# BACTERIA FROM BOVINE MASTITIS: SURVEY AND LITERATURE REVIEW

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**Abstract**

Subclinical bovine mastitis is the most prevalent and responsible for the loss of 70% in production, reducing milk secretion by 45%. Clinical mastitis shows clinical signs, in addition to reducing production. The objective of this work was to study bovine mastitis in the literature and conduct research on bacteria in cases of mastitis in four dairy farms in the region of Pelotas - RS - Brazil. In addition to bibliographic research, pathogenic aerobic bacteria were isolated and identified in 94 milk samples with clinical mastitis (n = 12) or subclinical (n = 82) with positive CMT (+ + +). The samples collected and previously identified in test tubes were cultured in blood agar and oven 36°C/24 h, and reading the results performed within 24h to 36°C aerobically. Bacterial growth was observed in 62 (65.9%) samples: 12 (19.4%) clinical mastitis and in 50 (80.6%) subclinical mastitis. After identified biochemical characterization: Coagulase negative *Staphylococcus* sp. (n = 29), *Staphylococcus* sp. Coagulase positive (n = 6), *Corynebacterium* spp. (n = 16), *Streptococcus* spp. (n = 7), *Streptococcus uberis* (n = 4). The knowledge and identification of the etiological agent is important for the correct treatment and implementation of mastitis control and prevention methods and for herd monitoring.

**Keywords:** Mastitis, Card Mastitis Test, CMT, Somatic Cells Count, SCC.

## Introduction

Milk is considered a complete food with high nutritional value, composed mainly of water (87.3%), proteins (3.3 to 3.5%), lipids (3.5 to 3.8%), mineral salts (0.7%), vitamins and lactose (4.9%) (SGARBIERI, 2005). Being rich in proteins, vitamins, fats, carbohydrates and minerals is an important source of nutrients for humans and several bioactive compounds that are highly significant for several biochemical and physiological functions (MARTINS; ANDRADE, 2011; ORTEGA-ANAYA; JIMÉNEZ-FLORES, 2018; KHAN et al., 2019). And so, it can be better used by the body, it should be of excellent quality, thus ensuring efficiency (MENDONÇA et al., 1999). Milk and derivatives are excellent means for the development of desirable microorganisms, and other pathogenic and deteriorating microorganisms, as well as the milk microbiota has a direct influence on the health of bovine mammary glands, thus requiring care with their production: milking, processing and storage

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(GUIMARÃES; LANGONI, 2009; PANG et al., 2018). The microorganisms originate from the milk of infected udder, and the surface thereof or a variety of other sources environment (GODKIN; LESLIE, 1993; METZGER et al., 2018).

Mastitis is characterized by an inflammation of the mammary gland related to physical, chemical, thermal, or microbial, and 90% of mastitis are caused by bacteria (RAMALHO et al., 2012). This disorder can be classified as a form of presentation as clinical or subclinical (PHILPOT; NICKERSON, 1991). The clinical presentation is characterized by visible signs, while the subclinical form displays a degree of difficulty to identify because there is no clinical changes in the mammary gland that can be observed macroscopically (MIRA et al., 2013). A diagnosis of clinical mastitis is based on clinical observations with identification of the infectious agent. The subclinical form is usually the most prevalent accounting for approximately 70% of production losses may reduce the secretion of milk by 45% (FONSECA; SANTOS, 2000). In this case it is necessary to use other methods of diagnosis, somatic cell count (SCC) and somatic cell score (California Mastitis Test - CMT) are common diagnostic tests for the detection of subclinical mastitis (HARMON, 1994; MACHADO et al., 2000; DELLA LIBERA et al., 2011; ADKINS; MIDDLETON, 2018). In Brazil it can be said that subclinical mastitis is present in all dairy herds (MACHADO et al., 2000).

The pathogens involved in the etiology of bovine mastitis can be classified into "major" and "minor". The first category includes agents that cause higher SCC, significant changes in milk composition and, consequently, a great economic impact, the main ones being: *Staphylococcus aureus*, *Streptococcus agalactiae*, Coliforms, *Streptococcus* spp., *Enterococcus* spp., *Pseudomonas* spp., *Actinomyces pyogenes* and *Serratia* spp. Negative Coagulase (NC) *Staphylococcus* and *Corynebacterium bovis* pathogens are considered "minor" and promote moderate inflammation with lower SCC (HARMON 1994). Considering the source of infection, the agents of mastitis are classified, infectious (primary mastitis), such as: *Streptococcus agalactiae*, *Staphylococcus aureus*, *Mycoplasma* spp., *Corynebacterium bovis*, NC *Staphylococcus*, or environmental (secondary mastitis) such as *Escherichia coli*, Coliforms, *Streptococcus uberis*, *Enterococcus* spp., *Streptococcus equinus* (formerly *S. bovis*) and other environment *Streptococcus* spp. (COSTA et al. 1998, MENDONÇA et al. 1999; HEIKKILÄ et al., 2018).

The increase in somatic cells is the main feature used for the diagnosis of subclinical mastitis, other factors may however result in this change. There are several tests that assess Somatic Cell Count (SCC) of milk from these tests highlight the CMT, WMT (Wisconsin Mastitis Test) and Electronic Somatic Cell Count (SCC) (RUPP et al., 2000). The cultivation

of milk samples from individual mammary quarters is used to identify an etiologic agent, especially in cases of positive CMT (BRAMLEY et al., 1996; SEARS et al., 1993). In times of automatization of dairy cow husbandry, objective tools for mastitis diagnostic are demand and moderns studies about diagnose show the true positives quarters on CATH ELISA or handheld dynamometer and an infrared thermometer to diagnose and score clinical and subclinical mastitis (MAHMMOD et al., 2013; WOLLOWSKI et al., 2019; PUGGIONI et al., 2020) but he results obtained in the investigation of the causal agents of mastitis are fundamental to the understanding of the specific problems of the herds, to guide rational measures to control mastitis and suggest changes regarding the management adopted, including performing susceptibility testing to indicate best medication for treatment (RADOSTITIS et al., 1994; BRAMLEY et al., 1996). The use of diagnostic tests indirectly leads to a reduction in mastitis cases, providing thus a higher quality milk product (MIRA et al., 2013).

The objective of this work was to study clinical and subclinical bovine mastitis in the literature and conduct research on bacteria frequency in cases of mastitis in four dairy farms in the region of Pelotas - RS - Brazil.

## Materials e Methods

### Dairy Farm

Four Jersey and Dutch dairy farms in Pelotas - RS were studied during the six-month period (January to June) to verify cases of clinical and subclinical mastitis (CMT + ++). The four farms milking was performed twice a day, with a mean interval of 12 h. During visits was evaluate all lactating cows and the four mammary quarters.

The clinical mastitis was diagnosed by testing "Dark Background Test" with five first jets of milk and observation lumps. Subclinical mastitis was tested with California Mastitis Test (CMT). The test CMT is determined according to the degree of gelation or viscosity of the mixture of equal parts milk and reagent (2 mL) in the appropriate tray. The results may be negative or positive scores, the latter being classified in three levels (+, + +, + + +). In this case were selected for the analysis process microbiological those with scores + + +, as well as cases of clinical mastitis with the presence of clinical signs.

### Milk Sampling

After pre-dipping, "Dark Background Test" and CMT, from the mammary quarter positive to clinical or subclinical mastitis, 10 mL of milk was collected in sterile tubes. Before was made disinfection of teat orifice with cotton soaked in alcohol 70°GL, identified for each

mammary quarter and shipped immediately after collection, to the Regional Laboratory Diagnostics - Faculty of Veterinary (UFPel) under refrigeration in insulated containers with ice.

**Microbiology**

In the laboratory, samples were plated on blood agar (8% defibrinated sheep blood), the reading of the plates held at 18 to 24 h in an oven maintained at a temperature of 36°C in aerobic atmosphere. The samples which were subjected to bacterial growth making smear stained by Gram and subsequently to the appropriate biochemical tests such as catalase, coagulase, and assimilation of carbohydrates for their identification.

All samples were biochemically characterized in the Bacteriology Sector of the Regional Diagnostic Laboratory of the Faculty of Veterinary Medicine. The methodology used to follow the Cowan and Steel bacterial identification manual (STEEL et al. 1993). The samples were subjected to the Catalase test to differentiate *Staphylococcus* spp. (positive catalase) from *Streptococcus* spp. and *Enterococcus* spp. (both negative catalase). Catalase positive samples were submitted to MR-VP (Methyl Red, Voges-Proskauer), Coagulase test and biochemical analyses against Ribose, Nitrate, Galactose, Maltose, Mannitol and Trealose, in addition to polymyxin resistance.

CAMP test was performed for catalase negative samples and Esculin, Inulin, Mannitol, Salicin, Sorbitol and Trealose tests. These samples were placed in BHI broth (Brain Heart Infusion), manufactured by Laboratórios Conda S.A. + 6.5% NaCl to differentiate *Streptococcus* spp. (no growth) from *Enterococcus* spp. (there is growth). After carrying out the tests, the samples were placed in the oven at 37 °C and after 24 h the reading was done.

The study was conducted with frequency analysis of cases of clinical and subclinical mastitis and frequency of microorganisms observed. The total number of mammary quarters analysed was calculated the percentage of clinical mastitis, subclinical (CMT + + +), non-infectious and infectious and the data were analysed descriptively.

**Results e Discussion**

In the four dairy farms studied, 94 (quarter mammary) cases of mastitis were observed, 82 (87.2%) cases of subclinical mastitis (CMT + + +) and 12 (12.8%) cases of clinical mastitis. The bacterial isolation was observed in 62/94 (65.9%) samples: 12 (19.4%) originated from clinical mastitis and in 50 (80.6%) from subclinical mastitis. Considering only subclinical mastitis (n = 82) bacterial isolation was 50, representing 60.8% (Table 1).

Table 1 – Distribution of clinical and subclinical mastitis frequency in four dairy farms in the city of Pelotas - RS

| Mastitis | N | % |
|----------|-----|-------|
| Clinical | 82 | 87.2% |
| Subclinical | 12 | 12.8 |
| TOTAL | 94 | 100 |

Zeryehun and Abera (2017) concluded that prevalence of mastitis particularly the subclinical mastitis was major problem of dairy cows when observed prevalence of clinical and subclinical mastitis and quarter level prevalence for clinical and subclinical mastitis were 12.5% and 51.8% at cow level. Clinically, 101 (6.6%) quarters which belong to 75 (19.5%) animals were found to be with blind teat.

Philpot and Nickerson (1991) indicate that 90% of mastitis are caused by bacteria. In addition to these pathogens, fungi, yeasts, algae, and viruses may also be involved in the etiology of the disease, but the occurrence is low. According to Ribeiro et al. (2003) infectious subclinical mastitis is one that presents positive test result for CMT, or other tests indicative and confirmed by microbial growth. This test was chosen because it is one of the most popular and practical tests for the diagnosis of subclinical mastitis, which analyzes separately the mammary glands of lactating cows (PHILPOT, 1994; DELLA LIBERA et al., 2011; PADUCH et al. 2013; GODDEN et al., 2017).

For some years, the frequency of clinical and subclinical mastitis in dairy cattle has been studied worldwide. It seeks to understand which microorganisms involved in each one to better understanding the origin and prevention of this disease. In relation to subclinical mastitis, like the results observed in this study, other authors did not have 100% of bacterial isolation in CMT positive milk samples. Costa et al. (1986) reported bacterial growth at 88.8% between 2533 CMT positive milk samples, originating from 32 dairy properties, eighteen municipalities of the state of São Paulo- Brazil.

Results higher than those cited in this paper in relation to subclinical mastitis. Filippsen et al. (1999) showed that of 1.319 mammary quarters with CMT positive, 670 (50.0%) presented in isolation microbiological, and Brito et al. (1999) analyzed samples 6315, found that in 57.6% of bacteria were isolated. Beloti et al. (1997) working with 295 milk samples from subclinical mastitis, bacterial growth obtained in 223 (75.6%) samples. These results are lower than those obtained in the present study. At the same time Costa et al. (1995)

reported 46.5% positive mammary quarters the CMT showed bacterial isolation.

The different studies show frequency of mastitis in dairy farm, isolation frequency in subclinical and clinical mastitis and which microorganisms involved (KAKI et al., 2019; AMBROGGIO et al., 2018). Etiological agents may vary from place to place, depending on the weather; animal and livestock species and include a wide variety of gram-positive and gram-negative bacteria; fungal (DEB et al. 2013). Sargeant et al. (1998) describe the frequency of occurrence of clinical mastitis in dairy herds in Ontario. In 65 dairy farms involved in a 2-year observational study with 2840 cows and heifers, 19.8% of cows experienced one or more cases of clinical mastitis during lactation. Environmental, contagious, and minor pathogens were all associated with cases of clinical mastitis.

A study conducted by Ribeiro et al. (2001a) observed that the total of 9.079 with a positive reaction to CMT, 4.952 (54.5%) showed microbial growth. Ribeiro et al. (2001b) showed that of 9.150 samples with positive CMT, 4.988 (54.5%) had bacterial isolation. In another study Ribeiro et al. (2001c) found that of 1.554 samples positive to the CMT, 596 (38.4%) presented microbial isolation. Confirming that not all milk samples positive present CMT bacterial growth. The same author notes that the greater the intensity of the reaction to CMT greater the number of samples positive microbiologically. This justifies the high percentage of this work insulations in cases of CMT positive, since it was only evaluated score + + +. To Brito et al. (1999), the number of bacterial isolates increased with the intensity of the reaction to CMT. Regarding 32 (34.1%) samples with CMT + + + found in this study, which showed no bacterial isolation, can be explained by some physiological interferences. There are several factors that can influence the variation of SCC being cited: the order of birth, lactation, month and season (SCHEPERS et al., 1997; LAEVENS et al., 1997; SOUZA et al., 2005; CUNHA et al., 2008). For this reason, the CMT test should be performed as presumptive test for mastitis, just as it is necessary to repeat the microbiological examination in these cases. The inflammation may also be present without an infectious process in the gland, due to traumatic injuries, assaults by chemical agents, among other factors. The microbial agent may further have already been eliminated by the natural way of self-healing processes, resulting in a negative culture (RIBEIRO et al., 2003; BRITO et al., 1999; SKRZYPEK et al., 2004; GOMES et al., 2011).

Costa et al. (2001), in their study, 8.116 samples were collected from the mammary glands negative CMT, and 45.1% were isolated microorganisms contagious. Moreover, the test samples positive CMT not always show microbial growth. According to the author, it is suspected that the agent is not removed continuously false negative result may occur. According to Sears et al. (1993) and Brito et al. (1999), samples of milk should be confirmed

microbiologically negative only after two or three analyzes.

Freitas et al. (2005) analyzed 984 quarters breast. Of these, 10 (1.0%) had clinical mastitis and 562 (57.1%) subclinical mastitis. Correlating the CMT test with microbiological examination, it was found that 562 of subclinical mastitis, only 356 (63.3%) were positive for microbiological examination. The positive results in CMT, but negative in microbiological examination may indicate an inflammatory etiology of nonbacterial, because according to Costa et al. (1996), the CMT is a helper method in good correlation with the microbiological examination, however, the inflammatory process may not be of infectious origin.

The results reported for the identification of subclinical mastitis by CMT suggest greater caution in the use of this test as an indicator of intramammary infection by the finding of large numbers of milk samples without microbial growth, since it is probably a non-infectious subclinical mastitis. And we still need to work proactively in mastitis control, it is a disease management and to make a proper prevention, it is necessary to consider the entire property management, mainly including hygienic measures (CARVALHO et al., 2002, SILVA et al., 1998). These results are worrying, since according to Prado et al. (1997) the subclinical form is responsible for the largest losses in the production and composition of milk.

The quality of raw milk is influenced by multiple factors, among which are zootechnical factors associated with management, health of the mammary gland, feeding, genetic potential of the herds and factors related to the obtaining and storage of the milk-milk milk (GUERREIRO et al., 2005). One of the causes that exert extremely harmful influence on the composition and characteristics of milk is a mastitis (Müller, Ernst 2002).

In cases of clinical mastitis in 100% of cases was bacterial isolation, indicating that there is always the microbial involvement when the clinical signs are apparent. After the biochemical characterization identified: Negative Coagulase *Staphylococcus* spp. (n=29), Positive Coagulase *Staphylococcus* spp. (n=6), *Corynebacterium* spp. (n=16), *Streptococcus* spp. (n=7), *Streptococcus uberis* (n=4) (Table 1). The identification of the etiologic agent is important for the implementation of methods of prevention and control and monitoring of the herd. The importance of diagnosing subclinical mastitis and apparently healthy, the use of CMT, is to discover which animals can be transmitters of infection by line or by hand milking the milker (BRAMLEY et al., 1996; HARMON 1994). These animals besides being sources of contamination, contribute by low milk quality and lower total volume produced, which reflects in economic losses to property (MOEINI et al., 2002). However, despite the bacteriological diagnosis to be crucial, it is expensive and time consuming, not being applicable to herds with large numbers of animals. Several simplifications have been studied

in order to eliminate these difficulties, the use of special culture media and creating schemes presumptive identification (RIBEIRO et al., 2003; FIGUEIREDO, 1995).

Table 2 – Microrganisms isolated from milk of cows with mastitis in Pelotas - Rio Grande do Sul

| Microrganisms | N | % |
|---|---|---|
| Coagulase negative *Staphylococcus* spp. | 29 | 46,8 |
| Coagulase positive *Staphylococcus* spp. | 6 | 9,7 |
| *Corynebacterium* spp. | 16 | 25,8 |
| *Streptococcus* spp. | 7 | 11,3 |
| *Streptococcus uberis* | 4 | 6,4 |
| TOTAL | 62 | 100 |

The species isolated in this study belonged to the classification of major pathogens (53.2%) and minor (46.8%), environmental (11.3%) and infectious (88.7%) and primary (27.4%) and secondary (72.6%). The most common microorganisms in cases of bovine mastitis are *Staphylococcus aureus*, *Staphylococcus* spp., *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Corynebacterium* spp., *Escherichia coli*, *Nocardia* spp. *Prototheca* and *zopffi* (BRABES et al., 1999; BIRHANU et al., 2017; SULEIMAN et al., 2018; ROCHA et al., 2019; BIANCHI et al., 2019; NAUSHAD et al., 2019).

Among the contagious pathogens, *Staphylococcus aureus* is the most frequent and prevalent in cases of bovine mastitis. It causes clinical and subclinical intramammary infection with serious economic loss and herd management problems in dairy cows (ZSCHÖCK et al., 2000; Kerro Dego et al., 2002). According Gentilini et al. (2002) currently CN *Staphylococcus* have received greater importance as causative agents of intramammary infections in dairy cattle worldwide (KROL et al., 2016; RASPANTI et al., 2016; HOEKSTRA et al., 2018; MAHMMOD et al., 2018; RONCO et al., 2018; MACFADYEN et al., 2019). The *S. aureus* is known worldwide as a causative agent of various purulent diseases in both humans and animals (BEAN; GRIFFIN, 1990) is also one of the most important causes of food poisoning (PENNA et al., 1998) due to production of various enterotoxins (OMOE et al., 2002). Thus, the milk and dairy products are important vehicles of *S. aureus* and are commonly involved in outbreaks of food poisoning (BERGDOLL, 1989).

Although most articles reviewing belong to Brazilian studies in other countries, such as Sweden, the isolated species are the same: The most common isolates of 590 bacteriological diagnoses were *Staphylococcus aureus* (19%) and NC *Staphylococcus* sp.

(NCS 16%) followed by *S. dysgalactiae* (9%), *S. uberis* (8%), *Escherichia coli* (2.9%), and *Streptococcus* spp. (1.9%) (PERSSON et al., 2012). The distribution of the most commonly isolated bacteria considering only bacteriological positive samples were: *S. aureus* (31%), NC *Staphylococcus* spp. (27%), *S. dysgalactiae* (15%), *S. uberis* (14%), *E. coli* (4.8%), and *Streptococcus* spp. (3.1%).

The correct identification of *S. aureus* from bovine mastitis is important both from the epidemiological point of view as the prevention of infections, including immunoprophylaxis (BRITO et al., 2002; SOUZA et al., 2019). In Pernambuco, Pinheiro de Sá et al. (2000) found that the most common microorganisms in cases of bovine mastitis were *Staphylococcus* spp. (32.6%) and *Corynebacterium* spp. (13.2%), but among *Staphylococcus* isolated, the species *S. aureus* was the most prevalent (66.4%).

In 2001, a nationwide survey was conducted in Finland to estimate prevalence of bovine mastitis, distribution of mastitis pathogens. The results were compared with those of a previous survey done in 1995. The prevalence of mastitis continued to decrease from 38% in 1995 to 31% in 2001. This mainly resulted from increased prevalence of *Corynebacterium bovis*. NC *Staphylococcus* spp. remained the most common bacterial group, comprising almost one-half of the pathogens isolated, whereas the relative number of *Staphylococcus aureus* isolations decreased from the time of the previous study. Compared with the other Nordic countries, penicillin resistance among the *Staphylococcus* spp. was still at a relatively high level in Finland (52.1 and 32.0% for *Staphylococcus aureus* and CN *Staphylococcus*, respectively) (PIKALA et al., 2001).

Mastitis causing CN *Staphylococcus* and *Staphylococcus aureus* are compared to Taponen; Pyörälä (2008). *Staphylococcus* spp. are the bacteria most commonly isolated from bovine mastitis, and CNS are now predominant over *S. aureus* in most countries. CNS include various species, but only a few prevail in bovine mastitis. *S. aureus* can cause clinical mastitis, but often causes subclinical mastitis, which remains persistent and increases milk somatic cell count. CNS, traditionally regarded as minor pathogens, seem to lack the ability to cause severe mastitis. CNS can, however, persist in the mammary gland and moderately increase milk somatic cell count.

Freitas et al. (2005) noted that the most prevalent agents in bovine mastitis in Pernambuco (Brazil) were 36% NC *Staphylococcus* spp., *Corynebacterium* spp. 34.8% and *S. aureus* 13.6%.

Besides the problems with the infected animals, the study of microorganisms in milk also reveals knowledge about its index of microbial contamination that can be used in the prosecution of its intrinsic quality and sanitary conditions of its production and herd health.

Considering the potential to multiply the bacteria in the milk can cause chemical changes such as degradation of fats, proteins or carbohydrates, which may render the product unfit for consumption and industrialization (COUSIN, 1982).

It has questioned the interpretation of the CMT as diagnosing subclinical mastitis, being a subjective test with false-positive or false-negative (MARTIN et al., 1994; CASURA et al., 1995). However, even with some positive results in CMT, but that does not indicate an infectious process, the articles reviewed indicate that this test should be routinely performed for measures in the handling of animals and milking line are adopted to ensure maximum production and quality of milk (BARBOSA et al., 2002; OLIVEIRA et al., 2011).

**Conclusion**

Based on the research about clinical and subclinical mastitis, microorganisms more frequent and prevalent in mastitis cases, and similarity between positive CMT and isolations of bacterial, these results were able to visualize, in the region of Pelotas - RS, a frequency of clinical and subclinical mastitis in four dairy properties in the period of six months similar results also observed in the country and in the world. It was also possible to know the microorganisms involved in these two cases and confirm what has been happening in research from other locations. Besides, the results reinforce the importance of prevention and control of this disease.

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