., 2003). This infection is related to the absence of an immune response, which involves different host and bacterial agents (Zecconi et al., 2005). *E. coli* is one of the most important environmental bacteria. Mastitis caused by *E. coli* can be treated in a few days and is characterized by pain, inflammation of one or all of the mammary quarters, fever, and milk with clots and an abnormal appearance (Oviedo-Boyso et al., 2007).

To control mastitis, disinfectants, such as sodium hypochlorite, chlorine, iodine-based gel, iodophor solution, dodecylbenzene sulfonic acid, chlorhexidine, phenolic compounds, and alcohol are used to prevent new infections, however, in the presence of infections, in most cases, the animal is culled or antibiotics are used. The use of antimicrobials occurs especially at two-time points. Firstly, clinical mastitis is commonly treated by antibiotic ointments in the mammary gland cavity (local treatment) in lactating cows. When severe mastitis is observed, parenterally antibiotics are also administered. The second time point corresponds to the use of local antibiotics on the day of drying-off (Krömker and Leimbach, 2017).

The use of antibiotics aims to eliminate infections, which implies the presence of antimicrobial concentrations in the udder higher than or similar to the minimum inhibitory concentration for the main pathogens. A bacteriological diagnosis and the appropriate selection of antibiotics according to the antibiotic sensitivity of the bacteria are fundamental for the effective treatment of mastitis (Jhambh et al., 2012). Thus, bacterial isolation and antibiograms are essential tools in the daily life of a dairy farm, since they document resistance or susceptibility patterns to antimicrobial products. In addition to being useful for the confirmation of a clinical diagnosis, laboratory results might indicate management errors and suggest possible corrections, which could significantly reduce the incidence of relapses.

The objective of this study was to identify the main agents related to bovine mastitis and to evaluate their microbial resistance to antibiotics using an antibiogram and quality tests of the milk produced by grass-fed cows.

2. Materials and Methods

The study involved 280 Holstein and Girolando cows from a particular property specializing in dairy production in Camapuã city, MS, Brazil. The experiment was carried out in the summer period (January to March) and the cows were kept in rotational grazing on patches of Panicum maximum, cv. Mombasa, and cv. Tanzania, of 15 to 20 ha, with two days of occupation on each patch. The dairy farm's daily milk production was 2800 L. The cows were milked twice a day in the masonry milking room and the udders were washed and disinfected before and after milking as the main mastitis control method.

Every two weeks, between January and March, milk samples were collected from each

milking for quality analysis and the identification of the microorganisms in the milk.

For collection, antisepsis of the mammary ceilings was performed by washing with soap and water, drying with paper towels, and disinfecting the ceiling ostium with ethyl alcohol at 70º GL. Subsequent CMT tests for the detection of subclinical mastitis were performed. Samples for bacteriological examinations were collected from the mammary quarters of CMT-positive cows in sterile tubes and stored under refrigeration until further analysis. The microbiological analyses were performed at the Laboratory of Microbiology at the Dom Bosco Catholic University (UCDB), Campo Grande, MS. Aliquots of 0.01 mL of milk were used for surface cultures of Manitol Agar, MacConckey Agar, and Nutrient Agar, incubated in a bacteriological oven at 37ºC. Readings were taken after 24 and 48 h of incubation.

The morphological characteristics of the colonies were initially observed, such as size, type, staining, and the presence of hemolysis. The antibiogram was constructed by means of antibiotic diffusion disks with samples of bacterial culture (*Escherichia coli* and *Staphylococcus aureus*), previously grown in liquid medium.

The antimicrobials, impregnated on a filter paper disc, were placed over the culture medium inoculated with the bacteria. Eight antibiotics were assessed: sulfazotrim (25 μg), penicillin (10 μg), streptomycin (10 μg), vancomycin (30 μg), chloramphenicol (30 μg), tetracycline (30 μg), amoxicillin (10 μg), and gentamicin (10 μg).

The susceptibility interpretation was based on the measure of the inhibition halo of bacterial growth formed around the disc, in millimeters (Table 1).

Table 1. Classification of bacterial resistance as a function of halo size, in millimeters (mm), for the different antibiotics evaluated

Antibiotic	Classification of bacterial resistance (mm)	
	Resistant	Sensitive
Sulfazotrim (25 μg)	<10	>16
Penicillin (10 μg)	<28	>29
Streptomycin (10 μg)	<13	>23
Vancomycin (30 μg)	<14	>17
Chloramphenicol (30 μg)	<12	>18
Tetracycline (30 μg)	<14	>19
Amoxicillin (10 μg)	<19	>20
Gentamicin (10 μg)	<12	>15

Tests of 70% alcohol and methylene blue were performed to evaluate milk quality. In the 70% alcohol test, 1 mL of alcohol was used for each mL of sample, and the formation of clots in the milk was observed for up to 6 h. In the methylene blue test, an indicator substance (dye) was used, which, when reduced, becomes colorless when in contact with bacterial culture, since it becomes an electron acceptor. The rate of transformation is directly proportional to the concentration of bacteria in the medium, which enables the evaluation of milk quality based on the level of reductase, according to the methodology described by Tronco (2013), with a poor quality sample having a discoloration time of less than 20 min and a good quality sample over 5.5 h (BRASIL, 2018).

This work is in accordance with the ethical principles established by National Council for the Control of Animal Experimentation (CONCEA) and approved by Committee on Ethics in Animal Use at Federal University of Mato Grosso do Sul (Protocol n. 802/2016).

The statistical analysis was descriptive, calculating the absolute and relative frequencies (Sampaio, 2002). The measurements of inhibition halos of bacterial growth inhibition were subjected to an analysis of variance and compared with a Tukey test ($p<0.05$), using Sistema de Análises Estatísticas e Genéticas, SAEG (VIÇOSA, 1999).

3. Results and Discussion

3.1 California Mastitis Test (CMT) and Microorganisms Associated

Of the 280 cows evaluated, 48 presented a positive result for the CMT test (17.14%). Of these, 18 were positive for *Escherichia coli* (37.5%) and 20 were positive for *Staphylococcus aureus* (41.65%). The others positive samples (20.85%) did not present bacterial growth and were characterized as false positives, probably due to the low accuracy of the CMT method or to elimination by the animal's own defenses, such as phagocytosis, which could produce a negative result (Langoni et al., 2017).

The bacterial infection index (17.14%) can be considered high, and close to the 20.48% obtained by Pardo et al. (1998) in which of a total of 83 cows examined, 17 presented clinical mastitis. Oliveira et al. (2011) evaluated 237 crossbred dairy cows and found that of the 935 mammary quarters evaluated, 6.6% had subclinical mastitis, 1.3% had clinical mastitis, and 92.1% were negative. The isolated bacteria from clinical mastitis cases were *Staphylococcus* spp. coagulase negative (25%), *Staphylococcus aureus* (16.7%), *Streptococcus* spp. (8.3%), and *Corynebacterium* spp. (8.3%). In contrast to the obtained results, the presence of *Escherichia coli* was not observed in cases of clinical mastitis.

Langoni et al. (1991) observed a higher frequency of the bacterial agent *Staphylococcus* sp. than the other bacterial agents, corresponding to 35.53%. Similar results were also found by Barbalho and Mota (2001) who demonstrated that bacteria of the genus *Staphylococcus* sp. corresponded to 38.76% of the total agents isolated from milk samples.

Some authors report that the most important microorganism that causes mastitis is *Staphylococcus* spp., especially since *S. aureus* is responsible for one-third of the cases of

clinical and subclinical mastitis (Li et al., 2017; Yadav and Kumar, 2012). These findings corroborate those presented in this study, which demonstrate that *Staphylococcus aureus* was the main agent isolated (41.65%), followed by enterobacteria (37.50%).

Andrade et al. (2005) studied 2823 breeding females of the Holstein and Gir breeds in Brazil and reported that the main pathogens found in the herd were Gram-positive cocci (41.3%) and Gram-negative rods (52.6%), with *Staphylococcus aureus* and *Escherichia coli* being the main pathogens found in uterine infections. *In vitro* tests demonstrated that these microorganisms showed a higher susceptibility to chloramphenicol, gentamicin, and neomycin. Among the antibiotics tested in this study for Gram-positive bacteria (*Staphylococcus aureus*), sulfazotrim showed the best results, with a larger inhibition halo (25.78 mm) than the other treatments (Table 2).

Table 2. Growth inhibition (halo inhibition) of Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) isolated from cows positive for the CMT test (California Mastitis Test) using different types of antibiotics

Antibiotic	Bacterial growth inhibition (halo of inhibition, mm)		
	Gram-positive	Gram-negative	P
Sulfazotrim (25 μg)	25.78 ± 1.08 $^{a\,A}$	25.67 ± 0.33 $^{a\,A}$	NS
Penicillin (10 μg)	6.67 ± 2.26 $^{d\,A}$	nh ^{B}	0.0335
Streptomycin (10 μg)	13.89 ± 0.79 $^{c\,A}$	12.00 ± 0.52 $^{d\,A}$	0.0990
Vancomycin (30 μg)	16.89 ± 0.68 $^{bc\,A}$	nh ^{B}	0.0001
Chloramphenicol (30 μg)	20.22 ± 1.05 $^{b\,A}$	18.50 ± 0.74 $^{b\,A}$	0.2667
Tetracycline (30 μg)	nh ^{B}	15.00 ± 1.96 $^{c\,A}$	0.0001
Amoxicillin (10 μg)	13.40 ± 1.67 $^{c\,A}$	nh ^{B}	0.0002
Gentamicin (10 μg)	16.44 ± 0.58 $^{bc\,A}$	14.17 ± 1.25 $^{c\,A}$	0.0520
P	0.00001	0.00001	

Means with different lowercase letters in the same column are significantly different ($P<0.05$). Means with different uppercase letters in the same column are significantly different ($P<0.05$).

3.2 Susceptibility of Microorganisms Associated With Mastitis

The susceptibility of bacteria was 100% to sulfazotrim, 77.7% to chloramphenicol, and 66.6% to vancomycin, the other antibiotics did not have significant results (Table 3). According to Andrade et al. (2005), the percentage of microorganisms' resistant to different drugs is a complicating factor for prophylactic and therapeutic regimens for the control of

uterine infections and consequently intra-mammary infections. This resistance is probably associated with the indiscriminate and incorrect use of antibiotics, often without determination of the susceptibility of the microorganisms to the drugs (Andrade et al., 2005), with the acquisition of antimicrobial resistance, and with the biofilm-forming ability of the bacteria (Taponen and Pyörälä, 2009).

Penicillin and tetracycline were the least effective antibiotics against Gram-positive bacteria, with a halo formation of 6.67 mm (Table 2) and the absence of a halo, respectively, which indicates 100% bacterial resistance to both treatments (Table 3). However, Aslantaş and Demir (2016) showed the resistance rates to penicillin (45.5%), tetracycline (33%), and amoxicillin (0.9%). Probably, these differences about the effectiveness of antibiotics is by continuous use. This act can cause antibiotics resistance when the rules about dose and applications are neglected. Gomes et al. (2016) and Saeki et al. (2011) found positive results in which the alcoholic extract of propolis was effective against Staphylococcus aureus from of animals with mastitis, presenting a halo between 6 and 18 mm, suggesting as an alternative to use of commercial antibiotics.

Table 3. Antibiogram of Gram-positive bacteria (*Staphylococcus aureus*) isolated from cows positive for the CMT (California Mastitis Test) from a dairy farm located in Camapuã, MS, Brazil

Antibiotic	Resistant (%)	Intermediate (%)	Sensitive (%)
Sulfazotrim (25 μg)	0	0	100
Penicillin (10 μg)	100	0	0
Streptomycin (10 μg)	33.3	33.3	33.3
Vancomycin (30 μg)	22.2	11.1	66.6
Chloramphenicol (30 μg)	0	22.2	77.7
Tetracycline (30 μg)	100	0	0
Amoxicillin (10 μg)	33.3	55.5	11.1
Gentamicin (10 μg)	0	66.6	33.3

In addition, the higher production in summer increases the susceptibility of animals to the entry of microorganisms, due to the humidity and high temperature conditions that favor the development of pathogens, combined with increased contact with animals.

Penicillin, tetracycline, amoxicillin, and streptomycin are frequently used for the treatment of mastitis and had already been used in previous treatments, which led to inexpressive results on the *Staphylococcus aureus* samples, which might be related to the resistance induced by previous administrations (Table 3). Ribeiro et al. (2009) in a study on pathogenic microorganisms in bovine milk, found the highest resistance rates of 53.5% when the strains

were submitted to penicillin. Similarly, Ren et al. (2020) investigated the prevalence and antimicrobial susceptibility of *Staphylococcus aureus* from subclinical bovine mastitis in dairy farms located in China. The authors found the resistance rates to penicillin, tetracycline, and chloramphenicol were 58.5, 18.5, and 1.5%, respectively.

In the antibiogram constructed by Oliveira et al. (2011) in contaminated milk from 234 crossbred cows in North region of Brazil, 100% of the isolates of *Staphylococcus* spp. coagulase negative, *S. aureus*, *S. intermedius*, and *Streptococcus spp.* were sensitive to sulfazotrim. However, *Corynebacterium* spp. was 100% resistant to the same antimicrobial. Cephalothin, cefoxitin, and gentamicin were effective against bacteria isolated from the genus *Staphylococcus* spp., which represented the majority of mastitis agents.

The use of antibiotics is the most widely used treatment of mastitis, however, the growing concern about the presence of antibiotic residues in milk and the emergence of resistant bacterial strains has stimulated the search for alternative means to reduce or eliminate such problems. The use of antibiotic therapy in the control of subclinical mastitis during lactation and its possible consequences should be studied over a period of more than 30 days. Then, it would be possible to obtain more precise information about the variation in milk production, as well as whether or not reinfections were important for the study of the cost-benefit of treatment (Zafalon et al., 2007).

When evaluating the in vitro activity of the alcoholic extract of propolis against agents of bovine mastitis, Loguercio et al. (2006) found 94.4% of Staphylococcus sp. and 85.2% of Streptococcus sp. were susceptible to propolis ethanolic extract. Similarly, Gomes et al. (2016) evaluated the in vitro antibacterial activity of brown propolis, by determining the minimum inhibitory concentration. The alcoholic extract of propolis was obtained from 35g of crude propolis macerated in 65mL of cereals alcohol. The authors found the alcoholic extract of propolis 35% showed antibacterial action with minimum inhibitory concentration ranging from 4.5 to 18.9mg / mL for Escherichia coli. For Gram-positive bacteria, Gomes et al. (2016) showed the minimum inhibitory concentration of the alcoholic extract propolis ranged from 2.25 to 18.5 mg/mL. The smallest minimum inhibitory concentration of propolis alcohol extract capable of inhibiting growth of Streptococcus spp. of bovine origin was 2.25mg/mL. For Staphylococcus bacteria, also from bovine origin the MIC was 9.3mg / mL, indicating greater resistance of this genus to the propolis extract when compared to the genus Streptococcus, which is expected, since bacteria of the genus Staphylococcus have greater ability to develop resistance to compounds in general, being used as a microorganism reference in stress tests (Gomes et al., 2016).

The sulfazotrim antibiotic presented similar efficacy compared to the propolis extract for *Staphylococcus*. However, only 37% of the *Streptococcus* bacteria were susceptible to the antimicrobial. The use of gentamicin was approximately 89% efficient against *Staphylococcus*, while penicillin was only 47.2%. Ribeiro et al. (2009) also found a high efficacy, equal to 76.3%, for gentamicin against the isolates. Similarly, in work of Aslantaş and Demir (2019) all isolates of *Staphylococcus* were susceptible to vancomycin and gentamicin. Our results showed 66.6% of resistance for vancomycin and 33.3% for gentamicin (Table 3).

The Gram-negative bacteria (*Escherichia coli*) showed 100% sensitivity to sulfazotrim and 66.6% to chloramphenicol (Table 4). The other antibiotics did not present meaningful results, and penicillin, vancomycin, and amoxicillin were completely ineffective in the treatment of Gram-negative bacteria with 100% bacterial resistance (Table 4), indicated by the absence of inhibition halo formation (Table 2).

Table 4. Gram-negative bacteria (*Escherichia coli*) isolated from cows positive for the CMT (California Mastitis Test) from a dairy farm located in Camapuã, MS, Brazil

Antibiotic	Resistant (%)	Intermediate (%)	Sensitive (%)
Sulfazotrim (25 μg)	0	0	100
Penicillin (10 μg)	100	0	0
Streptomycin (10 μg)	83.3	16.6	0
Vancomycin (30 μg)	100	0	0
Chloramphenicol (30 μg)	66.6	33.3	0
Tetracycline (30 μg)	16.6	83.3	0
Amoxicillin (10 μg)	100	0	0
Gentamicin (10 μg)	16.6	33.3	50

Rangel and Marin (2009) found that *E. coli* isolated from milk samples from cows with mastitis presented 92.2% resistance to the antibiotic tetracycline and 90.5% to streptomycin.

Silva et al. (2010) carried out studies on the etiology of mastitis in sheep in the north region of Brazil, in addition to establishing the sensitivity profile of the bacteria isolated to antimicrobials. The bacteria isolated were *Staphylococcus* spp. coagulase negative (42.9%), *Staphylococcus aureus* (9.52%), *Streptococcus* spp. (4.76%), and *Escherichia coli* (4.76%) for the clinical mastitis cases. In the antibiogram, 100% of the isolates of *Staphylococcus* spp. negative coagulase was sensitive to amoxicillin, cephalothin, cefoxitin, enrofloxacin, florfenicol, gentamicin, kanamycin, neomycin, oxacillin, and penicillin/novobiocin and 91.7% was sensitive to penicillin, sulfazotrim, and tetracycline. One hundred percent of the *S. aureus* isolates was sensitive to cephalothin, cefoxitin, florfenicol, gentamicin, penicillin/novobiocin, and sulfazotrim. The isolates of *Streptococcus* spp. were 100% susceptible to amoxicillin, cephalothin, cefoxitin, florfenicol, penicillin G, and penicillin/novobiocin; 100% resistant to enrofloxacin, streptomycin, gentamicin, kanamycin, and neomycin; and 50% resistant and 50% intermediate to sulfazotrim. Of the *Escherichia coli* isolates, 66.7% was resistant to ampicillin, cephalothin, florfenicol, and tetracycline; 33.33% was sensitive to enrofloxacin and sulfazotrim; 33.3% had intermediate sensitivity to cefoxitin, enrofloxacin, streptomycin, kanamycin, neomycin, and sulfazotrim; and 33.3% was resistant to cefoxitin, streptomycin, kanamycin, and neomycin.

3.3 Tests of 70% Alcohol and Methylene Blue

Approximately 15% of the samples subjected to the 70% alcohol test coagulated in between 4 and 6 h. In the methylene blue test, no change in the milk was observed during the first 20 min to 2 h. However, between 2 and 5.5 h, there was a high occurrence of positive samples, equivalent to 90% of the material collected (Table 4).

Even with the high infection rate, milk quality was not compromised, since in the 70% alcohol test, only 15% of the samples coagulated in less than 4 h, indicating a only small degree of alteration (Table 5).

Table 5. Classification of milk by alcohol and methylene blue tests as a function of the time of coagulation and bleaching from a dairy farm located in Camapuã, MS, Brazil

Milk classification	Alcohol 70 % test		Methylene blue test	
	Coagulation	% of cows	Bleaching	% of cows
Poor	<20 min	0	<20 min	0
Bad	20 min–2 h	0	20 min–2 h	0
Regular	2 h–5.5 h	15	2 h–5.5 h	90
Good	>5.5 h	85	>5.5 h	10

The formation of milk clots is related to the reduction in kappa-casein by the epithelial cells of the infected mammary glands, due to the degradation of proteins by proteinases originating from bacteria, leucocytes, or the blood which, consequently, causes a loss of stability of the caseins (Yang et al., 2009).

In the methylene blue test, 90% of the samples presented reductase activity between 2 and 5.5 h (Table 5), which indicates that the sampled milk can be considered of regular to good quality, according to the 90 min limit regulated by the Ministry of Agriculture (BRASIL, 2018). Although milk quality is evaluated by different analyses (BRASIL, 2018), for an adequate milk quality improvement program, it is necessary to identify the causative agents and the level of resistance to the various antibiotics available, since the bacterial count is related to the hygiene of milking, antimicrobial therapies, equipment cleaning, milk cooling, and labor education and awareness of the problem.

Thus, it can be suggested that of the 2800 L of milk produced daily on the farm, 13.57% (approximately 380 L) would be of compromised quality, since the presence of Gram-positive or Gram-negative bacteria has been identified, which would contaminate the rest of the production when mixed in the chiller, thus reducing the quality of the milk obtained from this property.

4. Conclusions

Staphylococcus aureus and *Escherichia coli* were the most frequent bacteria identified in the milk samples and the most effective antibiotics *in vitro* against these pathogens were sulfazotrim (25 μg) and chloramphenicol (30 μg). The use of penicillin (10 μg), vancomycin (30 μg), tetracycline (30 μg), or amoxilin (10 μg) was not effective in inhibiting mastitis-causing pathogens *in vitro*.

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