



Etiology of Subclinical Bovine Mastitis In Biblián- Ecuador

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Abstract | Mastitis is an inflammatory reaction of infectious, traumatic or toxic origin of the mammary gland tissue. It is a common pathology in dairy cows and of economic importance in the dairy industry worldwide. The objective of the study was to identify the microorganisms that cause subclinical bovine mastitis and their frequency in the Biblián-Ecuador canton. One thousand four hundred and forty mammary quarters were analyzed, coming from 360 Holstein cows in milk production. The California Mastitis Test (CMT) was used to qualitatively verify the increase in somatic cell count (SCC) in milk to indicate the presence of microorganisms causing bovine mastitis. Of the 1440 quarters studied, 175 were found positive by CMT, these were plated in duplicate and incubated at 37°C for 18 hours. The microorganisms were isolated and identified by MAL-DI-TOF. The analyses determined the presence of *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Lactobacillus mali*, *Moraxella osloensis*, *Kocuria salsiccia*, *Bacillus weihenstephanensis*, *Corynebacterium xerosis*, *Macrococcus canis*, *Chryseobacterium bovis*, *Rothia endophytica*, among others. However, *Staphylococcus chromogenes*, *Lactobacillus mali* and *Staphylococcus aureus* were the most frequent bacteria in cases of subclinical bovine mastitis, reaching 23.4, 10.9 and 9.5% presence, respectively; Likewise, it was found that *S. chromogenes* and *L. mali* are more frequent in mechanical milking systems; *S. aureus* and *S. uberis* are present in equal frequency in mechanical and manual milking; *Acinetobacter iwoffi* and *Kocuria salsiccia* were only recorded in mechanical milking of dairy herds in Biblián -Ecuador. The study results suggested to improve management and hygiene in dairy farms of study area.

Keywords | Bovine, Mastitis, Etiology, Biblián - Ecuador.

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INTRODUCTION

Mastitis is the most common disease affecting dairy cattle (Ruíz et al., 2016; Ji et al., 2022). It presents inflammation of the mammary gland, changes in the glandular tissue and chemical composition of milk (López, 2014). Therefore, it is highly prevalent and causes severe economic losses challenging to estimate (Chaves et al., 2017; Neculai et al., 2021; Maldonado et al., 2022; Aken

et al., 2022; Demil et al., 2022) ranging from 170.00 to 400.00 \$/cow/year (Araúz, 2011). For example, China reports losses range from 12,000 to 76,000 USD/farm/month (He et al., 2019), Egypt annually losses of 21,933,258.6 LE are reported (Azooz et al., 2020) due to decreased production and loss of quality (Year and Ramírez, 2016), therapeutic costs, discarding of young animals with high genetics (Bedolla, 2008; Bonifaz and Conlago, 2016; Mera et al., 2017) and negative effect on fertility (Pedroso and

Roller, 2017), with gestation losses of 89% between day 30 – 60 of pregnancy (Miranda et al., 2019).

Mastitis is the costliest of the infectious diseases of dairy cattle (Fernandez et al., 2012; Cuéllar, 2020; Puerto et al., 2021) affecting 16% of gross income and up to 20 -25% loss of total production (Vissio et al., 2015). The subclinical form constitutes 70 to 75% of total economic losses, and affects between 15 and 40% of the mammary quarters (Araúz, 2011; Bobbo et al., 2017), costing \$ 108-252/cow/year (Aken et al., 2022). It is caused by multiple animal-dependent and independent factors (Mora et al., 2015); infectious agents are the most important aetiological agents (Martínez et al., 2015; Dalanezi et al., 2020), considering trauma, injuries, allergies, toxic substances and neoplasms as secondary causes (Bedolla, 2008; Peláez, 2015). However, a mineral deficit reduces the activity of immune cells, causing immunosuppression, which is a predisposing factor for udder inflammation (Sugrue et al., 2019).

The recognition of bovine mastitis's aetiology is essential, considering that pathogenic microorganisms have overcome a series of hierarchical barriers that result in zoonotic transmission from cattle to humans through the consumption of raw milk and meat (De Jesus et al., 2020; Maity and Ambatipudi, 2020). On the other hand, several publications from different geographical environments describe the resistance of pathogens to antibiotics in recent decades due to the indiscriminate and irrational use of antibiotics to treat the disease (Gao et al., 2012; Klibi et al., 2018; Castro, et al., 2020; Cheng and Han, 2020; O'Dea et al., 2020; Goulart and Mellata, 2022; Naranjo and Slowey, 2022; De Oliveira et al., 2022) perhaps due to the lack of knowledge at the producer level for rapid and efficient tests for the recognition and sensitivity of pathogens to antimicrobials.

In Ecuador, there is scarce information on the incidence, prevalence and recognition of etiological agents (Reyad, 2015); however, it is clear that the pathology constitutes one of the causes of low productive yields in the dairy industry, registering 7.11 litres/cow (INEN, 2017), with a contribution of 10. 40% of the Gross Domestic Product (GDP) of the national total. These figures show that milk production in the country is critical for food security and sovereignty since milk is considered a complete food of national priority (Ruíz et al., 2016), with 75% of production going to the sale of fresh and industrial milk (González and Vidal, 2021). This represents a consumption of 110 litres/inhabitant/year, an amount that is still below the FAO and WHO recommendations of 180 litres per capita per year (CIL, 2021), 12.86% for the production of cheese, butter, yoghurt and other dairy products, 6.86% for family consumption, 3.67% for feeding calves raised in buckets, and 1.70% for other purposes (Campaña and Aguilar, 2021). It

is therefore, current study was designed to identify the frequency and microorganisms that cause subclinical bovine mastitis in the Biblián-Ecuador.

MATERIALS AND METHODS

The study was carried out in Ecuador, Cañar province, Biblián canton, located at an average altitude of 2,608 meters above sea level, with temperatures ranging between 2 and 14°C and annual rainfall between 750 and 1000 mm/year (PDOT-BIBLIAN 2014). Livestock farms are semi-intensive, with manual or mechanical milking and two milkings/day, with forage mixtures of perennial ryegrass, annual ryegrass, blue grass, white clover and red clover.

Three hundred and sixty female Holstein Friesian cross-bred cattle were selected between day 30 and 150 of production, with 2 to 3 calvings. The early detection of subclinical mastitis was carried out during the months of June, July and August, the reagent for the diagnosis of bovine subclinical mastitis (CMT) from the commercial company LIFE - Ecuador was used. Following the process described by (Mellenberg and Roth, 2022), the udder was washed and dried, the first jet of milk was discarded and 2 ml of milk from each mammary quarter was collected on the test paddle, mixing with the Mastitest®-CMT reagent in equal parts, and shaking the paddle with circular movements for 2 minutes. Finally, the reaction created between sodium alkylarylsulfonate and the number of somatic cells (SC) present in the milk (gelation) was observed, classifying this reaction according to the method described by Smith in 1990, cited by (Bedolla et al., 2007). Obtained qualitative results for each quarter (Traces, +, ++ and +++) were explained in Table 1, adjusted under the interpretation proposed by (Pastor and Bedolla, 2008).

Table 1: Interpretation of the California Mastitis Test.

Interpretation cells/MI	Reaction	Number of somatic cells
Negative	No evidence	0-200.000
Traces	Mild precipitation	201.000-400.000
+	No gel formation	401.000 - 800.000
++	Thick mixture	801.000 - 1'500.000
+++	Central peak formation	> a 1'500.000

Samples that tested positive for mastitis in the CMT test were recollected in triplicate

Samples that tested positive for mastitis in the CMT test were recollected in triplicate into sterile 10 ml Falcon tubes and identified before being transported in a refrigerator

at 4°C for somatic cells count (SCC) and microbiological testing. The somatic cell count was performed using the Ekomilk Scan CS analyzer (Ekomilk brand, Ultra Pro model, serial: SN S00802306, Bulgaria), using the method recommended by (INEN, 2012).

The samples for microbiology were sown in Petri dishes in duplicate on nutrient agar and subsequently incubated at 37°C for 24 hours, verifying the growth of bacterial colonies. Subsequently, the colonies were isolated by morphological difference and the colonies were purified in three rounds to ensure purity. Colonies were then plated for the last time on blood agar and incubated for 18 hours at 37°C for the identification process using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry technique. This technique has become a reference resource for the identification of microorganisms in clinical microbiology services because it is quick, simple and more reliable than the conventional biochemical identification technique (Barreiro et al., 2010; Vega et al., 2012; Cattani et al., 2015; Siller et al., 2017; Costa et al., 2022).

A descriptive observational analysis of the data was performed using simple absolute frequencies (number of cases) and relative frequencies (percentage), which are presented in frequency tables. The prevalence of microorganisms causing bovine subclinical mastitis was calculated as a proportion using as a numerator the number of positive cases for mastitis and as a denominator the number of isolates of each microorganism, using the formula described by (Rodríguez, 2020).

Prevalence = $\frac{\text{Number of isolates of a pathogen}}{\text{Total number of isolates}}$ X 100

RESULTS

Of a total of 1440 mammary quarters analyzed,, 175 were detected by CMT field test and confirmed by somatic cell counting with subclinical bovine mastitis. Nineteen bacteria were identified responsible for MSB, including pathogenic, skinresident and environmental bacteria. *Staphylococcus chromogenes* is the most prevalent among the environmental microorganisms, with 23.4%. On the other hand, *Staphylococcus aureus* and *Streptococcus agalactiae* were the aetiological agents responsible for contagious bovine subclinical mastitis in the dairy herds of Biblián canton, with a prevalence of 9.5% and 0.5%, respectively, as shown in Table 2.

On the other hand, the study identified *Staphylococcus chromogenes*, *Staphylococcus epidermidis* and *Kocuria salsiccia*, resident bacteria of the skin and mucous membranes of both humans and mammals, which can be considered

Table 2: Etiology of bovine subclinical mastitis in Biblián-Ecuador

Microorganisms	Isolated (Number)	Prevalence %
<i>Staphylococcus aureus</i>	13	9.5
<i>Streptococcus agalactiae</i>	1	0.7
<i>Streptococcus uberis</i>	10	7.3
<i>Staphylococcus chromogenes</i>	32	23.4
<i>Staphylococcus haemolyticus</i>	4	2.9
<i>Staphylococcus xylosus</i>	2	1.5
<i>Staphylococcus sciuri</i>	2	1.5
<i>Staphylococcus epidermidis</i>	9	6.6
<i>Lactobacillus mali</i>	15	10.9
<i>Moraxella osloensis</i>	4	2.9
<i>Kocuria salsiccia</i>	11	8.0
<i>Bacillus weihenstephanensis</i>	2	1.5
<i>Arthrobacter gandavensis</i>	1	0.7
<i>Acinetobacter iwoffi</i>	11	8.0
<i>Rothia terrae</i>	1	0.7
<i>Corynebacterium xerosis</i>	4	2.9
<i>Macroccus canis</i>	1	0.7
<i>Chryseobacterium bovis</i>	12	8.8
<i>Rothia endophytica</i>	2	1.5
	137	100.0

contaminants when found in clinical samples. However, since they were found in a considerable percentage of the samples, they were considered positive. Likewise, *Acinetobacter iwoffi* was found, an environmental type bacterium resistant to commonly used disinfectants and adaptable to environmental conditions.

The study also recorded *Lactobacillus mali* as the etiological agent of bovine mastitis. Its presence in dairy cattle is due to the supplementation of the feed ration with silage inoculated with Lactobacillus strains to increase the amount of lactic acid and lower the pH more quickly, consequently decreasing plant respiration and enzymatic activity, increasing dry matter losses and digestibility.

Analyzing the bacterial frequency causing subclinical bovine mastitis in dairy herds in the Biblián canton, depending on the milking system, it was observed that *S. chromogenes* and *L. mali* are more frequent in mechanical milking systems; *S. aureus* and *S. uberis* are present with equal frequency in mechanical and manual milking; *Acinetobacter iwoffi* and *Kocuria salsiccia* were only recorded in mechanical milking systems.

DISCUSSION

Staphylococcus aureus is the bacterium that has been reported in most of the studies carried out in dairy farms in the highland region of Ecuador as responsible for bovine mastitis. Bermeo (2014) reported that *Staphylococcus aureus* is the most prevalent microorganism in dairy farms suffering from bovine mastitis in the province of Azuay (Caraguay, 2012). Thus Bermeo (2014) indicates that *Staphylococcus aureus* is the most prevalent microorganism in dairy farms suffering from bovine mastitis in the province of Azuay. Caraguay (2012) identified *Staphylococcus* sp. and *Streptococcus* sp. in dairy farms in the province of Loja-Ecuador as pathogens responsible for bovine mastitis. Bonifaz and Conlago (2016) identified *Staphylococcus intermedius*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Staphylococcus epidermis*, *Escherichia coli*, *Micrococcus* and *Corynebacterium* sp. as aetiological agents of bovine mastitis in dairy farms in Cayambe, Quito-Ecuador.

Likewise, Andrade and Sánchez (2018) pointed out *S. aureus*, *Streptococcus* sp. *Bacillus* sp. *E. coli*, *Shigella* sp. *Klebsiella* sp. and *Enterobacter* sp. in cattle farms in the province of Bolívar-Ecuador, as responsible for this pathology. Cuzco (2015) determined *Staphylococcus aureus* and *Staphylococcus intermedius* as aetiological agents of bovine mastitis in dairy farms in the province of Chimborazo-Ecuador.

Rodríguez and Muñoz (2017) refer in their study to *Staphylococcus aureus* as the most prevalent microorganism in cases of bovine mastitis in Trujillo - Peru. In Colombia, many reserachers (Sánchez et al., 2028; Calderón and Rodríguez, 2008; Andrade et al., 2014; Cruz et al., 2007) include *S. aureus* as the most prevalent bacterium in this pathology, with 31.1, 29.09, 6.0, and 42%, prevalence respectively.

There is no doubt that *S. aureus* and *S. agalactiae* are bacteria found involved in cases of contagious bovine mastitis. However, their prevalence differs between regions, countries and herds (Ruiz et al., 2011) depending on factors such as udder hygiene, barn size, milking parlour hygiene (Ramírez, 2015), animal age, milker's hand washing and production volume (Ramírez et al., 2011) and lactation period, since the amount of lactoferrin (an enzyme that

sequesters free ferric ions present in milk) is low at the beginning of the lactation phase and rises as this phase decreases. Thus, it can be stated that the prevalence of *S. aureus* in cases of bovine mastitis during the first months of lactation will be higher, considering that this pathogen has a substantial amount of iron in milk for its growth (Pereyra et al., 2014).

Regarding the presence of *Staphylococcus chromogenes*, *Staphylococcus epidermidis* and *Kocuria salsiccia*, the finding coincides with those reported by Cabral et al. (2016). Tomazi et al. (2014) described *S. chromogenes* as one of the most prevalent microorganisms isolated from mastitis in dairy cows. Perhaps *Kocuria* spp. in bovine mastitis samples have been misidentified in clinical microbiology laboratories as coagulase-negative *Staphylococcus* (CoNS) based on their gram-reactive, catalase-positive and coagulase-negative properties (Kandi et al., 2016) and therefore, it is not reported as a pathogen of bovine mastitis.

Acinetobacter iwoffi was also present in raw milk samples from dairy cows with mastitis. The result coincides with the study of (Gurung et al., 2013), who found the bacterium in raw milk tanks from cows with bovine mastitis.

Mechanical milking systems exhibited a higher incidence of bacteria, such as *S. aureus*, *S. chromogenes*, *L. mali*, *Acinetobacter iwoffi* and *Kocuria salsiccia*. These results differ from those reported by Ávalos et al. (2022), who observed a greater presence of *S. chromogenes*, and *S. aureus* in mechanical goat milking systems. The difference could be attributed to inadequate milking practices, given that, in the dairy herds in the Biblián canton - Ecuador, producers use the same towel to dry the cows' udders. This procedure represents an important risk factor for the transmission of these microorganisms.

S. aureus is found in manual and mechanical milking systems, being more common in the latter. Similar findings were reported by Faria et al. (2005), who observed the presence of *S. aureus* in both milking systems. Furthermore, these results are supported by Ruiz et al. (2011), who reported a higher incidence of *Staphylococcus* spp. and *Streptococcus* spp. in mechanical milking systems.

CONCLUSION

Cases of subclinical bovine mastitis in Biblián canton, province of Cañar-Ecuador, are caused mainly by *Staphylococcus chromogenes*, *Lactobacillus mali* and *Staphylococcus aureus*, reaching 23.4%, 10.9% and 9.5% of presence respectively, without discarding the importance of other bacteria such as *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*,

Staphylococcus xylosus, *Staphylococcus epidermidis*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Lactobacillus mali*, *Moraxella osloensis*, *Kocuria salsiccia*, *Bacillus weihenstephanensis*, *Corynebacterium xerosis*, *Macrococcus canis*, *Chryseobacterium bovis* and *Rothia endophytica*.

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CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

NOVELTY STATEMENT

The study managed to determine the etiology of subclinical bovine mastitis and its frequency in mechanical and manual milking systems in the Biblián canton, province of Cañar, Ecuador.

AUTHORS CONTRIBUTIONS

Experiments were designed by LRG and JCG, and the experiments were performed by MCC. Data analysis was accomplished by MCC and DGB. The manuscript was written by MCC and JCG.

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