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Original Article

Etiology and *in vitro* antimicrobial susceptibility profile of strains isolated from bovine mastitis in dairy herds from the midwest region of Santa Catarina state, Brazil

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A R T I C L E   I N F O

A B S T R A C T

Dairy farming is an important social and economic activity in the Midwest region of Santa Catarina State, Brazil. However, despite technological advances in the productive chain, mastitis is still considered the main disease of dairy herds, due its high economic impact. Bacterial pathogens are the main cause of this disease and failures in the treatment of clinical cases are attributed in part to the antimicrobial resistance of these pathogens. As an alternative, *in vitro* antimicrobial susceptibility tests of strains isolated from bovine mastitis cases have been carried out; the information generated contributes to strategic treatment measures, and also to the control of microorganism resistance. Therefore, the aims of the present study were to evaluate the etiology and *in vitro* antimicrobial susceptibility profiles of bacterial strains isolated from cases of bovine mastitis in dairy herds from the Midwest region of Santa Catarina State, Brazil. For this purpose, 345 milk samples were collected from 70 herds located in 16 different municipalities. The main pathogens isolated were: staphylococci (55.9%), streptococci (34.8%), *Corynebacterium* spp. (7.8%) and *Escherichia coli* (1.4%). The resistance rates of staphylococcus strains to ampicillin and to gentamicin were lower (24.9%) and higher (33%), respectively, than those obtained in other Brazilian studies. The resistance profiles of streptococcus strains were similar to those described in the literature, while *Corynebacterium* spp., showed high resistance rates to tetracycline (85.2%). Moreover, a considerable prevalence of staphylococcus (27.5%) and streptococcus (11.7%) multiresistant strains was verified. These data reinforce the need for regional characterization of microorganisms.

INTRODUCTION

Brazil has significant milk production, with 35 million tons of milk produced per year. Santa Catarina ranks fifth in milk production among the Brazilian states, where the Western region accounts for 75% of total production (IBGE/PPM 2015). These statistics highlight the economic and social impact of dairy activity in the region.

Despite technological advances in productivity, genetic quality and management of dairy herds, bovine mastitis still represents a major problem in the productive process. Mastitis is the most costly endemic disease of milk production in the world (HALASA et al., 2007). In Brazil, it is estimated that clinical or subclinical cases of mastitis lead to a 12 to 15% decrease in milk production (DIAS, 2007). Bacterial infections are the main cause of mastitis and the most common pathogens involved are staphylococci, streptococci, coliforms and *Corynebacterium* (MELLO et al., 2012; OLIVEIRA et al., 2011; PERSSON; NYMAN; GRÖNLUND-ANDERSSON et al., 2011). Therefore, antimicrobial drugs have been widely used for the treatment and control of the disease.

However, the use of a single treatment protocol is a challenge, considering the multifactorial causes of mastitis and the occurrence of resistant bacterial strains to antimicrobial drugs (BRADLEY, 2002; ERSKINE et al., 2004).

Temporal trends of antimicrobial susceptibility of pathogens causing mastitis have been described in several countries. According to a retrospective study with 2,778 bacterial strains evaluated between the years 1994 and 2000, there was an increase in the number of ampicillin-, penicillin- and erythromycin-resistant *S. aureus* strains (ERSKINE et al., 2002). Antimicrobial resistance is not a recent phenomenon, but is currently a critical health problem. For many decades and at different levels, bacteria usually involved in common infections have experienced an artificial selection pressure due to the abusive use of antimicrobials. This has contributed to the persistence of genetically resistant bacterial subpopulations, even to newly developed drugs, which present a major public health challenge. With the scarcity of new antimicrobials arriving on the market, measures to prevent a global health crisis are urgently required (OMS, 2012).

As a control measure, the *in vitro* antimicrobial susceptibility profiles of bacterial strains isolated from cases of mastitis have been determined (PERSSON; NYMAN; GRÖNLUND-ANDERSSON et al., 2011; MARTINI et al., 2017; NADER FILHO et al., 2007; XU et al., 2015).

Information on the occurrence of resistance is not only required locally, but also regionally and internationally, to detect changes that require intervention strategies. In order to fulfill these requirements, continuous monitoring systems are needed to verify changes in the occurrence of resistance, thereby optimizing treatment strategies and controlling the increase in resistance of microorganisms to antimicrobials (AARESTRUP, 2004). In this regard, we evaluated the etiology and *in vitro* susceptibility profiles of bacterial strains isolated from cases of mastitis in dairy herds of the Midwest region of Santa Catarina State, Brazil, in order to contribute to prophylactic and control measures in these herds.

MATERIALS AND METHODS

The present study was previously approved by the ethics committee on the use of animals (CEUA) at the Catarinense Federal Institute – *campus* Concórdia, Brazil, under registration number 34/2015.

Three hundred and forty-five milk samples were collected from cows with clinical or subclinical mastitis. The animals were from 70 dairy herds located in 16 municipalities of Santa Catarina state: Alto Bela Vista, Arabutã, Capinzal, Concórdia, Descanso, Ipira, Irani, Jaborá, Lindóia do Sul, Modelo, Peritiba, Pinhalzinho, Ponte Serrada, Presidente Castelo Branco, Seara and Xavantina (Figure 01).

Figure 01 – Spatial distribution of the municipalities where the dairy herds were sampled. The numbers of samples collected per municipality were classified into categories, which are represented by different colors, as shown in the legend.
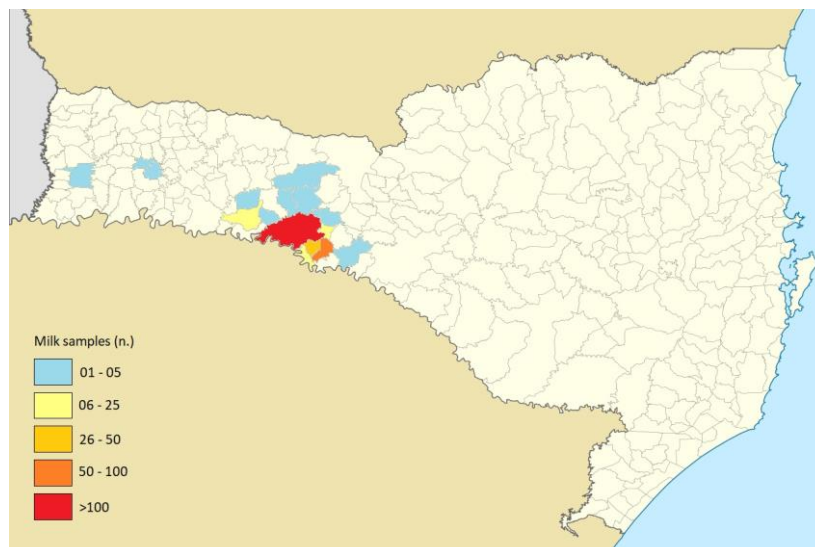
Source: Modified from https://commons.wikimedia.org/wiki/File:Brazil_Santa_Catarina_ location_map.svg#

Microbiological analyses were carried out at the Laboratory of Veterinary Microbiology at the Catarinense Federal Institute campus Concórdia. Aliquots of milk (10μL) were cultured in 5% Sheep Blood Agar and MacConkey Agar, followed by incubation at 37°C for 24 to 72 hours. The resulting growth of the

culture was monitored every 24 hours. Identification of the colonies was performed according to their morphological, microscopic and biochemical characteristics (MARKEY et al., 2013).

The antimicrobial susceptibility testing (AST) was carried out using the Kirby-Bauer disk-diffusion method (CLSI, 2012). Bacterial cultures of 18–24 hours were resuspended in saline solution to an equivalent turbidity of 0.5 McFarland standard. Using a sterile swab, the isolates were inoculated on Petri dishes containing Mueller Hinton (MH) agar for cultures of staphylococci and Escherichia *coli*, or MH Blood agar for cultures of streptococci and *Corynebacterium*. Subsequently, disks impregnated with antimicrobial drugs were applied according to the pathogen tested (Table 1).

Table 1 – Antimicrobial drugs tested according to the isolated bacterial groups.

Antimicrobial (concentration/disk)	Staphylococci	Streptococci	*Corynebacterium* spp.	*E. coli*
Amikacin (30µg)	-	-	-	X
Amoxacillin-clavulinic acid (20/10µg)	X	X	-	X
Ampicillin (10µg)	X	X	-	X
Cephalonium (30µg)	X	X	-	-
Cephapirin (30µg)	X	X	-	-
Cefuroxime (30µg)	X		-	X
Ciprofloxacin (5µg)	-	-	-	X
Clindamycin (2µg)	X	X	X	-
Erythromycin (15µg)	-	X	-	-
Gentamicin (10µg)	X	-	X	X
Levofloxacin (5µg)	-	-	-	X
Trimethoprim-sulfamethoxazole (25µg)	X	-	-	X
Tetracycline (30µg)	X	X	X	-

X: tested antimicrobial; -: not-tested antimicrobial.

After 18h of incubation at 37°C, the inhibition zone diameter (mm) was measured and the samples were classified as resistant (R), susceptible (S) or intermediate (I). Strains that showed any degree of resistance (R or I) to three or more classes of antimicrobials were classified as multiresistant (SCHWARZ et al. 2010). For data analysis, descriptive statistics were used, according to the frequency distributions obtained in microbiological tests.

RESULTS AND DISCUSSION

Staphylococcus was the most frequently isolated genus in the evaluated milk samples (55.9%) (Table 2). *Staphylococcus aureus* is still the most prevalent pathogen causing intramammary infections in dairy cattle worldwide (ABEBE et al., 2016; PETROVSKI et al., 2009; RIEKERINK et al., 2010; TAPONEN et al., 2017). In this study, the isolation rate for *S. aureus* was 40.3%. In Brazil, others authors found similar frequencies, ranging from 8.3% to 57.8% (COSTA KREWER et al., 2015; MARTINS et al., 2010; MELLO et al., 2012; LAFFRANCHI et al., 2001; OLIVEIRA et al., 2011). Besides *S. aureus*, other species of *Staphylococcus* that were earlier considered of minor importance are now accepted as relevant human and animal pathogens (GUIMARÃES et al., 2016). In the present study, four species of coagulase-negative staphylococci were identified: *S. haemolyticus, S. epidermidis, S. pasteuri* and *S. xylosus*, which were previously detected in mastitis milk samples (FREY et al., 2013; GUIMARÃES et al., 2016; SOARES et al., 2012; XU et al., 2015). The results obtained reinforce the importance of these pathogens as mastitis causing agents in dairy herds of the Midwest region of Santa Catarina State, having a direct implication for infection control measures, since the correct identification of the etiological agent is fundamental to establishing appropriate prevention and control measures (HENNEKINNE et al., 2010).

Table 2 – Frequency of pathogens isolated from samples of bovine mastitis milk collected in dairy herds of the Midwest region of Santa Catarina state.

Etiological agent	n. (%)
Staphylococci	193 (55.9)
Staphylococcus aureus	139 (40.3)
Staphylococcus haemolyticus	4 (1.2)
Staphylococcus epidermidis	2 (0.6)
Staphylococcus pasteuri	2 (0.6)
Staphylococcus xylosus	1 (0.3)
Staphylococcus spp.	45 (13.0)
Streptococci	120 (34.8)
Streptococcus uberis	75 (21.7)
Streptococcus. dysgalatiae	15 (4.3)
Streptococcus agalactiae	8 (2.3)
Streptococcus spp.	22 (6.4)
Corynebacterium spp.	27 (7.8)
Escherichia coli	5 (1,4)
TOTAL	345 (100.0)

The isolated strains of staphylococci showed higher resistance rates to tetracycline, gentamicin, trimethoprim-sulfamethoxazole and ampicillin (Table 3). In cattle production systems, antimicrobials like gentamicin, tetracyclines and penicillins are widely used (ECONOMOU; GOUSIA, 2015) and in Brazil, many veterinary medicines are based on these active principles, including intramammary formulations (MARTINI et al., 2017). Furthermore, the genetic basis for staphylococcal resistance to tetracyclines

(WENDLANDT et al., 2013), beta-lactams (LOWY, 2003) aminoglycosides (RAMIREZ; TOLMASKY, 2010) and trimethoprim-sulfamethoxazole (HUOVINEN, 2001) has been previously described. Therefore, the widespread use of antimicrobials in herds could contribute to increased artificial selection pressure on microorganisms, leading to the proliferation of drug-resistant subpopulations. This implies a lower cure rate of treated animals and also a serious risk to public health.

Table 3 – *In vitro* antimicrobial susceptibility profiles of bacterial strains isolated from milk samples collected from cows with mastitis in dairy herds of the Midwest region of Santa Catarina State.

	Staphylococci (%)			Streptococci (%)			*Corynebacterium* spp. (%)			*E. coli* (%)		
	S	I	R	S	I	R	S	I	R	S	I	R
AMC	96.8	0.0	3.2	91.2	0.0	8.8	-	-	-	-	-	-
AMI	-	-	-	-	-	-	-	-	-	100.0	0.0	0.0
AMP	74.6	0.5	24.9	94.8	0.0	5.2	-	-	-	-	-	-
CIP	-	-	-	-	-	-	-	-	-	100.0	0.0	0.0
CLI	82.2	0.0	17.8	63.8	0.0	36.2	100.0	0.0	0.0	100.0	0.0	0.0
CNM	94.8	0.0	5.2	90.9	0.0	9.1	-	-	-	100.0	0.0	0.0
CPR	94.8	0.0	5.2	90.9	0.0	9.1	-	-	-	100.0	0.0	0.0
CRX	96.9	0.0	3.1	95.7	0.0	4.3	-	-	-	100.0	0.0	0.0
ERI	-	-	-	75.7	4.3	20.0	-	-	-	100.0	0.0	0.0
GEN	67.0	0.0	33.0	-	-	-	100.0	0.0	0.0	100.0	0.0	0.0
LVX	-	-	-	-	-	-	-	-	-	100.0	0.0	0.0
SUT	67.8	0.0	32.2	-	-	-	-	-	-	-	-	-
TET	61.7	0.0	38.3	59.5	0.0	40.5	14.8	0.0	85.2	100.0	0.0	0.0

AMC: Amoxacillin-clavulinic acid 20-10 µg, AMI: Amikacin 30µg, AMP: Ampicillin 10µg, CNM: Cephalonium 30 µg, CPR: Cephapirin 30 µg, CRX: Cefuroxime 30µg, CIP: Ciprofloxacin 5µg, CLI: Clindamycin 2µg, ERI: Erythromycin 15 µg, GEN: Gentamicin 10µg, LVX: Levofloxacin 5µg, SUT: Trimethoprim-sulfamethoxazole 25 µg, TET: Tetracycline 30µg, S: Susceptible; I: Intermediate; R: Resistant; -: Not tested.

Resistance rates obtained from *Staphylococcus* strains to tetracycline (38.3%) and trimethoprim-sulfamethoxazole (32.2%) corroborate the results reported in previous studies, in which the resistance rates range from 0 to 76.7% for tetracycline and 0 to 57.14% for trimethoprim-sulfamethoxazole (BENGTSSON et al., 2009; COSTA et al., 2013; JAMALI; RADMEHR; ISMAIL, 2014; MARTINI et al., 2017; NERES et al., 2015; ROESCH et al., 2006; SURIYASATHAPORN, 2010). Similar values were found comparing the percentages of resistance to ampicillin (24.9%) and gentamicin (33%) with studies performed abroad, where the values reached 64% for ampicillin and 100% for gentamicin (PERSSON; NYMAN; GRÖNLUND-ANDERSSON, 2011; ROESCH et al., 2006; SAHEBEKHTIARI et al., 2011; SURIYASATHAPORN, 2010). However, when these values were compared with those obtained in studies conducted in Brazil, it was observed that the rates of resistance to ampicillin were below those observed in previous studies, 39.1%–95.8%; while gentamicin resistance was higher, 0–23.1% (MARTINI et al., 2017; MEDEIROS et al., 2009; NADER FILHO et al., 2007; NERES et al., 2015; OLIVEIRA et al., 2011). This may be due to regional differences in the use of these antimicrobials, producing different selection pressures on bacterial populations or even different genetic characteristics of resistance in strains from different regions, reinforcing the importance of the regional characterization of circulating strains. Aarestrup (2004) points out that information on the occurrence of antimicrobial resistant strains is necessary at the local, regional and international levels in order to detect changes that require differentiated intervention strategies.

Another relevant pathogen causing mastitis is *Streptococcus*, identified with a frequency of 1.8 to 14% in Brazilian herds (ANDRADE; HARTMANN; MASSON, 2009; MARTINS et al., 2010; MELLO et al., 2012; OLIVEIRA et al., 2011). In the present study, we found a higher frequency of this pathogen, 34.8% (Table 02), and *Streptococcus uberis* (21.7%) was the predominant species isolated. *S. uberis* is responsible for a significant proportion of mastitis cases and is considered an environmental pathogen (ABUREEMA et al., 2014; KHAN et al., 2003; UNNERSTAD et al., 2009). Among the risk factors that increase the incidence of *S. uberis* in the herd is the number of lactations. Zadoks et al. (2001) reported that after two lactations, the frequency of isolation of this pathogen in cows increased. A lack of hygiene in the environment has also been attributed as a risk factor for intramammary infections caused by *S. uberis* (TAPONEN et al., 2017). Galton (2004) observed that post-dipping may reduce the risk of intramammary infection by *S. uberis* by up to 94%. In a study conducted in dairy farms of the Midwest and Itajaí's High Valley regions in Santa Catarina state, Winck; Neto (2009) observed that post-dipping was performed in only 36.1% of the herds; furthermore, 73.1% of the farms showed a high total bacterial count (TBC), which indicates deficiencies in hygiene and cleanliness in the milk production.

The highest rates of resistance to streptococci were observed for tetracycline (40.5%), clindamycin (36.2%) and erythromycin (20%), corroborating the results

obtained in previous studies, in which 11.1 to 71.7%, 0 to 61.5% and 0 to 50% of *Streptococcus* isolates were resistant to tetracycline, clindamycin and erythromycin, respectively (DENAMIEL et al., 2005; DUARTE et al., 2004; EBRAHIMI; KHEIRABADI; NIKOOKHAH, 2007; GUÉRIN-FAUBLEÉ et al., 2002; OLIVEIRA et al., 2011; PITKÄLÄ et al., 2007; ROSSITTO et al., 2002).

Other isolated agents were *Corynebacterium* spp. (7.8%) and *E. coli* (1.4%). *Corynebacterium* is a pathogen that is frequently isolated in mastitis cases. In Brazil, the frequency of this agent ranges from 4.8 to 55.2%, while *E. coli* is isolated sporadically, in 0 to 3.2% of cases (BRITO et al., 1999; LARANJA; MACHADO, 1994; MARTINS et al., 2010; MELLO et al., 2012; OLIVEIRA et al., 2011). *E. coli* strains did not show resistance to any of the tested antimicrobials, while for *Corynebacterium* strains, a high resistance rate to tetracycline (85.2%) was reported (Table 02). Tetracycline resistance genes in *Corynebacterium* have been described in the literature (ROBERTS, 2005). However, antimicrobial susceptibility studies in *Corynebacterium* spp. isolated from mastitis cases are still scarce. Suriyasathaporn et al. (2010) observed that 100% of *Corynebacterium* spp. strains were gentamicin resistant, differing significantly from this study (0%).

Multiresistance was detected in 53 (27.5%), 14 (11.7%), 0 (0%) and 0 (0%) strains of *Staphylococcus*, *Streptococcus*, *Corynebacterium* spp. and *E. coli*, respectively. Resistance to various antimicrobial drugs results in failures in the standard response to treatment, leading to prolonged disease periods, increased health care expenditures, and risk of death (TANWAR et al., 2014). In addition, it is a public health problem, since bacteria of animal origin can serve as a reservoir of resistance, being transmitted to humans through the consumption of food and through direct or indirect contact with infected animals. Furthermore, antibiotic resistant strains can be disseminated to the environment through animal waste, contributing to the propagation of resistance genes in the environmental microbiome (ECONOMOU; GOUSIA, 2015).

CONCLUSION

Staphylococci, streptococci and *Corynebacterium* spp. have great relevance in the etiology of mastitis in dairy herds from the Midwest region of Santa Catarina state. The identification of multiresistant strains *in vitro* susceptibility profiles to antimicrobials, widely used in the region for the treatment of clinical cases of mastitis, is a warning that underlines the need for the regional characterization of circulating microorganisms, as well as the adoption of prophylactic and control measures.

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