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Bacterial identification, somatic cell count, antimicrobial profile and toxigenic *Staphylococcus* strains search from mastitic cow milk samples on small farms properties[1]

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ABSTRACT.- Lavor U.L., Guimarães F.F., Salina A., Mioni M.S.R. & Langoni H. 2019. **Bacterial identification, somatic cell count, antimicrobial profile and toxigenic *Staphylococcus* strains search from mastitic cow milk samples on small farms properties.** *Pesquisa Veterinária Brasileira 39(9):715-722*. Departamento de Higiene Veterinária e Saúde Pública, Universidade Estadual Paulista, Rua Prof. Dr. Walter Maurício Correa s/n, Campus Botucatu, Botucatu, SP 18618-681, Brazil. E-mail: helio.langoni@unesp.br

Bovine mastitis has a negative impact on milk production and can pose risks to public health. The present study aimed to evaluate the quality of bovine milk from small farms in the Botucatu/SP region. Somatic cell counts (SCC), identification of pathogens involved in mastitis, and sensitivity antimicrobial profile of staphylococci isolated were performed. The presence of enterotoxin encoding genes in isolates of staphylococci obtained from milk was investigated. Milk samples from individual mammary quarters of cows were submitted to the California mastitis test (CMT) and SCC. Of the 239 dairy cows from 21 dairy herds evaluated (mean = 11.4 animals/property), two cows (0.8%) presented clinical mastitis and 86 (35.9%) subclinical mastitis. Bacterial culture was performed in 177 quarter milk samples. Staphylococci were identified in 55 (31.1%), corynebacteria in 45 (25.4%), streptococci in 25 (14.1%) and coliforms in four (2.3%) milk samples. Average SCC from culture-positive samples was $1598x10^3$ cells/mL, in case of staphylococci was $1362x10^3$ cells/ml, streptococci was $2857x10^3$ cells/mL, corynebacteria was $976x10^3$ cells/mL and in the cases of coliforms $1161x10^3$ cells/mL were obtained. Staphylococci showed a high sensitivity (>95%) to cephalothin, cotrimoxazole, enrofloxacin, and gentamicin, with a 41.2% resistance to penicillin and 11.8% to oxacillin. Both coagulase positive (CPS) and negative staphylococci (CNS) carried genes encoding enterotoxins in 21.6% of the first group and 41.9% in the second. The *sea* gene was the most detected 45.8% (n=24) between them, followed by *seb* with 29.2% and *sec* with 25.0%. The *sed* gene was not identified. We highlight the potential risk to public health in the possibility of strains of *Staphylococcus* spp. enterotoxin-producing genes that can cause staphylococcal food poisoning.

INDEX TERMS: Bacterial identification, somatic cell count, antimicrobial profile, toxigenic, *Staphylococcus,* mastitis, cow, milk, farms, enterotoxins, public health, gene, *sea*, *seb*, *sec*.

RESUMO.- [**Identificação bacteriana, contagem de células somáticas, perfil antimicrobiano e pesquisa de linhagens toxigênicas de *Staphylococcus* em amostras de leite bovino de pequenas propriedades rurais.**] A mastite bovina impacta negativamente a produção leiteira e pode acarretar riscos à saúde pública. O presente estudo teve como objetivo a avaliação da qualidade do leite bovino proveniente de pequenas propriedades na região de Botucatu/SP. Foi realizada a contagem de células somáticas (CCS), identificação dos patógenos envolvidos nas mastites, e realizado o perfil de sensibilidade aos antimicrobianos dos estafilococos isolados. Pesquisou-se a presença de genes codificadores de enterotoxinas em isolados de estafilococos obtidos a partir do leite mastítico.

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Amostras de leite de quartos mamários individuais de vacas foram submetidas ao "California mastitis test" (CMT) e à CCS. Das 239 vacas em lactação provenientes de 21 rebanhos leiteiros avaliados (média = 11,4 animais/propriedade), dois (0,8%) animais apresentaram mastite clínica e, 86 (35,9%) mastite subclínica. 177 amostras de leite foram cultivadas em ágar sangue bovino 5% e ágar MacConckey e obteve-se 55 (31,1%) *Staphylococcus* spp., 25 (14,1%) *Streptococcus* spp., 45 (25,4%) *Corynebacterium* spp. e quatro (2,3%) coliformes. A média da CCS das amostras procedentes de todos os quartos mamários infectados avaliados foi de 1598x10^3 células/mL, enquanto que nos casos que foram isolados *Staphylococcus* spp. foi de 1362x10^3 células/mL, *Streptococcus* spp. 2857x10^3 células/mL, *Corynebacterium* spp. de 976x10^3 células/mL e nos casos de coliformes 1161x10^3 células/mL. Os estafilococos revelaram grande sensibilidade (>95%) à cefalotina, cotrimoxazol, enrofloxacina e gentamicina, com resistência de 41,2% à penicilina e 11,8% à oxacilina. Tanto estafilococos coagulase positivos (ECP) como negativos (ECN) revelaram genes codificadores de enterotoxinas em 21,6% do primeiro grupo e 41,9% no segundo. O gene *sea* foi o mais detectado 45,8% (n=24), seguido pelo *seb* com 29,2% e *sec* com 25,0%. O gene codificador da *sed* não foi identificado. Frente aos resultados, destaca-se o risco potencial à saúde pública pela possibilidade de veiculação de linhagens de *Staphylococcus* spp. carreadores de genes produtores de enterotoxinas, podendo ocasionar toxi-infecções alimentares.

TERMOS DE INDEXAÇÃO: Identificação bacteriana, contagem de células somáticas, *Staphylococcus,* leite, bovinos, propriedades rurais, mastite, enterotoxinas, saúde pública, genes, *sea*, *seb*, *sec*.

INTRODUCTION

Approximately 85% of the 1.3 million dairy farmers in Brazil can be classified as small producers. Low technification, poor management, poor sanitary control and inadequate hygiene conditions decrease the quality of the raw milk produced, posing a risk to consumers of the transmission of micro-organisms that cause zoonoses or possible toxi-infections.

Mastitis, the most prevalent disease in dairy cattle, affects the mammary gland leading to physicochemical and bacteriological changes in milk. Infectious causes are the most significant, both economically and in the public health interest. SCC is considered a good indicator of mammary gland health because it assesses the number of inflammatory cells present in milk. At counts above 200,000 cells/mL, fourth breasts are considered positive for mastitis (Dohoo & Leslie 1991).

Determining the etiology of mastitis enables the adoption of appropriate antimicrobial treatment. The differentiation of the contagious or environmental factors of the infection, the adoption of disease prevention and control measures are distinct for each group of microorganisms (Langoni et al. 1998, Radostits et al. 2007).

Despite the recent regulation of the dairy chain has led to transformations in the sector, such as the way of collection, with the bulk collection and the end of receiving uncooled milk, beginning of payment policies for quality, incorporation of technologies in the field and development of new products and industrial processes, the difficulties faced by small farmers in adapting their production to the new quality parameters established are still big (Carvalho 2010).

Due to the impossibility of meeting the pressures imposed by the market or lack of motivation due to the little consolidation of public policies in the dairy sector, many producers end up joining the informal market. Risking illegality, this producer can add to his production part of the income that would be appropriated by the intermediaries. (Silva & Tsukamoto 2001). As a result, this informally traded milk exposes consumers to greater risks, with serious public health implications.

Hygienic obtaining of milk, in addition to directly impacting product quality, increases the productivity of the herd by decreasing the prevalence of intramammary infections, resulting in direct and indirect economic gains for farmers (Langoni et al. 2011).

Staphylococcus is one of the main agents involved in the prevalence and persistence of mastitis in the dairy herd. (Fagundes & Oliveira 2004, Piepers et al. 2007). *Staphylococcus aureus* stands out for its difficult control due to its high resistance to the antimicrobial treatments used and the various virulence factors, causing long-lasting infections with low cure rates (Silva et al. 2014, Guimarães et al. 2017). Another important feature is the ability to produce enterotoxins, which can lead to poisoning by ingestion of thermostable toxins (Pinchuk et al. 2010) produced and released during their multiplication in food (Soriano et al. 2002).

Staphylococcal enterotoxins (SE) are a group of low molecular weight single chain proteins produced during all stages of bacterial multiplication, but especially during the half and end of the exponential phase (Soriano et al. 2002). Classic enterotoxins (A, B, C, and D) are the most common in bovine mastitis milk isolates. Their production is not restricted to *Staphylococcus aureus* species, but also to other staphylococci species such as coagulase negative staphylococci (CNS) (Radostits et al. 2007, Freitas Guimarães et al. 2013). The importance of the genus and its toxins is well elucidated in the literature, however, there is little information on the occurrence of genes that encode these toxins in the milk of animals from small dairy herds.

In order to reduce intramammary infections, it is essential to develop orientation and support activities for producers, such as extension activities, lectures and training directed to them. In order to achieve this reduction, it is recommended to improve the diagnostic techniques of clinical and subclinical mastitis, laboratory identification of the infectious agents involved, appropriate antimicrobial treatment of the affected quarters, cleaning and disinfection of the teats (pre and post-dipping) as well as the environment, in addition to the provision of good quality water to ensure the proper development of the inherent production activities.

This study aimed to investigate the aerobic microbiota of subclinical mastitis cases observed in small dairy properties, the relationship between the isolated species and the milk SCC of the respective breast quarters positive for the CMT test. It also aimed to identify the etiology of mastitis, as well as the potential risks to public health by detecting enterotoxin coding genes in strains of *Staphylococcus* spp. and established the antimicrobial sensitivity profile of staphylococci isolates obtained.

MATERIALS AND METHODS

Microbiological analyses, somatic cell count and molecular studies were performed at the Mastitis Research Center (NUPEMAS) of the Department of Veterinary Hygiene and Public Health, School of Veterinary Medicine and Animal Science, Unesp, Botucatu Campus/SP.

Dairy properties. The study was conducted in dairy herds whose daily production was less than 100 liters of milk. There were 21 distinct herds located in the rural area of Botucatu/SP. The number of lactating cows per herd ranged from 3 to 20, predominantly crossbred animals.

Mastitis diagnosis and sample collection. We screened all lactating cows from each herd. The diagnosis of clinical mastitis was based on clinical signs of breast inflammation (pain, flushing, heat and swelling) and/or changes in milk macroscopy (lumps, pus, blood or filaments) by proof of dark-bottomed cup or Tamis (Radostits et al. 2007). Subclinical mastitis was diagnosed with the aid of CMT (Schalm & Noorlander 1957). Tamis-positive milk samples with a CMT positivity score were collected aseptically, according to pre-established procedures (NMC 1999). Until microbiological examinations are performed immediately upon arrival at the laboratory, the samples were kept refrigerated (4 to 8°C) in isothermal boxes with recyclable ice.

Microbiological culture. The microbiological culture was performed according to standard procedures (NMC 1999). Initially, 10 microliters of each milk sample were grown in 5% bovine blood agar and MacConkey agar media, incubating at 37°C, observing microbial development every 24 hours for three days. The isolated colonies were picked in BHI (brain heart infusion) broth, studied morphologically for phenotypic characteristics (pigmentation and hemolysis), as well as morphologically by the Gram technique, and classified according to their ability to synthesize the coagulase enzyme in order to differentiate coagulase positive (CPS) from negative staphylococci (CNS). We used other biochemical tests to complement the classification of the other species and subspecies of CNS, such as the fermentation of sugars: mannitol, maltose, trehalose, xylose, arabinose, sucrose, lactose, xylitol, ribose and fructose. We observed urease and/or ornithine decarboxylase production, nitrate reduction and susceptibility to novobiocin (Cunha et al. 2007). For identification of enterobacteria, colonies isolated from MacConkey were subjected to biochemical tests of EPM, MILi and Simmons Citrate, where the production of gas, glucose, enzyme L-tryptophan deaminase (LTD), H2S, urea, motility, indole and lysine were evaluated (Trabulsi et al. 1999). Colonies suggestive of streptococci were submitted to catalase test, esculin hydrolysis and CAMP test, aiming to differentiate *Streptococcus agalactiae* from other species of mastitis-causing streptococci (Quinn et al. 2005).

Animals' SCC. In parallel to the above procedure, they were collected in a plastic bottle containing bronopol cell preservative (Bertrand 1996), Tamis or CMT-positive breast milk samples. SCC was performed on Somacount 300® equipment (Bentley, UK) by flow cytometry.

Microbial Sensitivity Profile. *Staphylococcus* spp. isolates were tested for microbial sensitivity by the disk diffusion method recommended by Bauer et al. (1966), on plates containing Mueller Hinton agar, arranged with the following antimicrobials: neomycin (30μg), gentamicin (10μg), penicillin (10UI), oxacillin (10μg), cephalothin (30μg), enrofloxacin (5μg) and cotrimoxazole (25μg). Interpretation of inhibition halos followed the Clinical and Laboratory Standards Institute (CLSI 2005) reference.

DNA extraction. *Staphylococcus* spp. were cultured in BHI broth, concentrated by centrifugation and DNA extracted using the Illustra Blood Genomic Prep Mini Spin Kit (GE Healthcare), as recommended by the manufacturer, after initial cell digestion with lysozyme (10mg/mL) and Proteinase K (20mg/mL).

Detection of enterotoxin coding genes. We used specific primers for the EEA (*sea*), EEB (*seb*), EEC (*sec*) and EED (*sed*) enterotoxin coding genes, according to Johnson et al. (1991). Reactions were performed in 0.2mL microtubes in a total volume of 25μL, with 10pmol of each primer, 1.0U Taq Platinum DNA polymerase (Invitrogen®), 200μM triphosphate deoxyribonucleotides, 1X PCR buffer, 0.75mM of MgCl2 and 3μL of sample. In all reactions negative controls were used with the replacement of nucleic acid by water. Incubation was performed on the Mastercycler gradient thermocycler (Eppendorf®).

Visualization of amplified products. Agarose gels were prepared in 2.0% concentration in 1X TBE buffer, stained with 1.0μL/mL SYBR Safe DNA gel stain (Invitrogen®). Products size were compared to the 100bp standard and subsequently photographed under UV transillumination.

Statistical analysis. The results were analyzed by general descriptive statistics, using the Windows Excel® program. Mixed linear models were used to compare the mean milk SCC between mammary glands infected with different pathogens. We used logistic regression models or Chi-square tests to test the association between *Staphylococcus* spp. and the antimicrobial sensitivity profile.

RESULTS AND DISCUSSION

Mastitis etiology

The results obtained for isolated species in the microbiological culture of 177 lactating cow's milk samples showed the occurrence of important mastitis pathogens and are presented in Table 1. The predominance of the genus *Staphylococcus* agrees with other prevalence studies conducted in both Brazilian dairy herds and in dairy farms in other countries (Nader Filho et al. 2007, Bolaños et al. 2014).

Corynebacterium spp. also plays an important role in the etiology of mastitis. *C. bovis* is responsible for significant reduction in milk production of infected fourth breasts. When evaluating the presence of *C. bovis* in dairy herds, Domingues et al. (1998) observed a decrease in milk production of up to 27.6%, while Zafalon et al. (1999) reported a 30.9% drop, in addition to observing a significant increase in milk SCC in animals with these bacteria.

Of the 31 isolates classified as *Streptococcus*, 67.7% (n=21) of these were classified as *S. agalactiae,* 25.8% (n=8) as *S. dysgalactiae* and 6.5% (n=2) as *S. bovis*. The main contagious agent of the genus Streptococcus and frequently isolated in the etiology of bovine mastitis is *S. agalactiae*. It is a mandatory and highly contagious intramammary microorganism (González et al. 1986) and can be efficiently eliminated with intramammary treatments during the lactation period.

Table 1. Bacterial isolation in milk samples from cows with clinical and subclinical mastitis in small dairy farms

Bacterial genus	Total (N)	Total %
Staphylococcus spp.	55	31.1
Corynebacterium spp.	45	25.4
No growth/contaminated*	34	19.2
Streptococcus spp.	25	14.1
Corynebacterium spp./*Staphylococcus* spp.	8	4.5
Staphylococcus spp./*Streptococcus* spp.**	5	2.8
Coliforms	4	2.3
Corynebacterium spp./*Streptococcus* spp.**	1	0.6
TOTAL	177	100

*No growth/contaminated = no bacterial growth samples or growth of three or more different microorganisms (NMC 1999).

Coliforms are important indicators of the hygienic and sanitary quality of food and frequently participate in breast infections. In the present study, only 2.3% (n=4) of the samples were found. Freitas Guimarães et al. (2013) and Ribeiro et al. (2009) also reported prevalence of Enterobacteriaceae-related mastitis in dairy cows.

The high prevalence of contagious microorganisms of the genera *Staphylococcus, Streptococcus* and *Corynebacterium* is due to the habitat of this group consisting of the mucosa and skin of the animals, indicating problems related to the operational hygiene of milking and possible chronicity of breast infection.

Regarding the biochemical characterization of staphylococci (Table 1), the main isolated species was *S. aureus* (n=21). We also observed the participation of CNS as important pathogens in bovine mastitis, as reported by Piepers et al. (2007).

Staphylococcus aureus and *S. intermedius* are causative agents of bovine mastitis, the latter being less frequently isolated in milk samples (Roberson et al. 1996, Oliveira et al. 2011, Freitas Guimarães et al. 2013). These species may be responsible for the occurrence of food poisoning outbreaks when associated with the presence of enterotoxin coding genes (Radostits et al. 2007, De Freitas Guimarães et al. 2013, Rall et al. 2014). *S. hyicus* is appointed as responsible for the production of exotoxin from scalded skin syndrome in pigs (Tanabe et al. 1996). Chenier & Lallier (2012) also attributed to this species some infections in laying hens. In this sense, the presence of other animals, such as swine and poultry on small dairy farms, may have contributed to the participation of this genus in the observed mastitis.

Regarding the species of CNS, the present study agreed with the scientific literature that indicated frequent participation of *S. hominis* and, to a lower frequency of *S. sciuri, S. capitis* (Radostits et al. 2007). *S. epidermidis* is more prevalent and persistent in human skin and mucous membranes. It can be inferred that its prevalence may be directly related to precariousness in the milking procedures in the visited properties, being necessary the confirmation from the bacteriological swab examination of the hands of the milkers.

Somatic cell count

Milk SCC reflects the intensity of the inflammatory response to breast tissue infection, and the values obtained in the present study are presented in Table 2. Defense cells, mainly neutrophils, migrate into the udder to eliminate the pathogen. In addition to the genetic characteristics and immunological capacity of animals, SCC is directly influenced by lactation period, month and season, order of birth, among others. However, the specific effect of pathogens and the state of breast infection are the main factors responsible for their variations.

The major pathogens (*S. aureus*, *S. agalactiae* and coliforms) cause mastitis, which results in large variations in milk composition and SCC, while secondary pathogens (CNS and *C. bovis*) cause moderate inflammatory process (Harmon 1994). The results obtained in this study corroborate those found by Souza et al. (2009) in which *S. agalactiae* was responsible for the largest increase in dairy cow SCC.

Comparison between the SCC values obtained within the *Staphylococcus* genus for the CPS and CNS groups revealed that the mean value of the first group ($1632x10^3$ cells/mL) was significantly higher than the average observed in cases of CNS ($1060x10^3$ cells/mL). However, the comparison of the observed maximum values, $7139x10^3$ CS/mL for CPS and $7357x10^3$ CS/mL for CNS, demonstrates the ability of the latter to elicit high intensity inflammatory processes.

Antimicrobial resistance

The genus *Staphylococcus* showed high sensitivity to most antimicrobials. Cephalothin, cotrimoxazole, enrofloxacin and gentamicin were the most effective antimicrobials (>95.0%). However, we noted worrying indexes of intermediate sensitivity or complete resistance, compared to some drugs, especially penicillin (41.2%), oxacillin (11.8%) and neomycin (5.8%) (Table 3).

Bacterial resistance is related to the existence of genes capable of encoding different biochemical mechanisms that give the microorganism the ability to resist the action of various drugs. It is the result of selective pressure on pathogens that occurs through the use or not of medicines (Tavares 2000).

Table 2. CPS and CNS strains isolated from milk of cows with mastitis in small dairy farms

Group	Species	N	% in the group	% in the genus	% in the isolates
	Staphylococcus spp.	9	24.3	13.2	6.3
CPS	*S. aureus*	21	56.8	30.9	14.7
	*S. hyicus**	4	10.8	5.9	2.8
	S. intermedius	2	5.4	2.9	1.4
	S. schleiferi	1	2.7	1.5	0.7
	Subtotal	37	100.0	54.4	25.9
	Staphylococcus spp.	8	25.8	11.8	5.6
	S. xylosus	9	29.0	13.2	6.3
CNS	*S. epidermidis*	6	19.4	8.8	4.2
	S. chromogenes	5	16.1	7.4	3.5
	S. warneri	2	6.5	2.9	1.4
	S. simulans	1	3.2	1.5	0.7
	Subtotal	31	100.0	45.6	21.7
	TOTAL	68	-	100.0	47.6

**S. hyicus* isolates obtained in this study were grouped together with other CPS for positive coagulase test results.

In addition to being a serious animal and public health problem, antimicrobial resistance results in direct financial losses to dairy farmers due to drug costs and milk disposal during treatment. They also lose dairy products and end consumers. When the recommended grace periods for antimastitics are not respected, the quality and safety of dairy products are compromised.

In a large study conducted in the state of Minas Gerais, Costa et al. (2013) evaluated the antimicrobial profile of 352 isolates of *Staphylococcus aureus* obtained from 35 different dairy properties. Resistance percentages obtained for cephalothin (0.28%), gentamicin (1.69%), neomycin (3.35%), enrofloxacin (<1.0%) and penicillin (34.1%) were very similar to our results.

Regarding penicillin, the staphylococcal strains isolated in the present study showed a sensitivity percentage of 58.8% (n=40) of isolates sensitive to this antimicrobial, 39,7% (n=27) of the isolates resulted in intermediate antimicrobial sensitivity and 1.5% (n=1) was resistant. In small dairy herds, low technification and probable deficiencies in therapeutic protocols could result in higher levels of microorganism resistance to penicillin, widespread antimicrobial agent in the treatment of mastitis in dairy herds (Vintov et al. 2003, Rabello et al. 2005). Contrary to what would be expected in isolates obtained from this type of herd, only one isolate resistant to the drug in question was observed.

Regarding oxacillin, the total 11.8% of intermediate sensitivity and resistance observed is like those obtained by Siqueira (2011) in bacteria of this genus isolated from samples of organic milk in the Botucatu region, in which 88% were sensitive to the drug.

The indiscriminate use of penicillin reported by the producers of this study may have contributed to the selective pressure of strains resistant to this antimicrobial, as compared the profiles obtained for groups of CPS and CNS. In the first, 48.6% of the isolates were considered sensitive and 51.4% were resistant to the drug, while the microorganisms of the CNS group presented 71.0% sensitivity and 25.8% resistance. The fact that CPS induces a more intense inflammatory response in cases of mastitis tends to use antimastitic treatments more frequently than CNS, and may lead to selective pressure and consequent resistance of microorganisms to the drug.

Enterotoxins

Of the 68 samples subjected to enterotoxin-encoding gene screening 30.9% (n=21) were positive for at least one type of classical enterotoxin encoding gene (*sea*, *seb*, *sec* or *sed*). The frequency of the CPS and CNS species, and the association with the genes are presented in Table 4.

Of the 37 CPS samples submitted to PCR, 21.6% (n=8) were positive for at least one of the classic enterotoxin coding genes and of the 31 CNS samples, the positivity was 41.9% (n=13). De Freitas Guimarães et al. (2013) obtained a significantly higher positivity of the CNS in relation to the CNS in the research of the same genes.

Considering only samples positive for the detection of enterotoxin coding genes, it was verified that the gene *sea* was the most prevalent with 45.8%, followed by *seb* with 29.2% and *sec* with 25% frequency. The *sed* gene was not detected in any of the samples. These results partially agree with those found in the scientific literature. Zoli et al. (2002) pointed out that sed-expressed enterotoxin is the second main type involved in food outbreaks, with bone poisoning being more frequent. Pinchuk et al. (2010) also associated food poisoning with *sed* detection.

The study by Pimentel et al. (2002) in composite milk samples revealed the production of some type of enterotoxin in 24.6% of CPS samples and 41.3% of CNS samples. The authors concluded that coagulase negative species produce enterotoxins more frequently, highlighting the lack of updated Brazilian

Table 3. Average SCC of milk from fourth breasts of subclinical mastitis cows according to bacterial genus

SCCx10^3 (CS/mL)	STA	STR	COR	COLIF.	STA/STR	COR/STA	COR/STR
Average	1362	2857	976	1161	3707	1904	-
Mean	579	1456	501	465	4501	934	608
Maximum	7357	9999	6554	3369	9999	4655	-
Minimum	124	175	42	319	375	539	-
N	55	25	45	4	5	8	1
%	38.5	17.5	31.5	2.8	3.5	5.6	0.7

STA = *Staphylococcus* spp., SRT = *Streptcoccus* spp., COR = *Corynebacterium* spp., COLIF. = coliform groups, STA/STR = genus *Staphylococcus* spp. and *Streptococcus* spp., COR/STA = genus *Corynebacterium* spp. and *Staphylococcus* spp., COR/STR = genus *Corynebacterium* spp. and *Streptococcus* spp.

Table 4. Sensitivity profile of *Staphyloccocus* spp. against different antimicrobials

Antimicrobial	*Staphylococcus* spp.					
	Sensitive		Intermediate		Resistant	
	N	%	N	%	N	%
Cephalothin (30µg)	67	98.5	1	1.5	0	0.0
Cotrimoxazole (25µg)	66	97.1	1	1.5	1	1.5
Enrofloxacin (µg)	65	95.6	1	1.5	2	2.9
Gentamycin (10µg)	66	97.1	2	2.9	0	0.0
Neomycin (30µg)	64	94.1	2	2.9	2	2.9
Oxacilin (10µg)	60	88.2	5	7.4	3	4.4
Penicilin (10UI)	40	58.8	27	39.7	1	1.5

Table 5. Frequency of enterotoxin coding genes in CPS and CNS isolated from milk samples from small dairy farms

CPS	Samples results		Genes			
	Negatives	Positives	*sea*	*seb*	*sec*	*sed*
Staphylococcus spp.	8	1	1	-	-	-
S. aureus	17	4	3	-	2	-
S. hyicus	2	2	1	1	-	-
S. schleiferi	-	1	-	-	1	-
S. intermedius	2	-	-	-	-	-
Subtotal	29	8	5	1	3	0
CNS	Negatives	Positives	*sea*	*seb*	*sec*	*sed*
Staphylococcus spp.	4	4	2	2	1	-
S. chromogenes	2	3	-	2	1	-
S. xylosus	6	3	1	2	-	-
S. epidermidis	4	2	2	-	1	-
S. warneri	1	1	1	-	-	-
S. simulans	1	-	-	-	-	-
Subtotal	18	13	6	6	3	0

legislation (RDC no. 12/2001 of the National Health Surveillance Agency, Anvisa) that advocates for only CPS counting for risk assessment of enterotoxin production (Brasil 2001).

Nevertheless, as reported by Faccioli (2010), a high prevalence of enterotoxin coding genes does not necessarily correspond to high enterotoxin expression. Carmo et al. (2009) evidenced the presence of natural mechanisms capable of inhibiting enterotoxin production in milk contaminated with enterotoxigenic *S. aureus* strains. Factors such as bacterial growth conditions, presence of glucose, pH of the medium and even regulatory systems of the microorganism itself interfere with gene expression (Cunha et al. 2007).

The results in Table 5 showed that in both CPS and CNS strains, there are isolates that have genes encoding two types of staphylococcal enterotoxins. In *S. aureus* strains, the presence of two enterotoxin coding genes, *sea* and *sec*. In the CNS strains, *S. epidermidis* revealed the *sea* gene in association with *sec*. Another isolate from *Staphylococcus* spp. presented the *seb* and *sec* genes, reinforcing that it is possible that certain staph strains express concomitantly more than one enterotoxin-producing gene.

We showed the importance of milk in the circulation of staphylococcal strains with enterotoxigenic potential that may, under favorable conditions, lead to food poisoning. In the context of family farming, this potential risk is even more worrisome because milk and dairy products are ingested by producers and their families, in addition to the clandestine trade in milk.

CONCLUSIONS

The results of the present study revealed that there is great variability in the etiology of mastitis in dairy herds of small rural properties.

The high frequency of isolation of contagious species of the *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp. in the milk of the animals evidences the greater importance of contagious mastitis in the herds evaluated. SCC showed significantly different values according to the pathogen responsible for inflammation of the mammary gland.

In the antimicrobial sensitivity profile, the CPS presented higher resistance when compared to the CNS. The presence of enterotoxin coding genes A, B and C in *Staphylococcus* spp. demonstrated the potential risks to public health due to the possibility of food poisoning through the consumption of raw milk from this type of herd.

The multiple etiology of mastitis observed in the small dairy farms analyzed in the study reinforces the importance of microbiological evaluation before the adoption of milk quality control and monitoring programs. Therefore, we emphasize the importance of the implementation of indirect diagnostic tests, such as CMT or SCC, for the detection of subclinical mastitis for subsequent microbiological examination and success in control programs.

Conflict of interest statement.- The authors have no competing interests.

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