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Identification of etiological agents of bovine clinical mastitis and the source of infection on a farm in Córrego Fundo, Minas Gerais

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ABSTRACT
Bovine mastitis is a multifactorial inflammatory disease of the mammary gland that has a significant economic impact on the dairy industry, resulting in high treatment costs, loss of production and milk quality, as well as adverse effetcs on health and well-being of cows. This study aimed to identify the causative agents of clinical mastitis on a farm in Córrego Fundo-MG, Brazil, and determine the source of infection. Microbiological analyses were conducted on milk samples from cows with clinical mastitis, as well as from various locations within the farm environment using the SmartColor® culture medium (OnFarm) to identify the microorganisms present in each sample. The results demonstrated that Streptococcus dysgalactiae and Escherichia coli were the primary causative agents of clinical mastitis and were the most frequent in the analyzed environment. The presence of these pathogens unequivocally indicates that the mastitis issue on this farm is probably environmental. This can be attributed to the behavior of the agents involved, the way mastitis manifests itself, and the identification of the pathogens in farm environment.

Keywords: Escherichia coli, Mammary gland, Milk, Environmental mastites, Streptococcus dysgalactiae.

1 INTRODUCTION

Mastitis is an inflammation of the mammary gland commonly caused by a multifactorial infection, in which bacteria are responsible for the majority of cases. The complexity of the disease is related with inflammation and intensity of the pathology, which are related to external environment, pathogenicity of the infectious agents, and animal's condition (Bressan, 2000).

According to Halasa et al. (2007), mastitis has a significant economic impact on dairy herds, leading to increased production costs due to expenses with medications, veterinary medical services, milk and animal disposal, high labor requirements, and decreased milk production. Additionally, cow's immunity decreases, making it susceptible to various diseases. Mastitis, if left untreated, the condition can be fatal.

There are two forms of mastitis manifestation, clinical and subclinical (Adkins and Middleton, 2018). In the clinical presentation, alterations are classified into degrees. In mild cases, changes in the milk such as the presence of clots, blood, coagulation, and color changes are noticeable. In moderate cases, classical signs of udder infection are present: pain, swell, redness, and local temperature

elevation, along with milk modifications. Systemic signs can also appear, with the cow showing severe symptoms such as fever, lethargy, dehydration, loss of appetite, and reduced milk production (Bradley, 2002).

The dark-bottomed cup test, also known as the strip cup test, is an extremely important visual diagnostic method for identifying milk alterations in first milk streams. This test allows the observation of clots, blood, coagulation, color abnormalities, as well as texture abnormalities, such as watery milk. On some farms, due to the high milking throughput, producers have adapted this test by adding black rubber flooring to the milking area, along with good lighting, to facilitate diagnosis (Santos and Fonseca, 2019).

On the other hand, subclinical mastitis lacks clinical alterations (Santos and Fonseca, 2019). In this form of manifestation, there is a sharp reduction in productivity and milk quality, as the somatic cell count (SCC) exceeds the healthy value for the mammary gland: SCC > 200,000 cells/ml (Gonçalves et al., 2018). Diagnosis can be performed through electronic somatic cell counting and the California Mastitis Test (CMT), which assesses SCC by evaluating milk viscosity when reacting with a reagent solution (Schalm and Noorlander, 1956).

This type of manifestation is the most concerning, as it acts silently, resulting in 70% loss of the entire herd production, whereas clinical mastitis accounts for only 30% (Santos, 2001). According to de Sá et al. (2018), *Streptococcus agalactiae*, *Staphylococcus aureus*, *Mycoplasma* spp., and *Corynebacterium bovis* are the most relevant pathogens of subclinical mastitis, classified as contagious microorganisms.

There are two main reservoirs for these microorganisms, related to the transmission method of each agent. Contagious pathogens primarily reside in the udders of cows with or without mastitis, leading to contamination, mainly during milking (Costa, 1998). Also, the hands of milkers, multipurpose cloths used for teat drying, and incorrect milking hygiene are other significant sources of infection (Santos and Fonseca, 2000).

Environmental mastitis are often associated with clinical occurrences. The main agents involved are coliforms (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp.) and environmental streptococci (*Streptococcus uberis*, *S. dysgalactiae*). The transmission focus is the animal's living environment, particularly where moisture and organic matter (like mud and manure) are present (Santos and Fonseca, 2019).

Considering all the aforementioned points, the aim of this study was to identify, through microbiological analyses, the microorganisms causing clinical mastitis on a rural property in Córrego Fundo-MG and determine the possible infection sources of the identified bovine mastitis cases.

2 MATERIALS AND METHODS

The study was conducted from March to April 2023 on a dairy farm with 254 lactating cows, located in the municipality of Córrego Fundo-MG. The farm operates under the Compost Barn system, an intensive system where cows are housed in a ventilated barn with controlled feeding. When properly managed, this system can provide great comfort for the animals and even reduce their exposure to pathogens, as well as improve cow cleanliness, which subsequently can lower mastitis rates (Janni et al., 2007).

Mastitis control on the farm is carried out by farm staff during milking. This is achieved by identifying clots in the milk using the dark-bottomed cup test and by assessing changes in viscosity using the California Mastitis Test (CMT). Subsequently, cows showing signs of mastitis are segregated, and milk samples are collected for microbiological culture. The culture is conducted using the SmartColor® streak plate method (OnFarm, Piracicaba, Brazil), involving three differential chromogenic media (tri-plate) to identify mastitis-causing pathogens. Identification is based on the characteristics, type, and color of the formed colonies. This approach is well-suited and advantageous for on-farm microbiological culture due to its cost-effectiveness, quick results (24 hours of incubation), and user-friendly nature for farm staff (Santos and Fonseca, 2019) (Table 1).

Table 1. Description of mastitis-causing microorganisms presentation after growth on SmartColor® culture medium.

Growth phase	Microorganism	Description
SmartColor 1	*Streptococcus uberis*	Dark metallic blue
	Enterococcus spp.	Purple
	Lactococcus spp.	Light pink
	Streptococcus agalactiae / *Streptococcus dysgalactiae*	Turquoise blue/ Light blue
	Other Gram positive not *Staphylococcus* sp.	Other colors
SmartColor 2	*Escherichia coli*	Purple/Wine
	Klebsiella spp., *Enterobacter* spp.,	Dark blue
	Serratia spp.	Light bluish-green
	Pseudomonas spp.	Yellowish-green
	Yeast and *Prototheca* spp./	Small, white-grayish and dry
SmartColor 3	*Staphylococcus aureus*	Pink
	Staphylococcus não *aureus*	Other colors

Adapted from: Albuquerque, 2021.

Thus, cows that exhibited clinical mastitis were segregated, identified by the presence of visually detectable clots in the dark-bottomed cup test. After segregating the diseased cows, milk samples were collected from each animal in an aseptic manner, discarding the first three milk jets. The samples were appropriately refrigerated (<7ºC) and transported in insulated boxes to Laboratório de Microbiologia do UNIFOR-MG. The entire process was carefully executed to preserve sample integrity during transport and ensure the reliability of the obtained results.

Milk samples were aseptically inoculated with sterile swabs in a laminar flow hood, using the SmartColor® streak plate method. Subsequently, the samples were incubated at 37ºC for 24 hours to ensure sample integrity and purity, providing optimal conditions for the growth of present microorganisms.

Following inoculation, plate readings were conducted to identify the mastitis-causing agent, following the table above (Table 1). Notations were made for each positive animal, detailing the respective microorganisms found and their mode of contagion.

The following day, a new collection was carried out based on the identified causative agent, aiming to confirm the infection focus, using the same inoculation protocol mentioned above. The collection points on the farm were based on Santos and Fonseca (2019), who stated that environmental mastitis originates from the surroundings. Therefore, analyses were conducted in specific lots of the Compost Barn, where productive cows reside. Several milking points were also examined, including teat cups, mats, and collection containers, to identify potential contagious agents, aligning with Costa's assertion (1998). The sampled points are outlined in Table 2.

Table 2. Collection points in the analyzed environment and number of repetitions.

Collection points	Repetitions
Mastitis cow lot	2
High-production cow lot	2
High SCC cow lot 1	2
High SCC cow lot 2	2
Dirty teat cup	2
Clean teat cup	2
Milking parlor mat	2
Collector	2

Plate readings also followed the interpretation from the presented table (Table 1).

3 RESULTS AND DISCUSSION

Throughout the experiment, the occurrence of clinical mastitis was observed in 15 cows (5.9%) over a two-month period. This observation was confirmed by the presence of clots in the strip cup test. The monthly incidence of mastitis was approximately 2.95%, which falls within the acceptable rate.

According to Santos and Fonseca (2019), the acceptable rate for this herd of 254 cows is 3.54% per month, as they described that for every 100 cows, an acceptable rate is between 3-4%.

All milk samples with mastitis were streak-plated, revealing the growth of *Streptococcus agalactiae / Streptococcus dysgalactiae* and *Escherichia coli*. The identified microorganisms are causative agents of environmental mastites. These etiological agents are predominantly found in the environment in which the cows are exposed. The results obtained in this study align with the findings reported by Bradley (2002), who highlighted the presence of these agents as the main causes of clinical mastitis on dairy farms.

Continuing with the experiment, two sets of environmental analyses were conducted to assess microorganism growth at strategic points on the farm. As shown in Table 3, 43 microorganisms were found: *Staphylococcus* non-aureus (27.9%) - 12/43, *Streptococcus agalactiae / Streptococcus dysgalactiae* (23.25%) - 10/43, *Escherichia coli* (18.6%) - 8/43, *Streptococcus uberis* (16.28%) - 7/43, *Staphylococcus aureus* (4.65%) - 2/43, *Pseudomonas* spp. (4.65%) - 2/43, *Klebsiella* spp. (2.32%) - 1/43, and Other Gram-negative (2.32%) - 1/43.

Table 3. Etiological agents identified at different collection sites in the two sets of analyses in the study using the SmartColor® culture medium.

Collection site	Etiological agents found in the 1st analysis	Etiological agents found in the 2nd analysis
Mastitis cow lot	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Escherichia coli* *Staphylococcus* non-aureus	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Staphylococcus* non-aureus
High-production cow lot	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Staphylococcus* non-aureus Outros Gram negativo	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Staphylococcus* non-aureus
High SCC cow lot 1	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Streptococcus uberis* *Escherichia coli* *Staphylococcus* non-aureus	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Streptococcus uberis* *Escherichia coli*
High SCC cow lot 2	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Streptococcus uberis* *Escherichia coli* *Staphylococcus* non-aureus	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Streptococcus uberis* *Escherichia coli*
Dirty teat cup	*Staphylococcus* não *aureus* *Escherichia coli*	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Staphylococcus aureus* *Staphylococcus* non-aureus
Clean teat cup	*Streptococcus uberis*	No growth
Milking parlor mat	*Streptococcus uberis* *Escherichia coli* *Staphylococcus* não *aureus* *Pseudomonas* spp.	*Streptococcus uberis* *Escherichia coli* *Staphylococcus aureus* *Staphylococcus* non-aureus
Collector	*Klebsiella spp.* *Pseudomonas* spp. *Staphylococcus* non-aureus	*Streptococcus agalactiae/ Streptococcus dysgalactiae* *Staphylococcus* non-aureus

Staphylococcus non-aureus is present in approximately 28% (12/43) of the microorganisms in the analyzed environment. According to Santos (2011), this group is widely spread among dairy herds, although it is not associated with clinical mastitis cases on the property under analysis, as it is a contagious agent related to subclinical mastitis.

A study conducted in Egypt by El-Diasty et al. (2019) revealed a high resistance rate of this pathogen to various types of antimicrobials, including some beta-lactams and cephalosporins, commonly used for mastitis treatment. Therefore, adopting rigorous measures to prevent mastitis caused by coagulase-negative *Staphylococcus* species is crucial, as these contagious agents possess a high prevalence and persistence capacity (Supré et al., 2011).

Streptococcus agalactiae and *Streptococcus dysgalactiae* account for a significant portion of 23.25% (10/43), confirming the origin of one of these issues in question. *Streptococcus agalactiae* is a highly contagious pathogen (Fonseca et al., 2023). Although the agent is not solely confirmed by OnFarm culture, Radostitis et al. (2007) asserts that most mastitis cases caused by this etiological agent are subclinical, with sporadic cases of clinical mastitis.

Leelahapongsathon et al. (2016) conducted a study on a herd in Thailand and reported a low rate of spontaneous cure for cows with *S. agalactiae*-infected mastitis. Additionally, basic milking routines are effective in eliminating the microorganism, such as post-dipping and equipment cleaning. This corroborates the findings of this study, where the same contagious agent was not found in clean teat cups (Table 3), attesting to the fact that the herd is most likely infected by environmental agents.

Therefore, positive cows on the property are likely contaminated with *Streptococcus dysgalactiae*, as this microorganism is environmental and was present in 100% of the analyzed barn lots (4/4) (Table 3). Capellari et al. (2022) isolated 17.81% of this etiological agent in milk samples and emphasized the importance of proper management of the cows' living environment, as this microorganism originates from the cows' surroundings.

The high incidence of this environmental microorganism is probably related to the Compost Barn, often associated with the high humidity of the bedding where cows are housed. This aligns with Fonseca et al. (2023), who related the high incidence of environmental streptococci with elevated humidity and temperature in confinement. These are crucial parameters that must be daily controlled and linked to udder hygiene, subsequently reducing the number of mastitis cases caused by these microorganisms.

Escherichia coli was the second most frequent environmental microorganism on the property, accounting for 18.6% of the results (8/43). Wenz et al. (2006) state that this microorganism is also opportunistic, responsible for clinical disease manifestation. Clinical severity is established not only by the agent itself but also by its direct relation with the cow health status.

Santos and Fonseca (2019) described the transmission of this agent through contact of the teats with organic matter, as this pathogen naturally multiplies in environments that are rich in manure. This aligns with Wenz et al. (2006) observations that the manifestation is predominantly clinical, usually of short durantion, with symptom severity depending on the cow's immunity.

In addition to the above two studies, according to Wilson et al. (1999), mastitis caused by this agent has a high rate of spontaneous cure (>85%), especially when the animal's immunity is adequate. However, treatment is recommended in acute cases of the disease when sepsis and toxemia are present, posing an imminent risk to the animal health (Langoni, 2017).

The microorganism *Streptococcus uberis* was responsible for 16.28% (7/43) of the microorganisms found in the study. Wente et al. (2019), in their research, found these microorganisms in 50% of the analyzed environments (4/8), highlighting the environmental nature of the agent. Furthermore, contagious *S. uberis* was also found, as in this study, indicating the presence of the agent in clean teat cups after complete milking disinfection. Thus, environmental and contagious control approaches are necessary for this agent.

The contagious agent *Staphylococcus aureus* was detected in 4.65% (2/43) of the results. There is significant concern about this microorganism, as it is one of the most frequent pathogens involved in subclinical mastitis, besides having a high potential for biofilm formation and being resistant to various antimicrobials (Souza et al., 2020; Damasceno et al., 2020). However, the milking equipment cleaning protocol on this property proved effective, as this bacteria did not appear after sanitization.

Therefore, the adversity of mastitis on this farm study aligns with the work of Rohling and Rangrab (2021), substantiating the relationship between the use of intensive production systems and the higher prevalence of environmental agents associated with mastitis. Fávero et al. (2015) found that moisture and bedding density are closely linked to the occurrence of environmental mastitis, in with coliforms and environmental streptococci are usually the main involved pathogens. Additionally, Santos and Fonseca (2019) identified coliforms and environmental streptococci as the primary causes of mastitis associated with intensive production systems using organic bedding, such as the Compost Barn.

4 CONCLUSION

The etiological agents responsible for clinical mastitis identified in this study were *Streptococcus dysgalactiae* and *Escherichia coli*. These pathogens were found throughout the analyzed environment, conclusively indicating that the mastitis issue on this farm during the sampled period is of environmental nature. This is attributed to the behavior of the involved agents, the way mastitis manifests, and the identification of pathogens in the environment.

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