Identification and antimicrobial susceptibility of milk isolates from cows with subclinical mastitis in the northwest of Paraná State, Brazil

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Summary

Bovine mastitis is a multifactorial disease, caused mainly by bacteria, whose most traditional method of treatment is the use of antibiotics. However, incorrect administration of these drugs may lead to the emergence of resistant strains. The objective of this study was to identify the most common pathogens causing subclinical mastitis in the northwest State of Paraná, Brazil and establish their antimicrobial susceptibility profiles. A total of 507 foremilk samples were collected in 13 municipalities. Bacteria were identified and their profile of antimicrobial susceptibility were tested. The distribution of positive samples was not homogeneous among the cities. *Streptococcus* were the most frequently genus observed (61%), followed by *Staphylococcus* (28%) and other genera (11%). Among streptococci and staphylococci, *Streptococcus uberis* and *Staphylococcus aureus* were the most frequently identified species, respectively. Overall, 40% of the isolates showed resistance to at least one of the antibiotics used in this study. A high proportion of cefoperazone resistant *Staphylococcus* spp. and bacitracin resistant *Streptococcus* were observed. The isolated microorganisms showed little resistance to enrofloxacin. The results obtained show the importance of isolating the microorganism causing mastitis and testing its resistance to antibiotics before performing the treatment of the disease. .

Introduction

Dairy farming is constantly growing in the world. Global milk production reached nearly 906 million tons in 2020, and Brazil is accounting, on average, for 7% of this production (FAO, 2021).

Due to the economic rise of the dairy market worldwide, it is essential to guarantee the quality of the milk produced [Garcia *et al.* 2019]. One of the main factors responsible for reducing milk quality is mastitis, the most important disease of cattle and the most expensive pathology for the milk industry (Rudenko *et al.*, 2021).

Mastitis is an inflammatory disease of the mammary glands that causes changes in glandular tissue and may involve several pathogens (Sokolov *et al.*, 2021). Among the organisms involved, approximately 100 types were identified, such as bacteria, fungi, and viruses (Rudenko *et al.*, 2021). The primary causative agents of mastitis are usually Staphylococci and Streptococci, but it is often caused by more than one microorganism (Gonzalez-Peña *et al.*, 2020). The most common origin of mastitis is microbial infection, but authors reported that its occurrence may result from trauma, chemical irritation or due to allergies (Kibebew, 2017).

Mastitis has a direct impact on the profitability of dairy cattle, once it causes production decrease and changes in the composition of milk (Krewer *et al.*, 2013). In addition, this disease can be responsible for

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modifications in the parenchyma of the mammary gland, causing functional loss and even the death of the animal, generating losses that include the disposal of contaminated milk, treatment, increased labor and veterinary services, and the destination of contaminated animals (Pavlenko *et al.*, 2018). Mastitis can also become a threat to public health due to the spread of pathogens and toxins, or by the presence of antibiotic residues in milk (Costa *et al.*, 2013).

The adopted strategy for the treatment of mastitis is based on the differentiation between the clinical and subclinical types, the general health status of the animal as well as its clinical history. The treatment for this disease is the most frequent cause for the use of antibiotics in dairy farmers (McDougall *et al.*, 2017). The correct use of these drugs can eliminate or inhibit bacterial growth, but when incorrectly administered, resistant strains can be selected (Pérez *et al.*, 2020).

Bacterial resistance represents a risk to human and animal lives (Da Costa and Silva Junior, 2017), since pathogenic microorganisms can become resistant to all the antibiotics known, causing infections more difficult to treat. Due to the type of management carried out by the producers, each herd may present different resistant strains, and the individual evaluation is necessary in order to avoid the loss of effectiveness of antimicrobials and the environmental contamination by their residues (Martins *et al.*, 2007; Serwecińska, 2020).

Bacterial resistance is a global public health problem that, despite being difficult to solve, can be managed (Fariña, 2016). Some strategies that can be adopted to avoid the selection of resistant strains are the knowledge of the causative agent of mastitis, its sensitivity to antibiotics and the evaluation of the epidemiological data (Ashraf and Imran, 2020).

In this way, the objective of this study was to investigate the most common pathogens in the Parana state from subclinical mastitis and establish their antimicrobial susceptibility profiles.

Materials and methods

Study area and sampling

Ethics approval was obtained through the Ethical Committee on the Use of Animals of the Pitágoras-Unopar University. The study was carried out in 13 municipalities from the northwest State of Parana, Brazil: Amaporã, Cianorte, Goioerê, Janiópolis, Loanda, Moreira Sales, Planaltina do Paraná, Querência do Norte, Santa Cruz de Monte Castelo, Santa Isabel do Ivaí, Tapejara, Tuneiras do Oeste and Umuarama. Geographically, all of them have a humid subtropical climate (Cfa) according to the Köppen classification.

The 27 commercial dairy herds involved in this study included the producers from a dairy plant in Umuarama, selected for convenience based on the willingness to participate in this research. Most of them were small farms, based on family labor and mechanical milking, with an average of 16 ± 5.9 cows per farm and mean daily milk production of 14 ± 7.4 L/cow. Most herds were Holstein-Friesian breed type.

Animals and milk sampling

A total of 507 foremilk samples were aseptically collected following the National Mastitis Council guidelines (Oliver, 2004). Before milking, teat ends were cleansed with 70% ethanol and the first three squirts were discarded. A total of 10 mL of milk from each mammary quarter were collected in sterile tubes. The milk samples were cooled with ice packs and immediately transported to the laboratory.

Cows were selected based on at least two consecutive positive scores on California Mastitis Test (CMT), which was routinely performed weekly in the farms, when the survey was conducted (between November 2018 and April 2019).

Microbiological analysis

The microbiological cultures of foremilk samples were performed according to Koneman and Cury (2001), seeding 10 µL aliquots on blood agar plates with 5% defibrinated sheep blood, followed by incubation at 37 °C for 48 h in aerobic conditions. Samples leading to the isolation of more than two species were considered as contaminated and discarded. Samples presenting two different pathogens were recorded as a mixed infection.

Preliminary identification was performed by colony morphology, hemolysis, and Gram staining. Further, differentiation between genera and species were performed according to Oliver, (2004) and Koneman and Cury (2001). For Gram positive bacteria, catalase was checked to distinguish streptococci from staphylococci. Staphylococci were further characterized by using coagulase test (sheep plasma) and Mannitol salt agar. For streptococci identification, sorbitol and mannitol fermentation, and arginine and aesculin hydrolysis were performed. Streptococci with β-hemolysis were identified as *Streptococcus agalactiae*. *Corynebacterium* spp., *Bacillus* spp. and *Trueperella* spp. were characterized to the genus level. Other groups ("other *Streptococcus* spp.," "other *Staphylococcus* spp.") were used to assemble bacterial genus for which less than five members were isolated.

Antimicrobial susceptibility

The disk-diffusion method on Mueller–Hinton agar were performed following the Clinical and Laboratory Standards Institute (NCCLS, 1990), classifying the isolated pathogens as susceptible or resistant. The antibiotics tested were bacitracin, cefoperazone, cephalexin, cloxacillin, enrofloxacin, gentamicin, Penicillin G, and the combination of sulfamethoxazole and trimethoprim, all with concentration of 30 µg per disc.

Data analysis

Differences in the distribution of pathogens were tested using chi-square analysis at 5% significance level when the absolute frequency was greater than five and using Fisher's test otherwise. The proportion of isolates exhibiting resistant phenotypes was calculated and presented as prevalence of antimicrobial susceptibility, dividing the number of resistant isolates by the total tested

Results and discussion

Bacterial isolates

A total of 507 foremilk samples from quarters with subclinical mastitis were analyzed, and 336 (66%) of them showed microbiological growth in the culture media. The distribution of positive samples was not homogeneous among the cities ($X^2 = 199.6$; $p<0.01$), with a higher rate in Tapejara (89%), Cianorte (82%), S.ta Isabel do Ivaí (81%) and Umuarama (72%). Tuneiras do Oeste and Goierê showed the lowest rates (52% and 50%, respectively) **(Figure 1**).

The microbiological identification of the positive samples is presented in Table I. Streptococci were the most frequently genus observed (61% of the identified pathogens), followed by staphylococci (28%) and other genera (11%) ($X^2 = 131.38$; $p<0.01$). Among other genera, the most frequently found were *Bacillus* spp., *Trueperella* spp. and *Corynebacterium* spp.

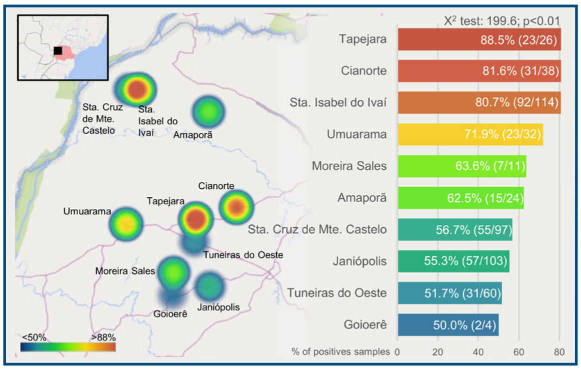

Figure 1. *Spatial distribution, absolute and relative frequency of foremilk samples with microbiological growth from dairy cows with subclinical mastitis in northwest Paraná, Brazil, between 2018 November and 2019 April.*

Most studies and literature reviews consider *Staphylococcus* spp. as the most frequent genus isolated from subclinical mastitis in dairy cows, followed by *Streptococcus* spp. (da Silva Duarte *et al.*, 2020; G.A. and J.M., 2011; Santos *et al.*, 2018). However, in our study we found a higher proportion of streptococci, what reinforces that the etiology of subclinical mastitis can differ regionally, and consequently shows the need for periodic monitoring for its control. Thus, we suggest that the causative agents of bovine mastitis depend on small-scale regional conditions. Amer *et al.*, (2018) argue that those differences may be attributed to animal breeds, management systems, and/or husbandry.
Among streptococci, *Streptococcus uberis* was the most frequently identified species (76%). This agrees with other studies that usually report *S. uberis* as the most frequently identified species in cattle (Contreras and Rodríguez, 2011; Valmorbida *et al.*, 2017). Other streptococci observed were *S. mutans* (13%), *S. sanguis* (10%) and *S. mitis*, *S. sobrinus* and *S. agalactiae* (1%).

The most observed species of the *Staphylococccus* genus was *S. aureus* (66%), followed by *S. epidermidis* (23%) and S. *hyicus* (11%). Among *Staphylococcus* spp., *S. aureus* is the most frequently pathogen associated with contagious intramammary infections of cattle herds worldwide. It is an agent that causes the greatest losses in dairy farming, presenting high pathogenicity and contagiousness (Hogeveen *et al.*, 2011). Furthermore, microorganisms of this genus pose risks to public health, as they can produce several virulence factors, especially considering their capacity to produce thermostable enterotoxins that can remain in milk, thus causing foodborne diseases in consumers (Viçosa *et al.*, 2010), in addition to the ability to transfer resistance genes to humans (Silva *et al.*, 2018).

Antibiotic susceptibility of bacterial isolates

The antimicrobial susceptibility was performed in 290 of the 336 isolates. Overall, 40% of the tested isolates showed resistance to at least one antibiotic used in this study (Table II). According to chi-square test, the antimicrobial resistance was proportional ($X^2=8.29$; $p=0.22$) between the antibiotics used and/or their association.

However, when categorized by genus, the antimicrobial resistance was not proportional between the categories of antibiotics for *Staphylococcus* spp., that showed higher resistance

Table I. *Occurrence of bacterial resistance to antibiotics in dairy cows from 57 articles published between 2009 and 2018 (n= number of isolates analyzed).*

	2010	2011	2012	2013	2015	2016	2017	2018
Amikacin	-	-	-	-	0.04a	0.04a	-	-
					(n=69)	(n=56)		
Amoxicillin	-	-	-	0.59a	-	0.71a	0.05b	0.50a
				(n=453)		(n=17)	(n=313)	(n=30)
Ampicillin	0.68de	0.27ab	0.78e	0.68de	0.47c	0.67cde	0.18a	0.48bcd
	(n=289)	(n=154)	(n=242)	(n=805)	(n=232)	(n=39)	(n=313)	(n=62)
Bacitracin	0.41b	-	-	0.08a	-	-	0.07a	-
	(n=188)			(n=194)			(n=846)	
Cephalexin	-	0.00a	-	0.01a		0.13b	0.74c	0.19b
		(n=65)		(n=453)		(n=56)	(n=869)	(n=32)
Cephalothin	0.13b	0.30b	0.00a	0.01a	0.13b	-	-	-
	(n=188)	(n=50)	(n=83)	(n=546)	(n=153)			
Cefoperazone	0.20a	-	-	0.50b	-	-	-	-
	(n=101)			(n=352)				
Ceftiofur	0.04a	0.02a	-	0.01a	-	-	-	-
	(n=101)	(n=65)		(n=546)				
Chloramphenicol	0.26b	-	0.14b	0.05a	0.00a	0.25b	-	-
	(n=188)		(n=180)	(n=194)	(n=69)	(n=32)		
Enrofloxacin	0.21b	0.02a	0.00a	0.01a	-	-	0.03a	0.09a
	(n=101)	(n=66)	(n=83)	(n=453)			(n=36)	(n=32)
Erythromycin	0.25cd	0.16bc	0.08ab	0.04a	0.39d	0.23abcd	0.20bc	0.72e
	(n=227)	(n=116)	(n=197)	(n=275)	(n=163)	(n=26)	(n=120)	(n=32)
Streptomycin	0.68b	-	-	0.12a	-	0.24a	-	-
	(n=188)			(n=453)		(n=46)		
Gentamicin	0.20d	0.03ab	0.06bc	0.02a	-	0.16cd	0.75e	0.87e
	(n=188)	(n=156)	(n=242)	(n=805)		(n=92)	(n=2100)	(n=30)
Neomycin	0.39b	0.03a	-	0.03a	-	0.43b	0.73c	-
	(n=101)	(n=118)		(n=546)		(n=56)	(n=1880)	
Norfloxacin	0.26b	0.07a	-	0.03a	-	0.12ab	-	0.09ab
	(n=188)	(n=88)		(n=282)		(n=26)		(n=32)

Data for the Amoxacillin + Clavulanic acid association in 2014 was omitted due to only one entry; a, b,c,d: Proportions followed by equal letters did not differ over the years by the chi-square test with 5% significance.

for cefoperazone ($X^2=23.14$; $p<0.01$). Cefoperazone is a third-generation cephalosporin which has bactericidal activity to most mastitis pathogens (Feßler *et al.*, 2017). The group of cephalosporins (mainly cefoperazone, cephalexin, and ceftiofur) is currently one of the main groups of antimicrobials commercially available for the intramammary treatment of mastitis during lactation and drying in production animals. Its widespread use in dairy farms is one of many factors that contribute to the selective pressure of microorganisms resistant to cefoperazone (Tomazi and dos Santos, 2020). Obviously, the development of antimicrobial resistance is not solely dependent on this widespread use. Further research focusing on the interactions of antimicrobial use and development of resistance would be appropriate for establishing preventive measures. The high proportion of cefoperazone resistant *Staphylococcus* sp. isolates suggested that resistant strains may be circulating in northwest Paraná across the investigated dairy farms.

A possible practical application of our results is that cephalexin, gentamicin and enrofloxacin continues to maintain its antibacterial activity against *Staphylococcus* genus in the studied area. In addition, is advisable to avoid cefoperazone therapy when the etiologic agent belongs to *Staphylococcus* genus to increase the cure rates. However, this statement has some limitations. It is important to point out that there is a close association between

Supplementary Table I. Sampling technique and representativeness of 57 articles published between 2009 and 2018 that met the inclusion criteria of the systematic review.

	Reference	State	Type of sampling
1	Alencar *et al*. (2014)	Rio de Janeiro	Purposive sampling, independent
2	Amorim *et al*. (2016)	Pernambuco	Purposive sampling, independent
3	Andrade *et al*. (2010)	Paraná	Convenience, random, independent
5	Assis *et al*. (2017)	Espírito Santo	Convenience, random, independent
6	Bandeira *et al*. (2013)	R.Grande do Sul	Purposive sampling, independent
7	Brito *et al*. (2014)	Maranhão	Convenience, random, independent
8	Carvalho *et al*. 2018	Maranhão	Convenience, random, independent
9	Casanova *et al*. (2016)	Santa Catarina	Purposive sampling, independent
10	Castro *et al*. (2012)	Rio de Janeiro	Quota sampling, independent
11	Chagas *et al*. (2012)	Minas Gerais	Convenience, random, independent
12	Costa *et al*. (2013)	Minas Gerais	Convenience, random, independent
13	Costa *et al*. (2013)	Santa Catarina	Snowball sampling, independent
14	Costa *et al*. (2015)	Santa Catarina	Convenience, dependend
15	Cunha *et al*. (2015)	Minas Gerais	Convenience, random, independent
16	deSantana Neres *et al*. (2015)	Sergipe	Convenience, independent
17	Dias *et al*. (2011)	Minas Gerais	Quota sampling, independent
18	Farias *et al*. (2013)	R.Grande do Sul	Purposive sampling, independent
19	Ferreira *et al*. (2010)	Piauí	Quota sampling, independent
20	Filho *et al*. (2016)	Paraná	Convenience, random, independent
21	Freitas *et al*. (2018)	R.Grande do Sul	Purposive sampling, independent
22	Gonçalves *et al*. (2018)	São Paulo	Purposive sampling, independent
23	Jardim *et al*. (2014)	Paraná	Convenience, random, independent
24	Jobim *et al*. (2010)	Paraná	Convenience, random, independent
24	Jobim *et al*. (2010)	R.Grande do Sul	Convenience, random, independent
24	Jobim *et al*. (2010)	Santa Catarina	Convenience, random, independent
25	Junior *et al*. (2015)	São Paulo	Purposive sampling, independent
26	Kaiser *et al*. (2015)	R.Grande do Sul	Purposive sampling, independent
27	Karach *et al*. (2016)	Paraná	Convenience, random, independent
28	Kolling *et al*. (2011)	R.Grande do Sul	Purposive sampling, independent
29	Krewer *et al*. (2013)	Bahia	Convenience, random, independent
29	Krewer *et al*. (2013)	Pernambuco	Convenience, random, independent
30	Lange *et al*. (2017)	Paraná	Convenience, random, independent
31	Martins *et al*. (2010)	Mato Grosso	Convenience,representative, independent
31	Martins *et al*. (2014)	Piauí	Purposive sampling, independent
32	Martins *et al*. (2015)	Goiás	Convenience, random, independent
33	Melo *et al*. (2013)	Pernambuco	Convenience, random, independent
34	Niero 2018	Santa Catarina	Purposive sampling, independent
35	Oliveira *et al*. 2009	Sergipe	Convenience, independent
36	Oliveira *et al*. (2010)	Pará	Convenience, random, independent
37	Oliveira *et al*. (2012)	Bahia	Convenience, random, independent
38	Oliveira *et al*. (2013)	Paraná	Simple random sampling, independent
39	Peters *et al*. (2016)	R.Grande do Sul	Purposive sampling, independent
40	Rall *et al*. (2014)	São Paulo	Purposive sampling, independent
41	Ribeiro *et al*. 2009	São Paulo	Purposive sampling, independent
42	Ruiz *et al*. (2011)	Pernambuco	Convenience, random, independent

	Reference	**State**	**Type of sampling**
43	Saab *et al*. (2014)	Paraná	Convenience, random, independent
44	Saeki *et al*. (2011)	São Paulo	Convenience, independent
45	Santos *et al*. (2010)	Paraná	Convenience, random, independent
46	Senhorelo *et al*. (2013)	Espírito Santo	Convenience, random, independent
47	Silva *et al*. (2011)	Bahia	Convenience, random, independent
48	Silva *et al*. (2012)	Pernambuco	Purposive sampling, independent
49	Soethe *et al*. (2015)	Paraná	Convenience, random, independent
50	Souza *et al*. (2016)	Minas Gerais	Convenience, random, independent
51	Ulsenheimer *et al*. 2018	R.Grande do Sul	Purposive sampling, independent
52	Valmorbida *et al*. (2017)	Santa Catarina	Purposive sampling, independent
53	Vesco *et al*. (2017)	R.Grande do Sul	Purposive sampling, independent
54	Zanette *et al*. (2010)	Santa Catarina	Quota sampling, independent
55	Zimermann *et al*. (2017)	Paraná	Purposive sampling, independent

Table II. *Absolute and relative frequency of pathogens isolated from milk of cattle with subclinical mastitis in northwest Paraná, Brazil, between 2018 November and 2019 April.*

Location (positive samples)	**Occurrence (%) of antimicrobial resistance in:**		
	Streptococci genus	***Staphylococci genus***	**Other genera**
Amaporã (15)	14 (93.3%)	1 (6.7%)	0
Cianorte (31)	13 (41.9%)	12 (38.7%)	6 (19.4%)
Goioere (2)	2 (100%)	0	0
Janiopolis (57)	25 (43.9%)	25 (43.9%)	7 (12.2%)
Moreira Sales (7)	2 (28.6%)	5 (71.4%)	0
St. Cruz do M.C. (55)	38 (69.1%)	7 (12.7%)	10 (18.2%)
St. Isabel do Ivaí (92)	62 (67.4%)	26 (28.3%)	4 (4.3%)
Tapejara (23)	16 (69.6%)	4 (17.4%)	3 (13%)
Tuneiras do Oeste (31)	19 (61.3%)	7 (22.6%)	5 (16.1%)
Umuarama (23)	14 (60.9%)	8 (34.8%)	1 (4.3%)
Total (336)	205 (61.0%)	95 (28.3%)	36 (10.7%)

seasonality, bacterial pathogens, and occurrence of antimicrobial resistance (Boireau *et al.*, 2018). Thus, the current resistance profile may differ from the findings above since this research was conducted between 2018 November and 2019 April. Another drawback is related to the geographical limitation, since from an epidemiological point of view, the sampling used in this research does not guarantee a significant sample at the regional level. Nevertheless, the identification of the microbiological agent is essential to the most appropriate choice of the drug to be used in therapy. When possible, etiology should be determined before treatment, and the spectrum of antimicrobial activity should be appropriate for the etiological agent (Ruegg, 2020).

Conclusions

Our results strengthen the knowledge of the microbiological agent and antibiotic-resistance patterns of pathogens isolated from subclinical mastitis in dairy cows at regional level.

More studies are necessary since the resistance pattern among pathogens differs regionally and could help to clarify the spread of drug-resistant strains of animal origin through environmental routes. The antibiotic treatment decisions should be based on resistance tests to avoid the spreading of resistant strains in dairy cattle populations of northern Parana and beyond.

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