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Etiology of Mastitis in Cattle in Zamora-Chinchipe, Ecuador

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ABSTRACT

Background: Cattle mastitis is a global sanitary and economic problem that affects human and animal health. Several microorganisms involved in its etiology, such as *Mycoplasma bovis,* make prevention and control more difficult. **Aim.** To deepen the bacterial etiology of cattle mastitis in Zamora-Chinchipe, Ecuador, where collected milk was observed to have Mollicutes. **Materials and methods:** A number of 247 milking cows were studied in 2015-2016 in herds from Zamora-Chinchipe province, selected in areas with adequate ecological characteristics. The California Mastitis Test (CMT) was run on milk samples from each cow, whereas Polymerase Chain Reaction (PCR) was performed to detect Mollicutes, and a bacteriological diagnostic was made to identify other pathogens. A comparison of proportions analysis was conducted to analyze the results of Mollicute presence, and CMT in the cows per cantons, and between the pathogens identified in the Mollicute-positive cows. Chi-square helped evaluate the relation between the Mollicute diagnostic results and CMT, and the pathogens identified. Logistic regression was useful to compare the prevalence ratio (PR). **Results:** The frequency of subclinical mastitis was found at high levels (81.4%) through CMT, and a frequency of 33.2% of *Mycoplasma* spp.-positive cows. In the bacteriological diagnostic, *Streptococcus* spp. (27.2%) and *Staphylococcus aureus* (22.1%) showed the highest frequencies ($P< 0.05$). *S. aureus* was detected with the highest frequency in the *Mycoplasma* spp.-positive cows (43.8%; $P<0.05$). **Conclusions:** The high infection caused by *Mycoplasma* spp. and *S. aureus* was significant, due to their potential repercussion on the clinical-epidemiological behavior of bovine mastitis in the province.

Keywords: Bovine mastitis, Mollicutes, *Mycoplasma*, PCR, *Staphylococcus* (*Source: Mesh*)

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INTRODUCTION

Cattle mastitis is a complex, multifactorial disease that causes inflammation of the parenchyma of mammary glands; the pathological changes produced in the tissues also lead to biological, physical, and chemical changes in milk, which change its composition, affecting a wide range of by-products, in addition to reducing milk production (Barreiro *et al.* 2017). It is known as the most common and costly dairy-related disease worldwide, and one of the main causes of the deterioration of biological efficiency and dairy cattle fertility (Dalanezi *et al*., 2020; Fernandes *et al*., 2021; Singh *et al.*, 2023).

Mastitis is also a health problem since the milk may transmit zoonotic microorganisms and antimicrobial-resistant pathogens widely and inappropriately used to control the disease (Paramasivam *et al*., 2023).

Among the mastitis-causing pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Actinomyces pyogenes*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, and enterobacteria such as *Escherichia coli*, *Klebsiella spp.* and *Enterobacter spp*. and *Mycoplasma* spp. (Cameron *et al*., 2017).

Concerns about the emerging character of *Mycoplasma bovis* based on reports from its presence in a larger number of countries in recent years (Citti and Blanchard, 2013; Ruegg *et al.*, 2017), as well as the diagnostic of Millicutes in bulk tank cattle milk (25.8%, 37/143) in the province of Zamora-Chinchipe, Ecuador. Though *M. bovis* was not confirmed (Ramírez *et al*., 2017), there is a need to conduct detailed studies on the related bacterial etiology to control the disease in herds.

MATERIALS AND METHODS

A total of 247 milking cows were studied in 2015-2016 in dairy herds in Zamora-Chinchipe province, selected from areas according to their ecological characteristics (Saa *et al*., 2012).

California Mastitis test

Following cleaning, 2 ml of milk was poured into the plate’s wells, then the same volume of reagent (CMT, 4% sodium sulfate lauryl, Nocar Mastitis Diagnostic, Medick). The sample was homogenized in a circular shaker for approximately 10-20 seconds, and the results were immediately read according to the level of jellification observed in the mix (Blowey and Edmonson, 2010).

Microbiological diagnostic

The first jets of milk from each nipple were discarded, then 10mL of milk from all the quarters were collected in sterile tubes, before thorough cleansing and disinfection using 70% ethanol (NMC, 2014).

All the milk samples were stored at 2-8ºC and transported to the Laboratory of Animal Health and Zoonosis at the Technical University of Loja, where the samples were stored at 4 ºC for further microbiological analysis.

Diagnostic of Mycoplasma spp. in milk samples

Mycoplasma spp. was detected through PCR, in MYCOLAB (Accredited Laboratory by ISO/IEC 17025, and Reference for the World Animal Health Organization (WAHO) for the detection of *Mycoplasmas*, at the National Center for Animal and Crop Health (CENSA), Cuba.

The detection of Mollicutes by PCR consisted of two primers MGSO and GPO-1, that amplified a 270 bp fragment corresponding to the conserved 16S ribosomal RNA (16S rRNA) (Van Kuppeveld *et al.*, 1998). The primers were synthesized at the Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba. The *Mycoplasma arginini* DNA was used as a positive control.

DNA extraction from milk samples

The milk sample was centrifuged (3000 rpm for 10 min), and the supernatant was removed. Then, 50uL of the precipitate was mixed with 200 µL of rupturing buffer (Tris HCl, 0.1M, pH 8.5, Tween 20, 0.05%, and K 0,24mg/mL proteinase). The mix was incubated for 1 hour at 60 ºC, and later, at 95 ºC for 15 minutes for DNA denaturing (Rossetti *et al.*, 2010).

Mix preparation, run conditions, and visualization of PCR products

The preparation of the mix for amplification was made in 25 µL final volume. The mix was made of 5 µL of the sample DNA, 1.5 µL from each primer (20 pmol), and 17 µL of the master mix (Promega). The reactions were performed using a DNA (Thermocycler REACTOR ThermoHybaid™), with a specific amplification program for each primer, as described by the previous authors.

The products were applied on 2% agarose gel (v/v). A 100 bp molecular weight marker was used as well (Promega, Madison, USA). The gel was stained with Etidium Bromide (0.5 µg/mL) for 15 minutes, and the results were observed employing an ultraviolet light transluminator.

Bacteriological diagnostic

Bacterial detection from the samples was made by pouring 15µL of milk and exhaustion on a Petri dish previously prepared with MacConkey agar, Mannitol Salt and Blood agar (Becton

Dickinson-Difco BBL™), and chocolate agar. All were incubated between 24 and 48 hours at 37 °C (NMC, 2014).

Additionally, sculine hydrolysis, hippurate, and inulin tests were performed, along with growing in sodium chloride (NaCl). The isolates showing Gram-negative bacilli in the Gram staining were immediately read (oxidase). Other tests were included, such as indole, triple sugar, urea, and citrate (Fernández *et al.*, 2010).

Statistical analysis

A comparison of proportions analysis (Castillo and Miranda, 2014) was performed to analyze the results of Mollicute presence, and CMT in the cows per cantons, and between the pathogens identified in the Mollicute-positive cows. Chi-square was performed to evaluate the relation between the Mollicute diagnostic results and CMT, and the pathogens identified in the bacteriological study. Logistic regression was used to compare the Prevalence Rate (PR) of each pathogen detected by bacteriology (Corp I, 2012).

RESULTS AND DISCUSSION

Overall, 247 samples of cow's milk were CMT-positive in 201 cows (81.4%), which demonstrated the high frequency of subclinical mastitis (SCM) in the provincial dairy herds. Moreover, 33.2% (82/247) of cows were positive for Mollicutes (Table 1).

In Paquisha and Yacuambí, Mollicute-positive cows were not detected, though CMT produced positive cases. Still, in both cantons, a lower number of animals were selected due to farmer refusal.

In Sao Paulo, 16.4% (11/67) Mollicute-positive herds were found, and only one tested positive for *M. bovis* (Manzi *et al.*, 2018), but other mycoplasma species are thought to cause mastitis. Although *M. bovis* is one of the most pathogenic species and the most commonly isolated one during pneumonia, arthritis, and mastitis caused by this genus in cattle, there are other species, such as *M. californicum*, *M. canadense*, *M. bovigenitalium*, *M. alkalescens*, *M. arginini*, *M. bovirhinis*, and *M. dispar*, which might cause infections in dairy cattle (Deeney *et al*., 2021).

M. bovis was not detected in Mollicute-positive bulk tank cattle milk in Zamora-Chinchipe, where other mycoplasma species have been considered (Ramírez *et al.*, 2017).

The lack of association between CMT and Mollicute results through Chi-Square (p<0.05) could be explained by the apparent involvement of other pathogens in the etiology of mastitis.

Table 1. CMT and Mollicute results in dairy cow herds from Zamora-Chinchipe, Ecuador

Cantons	California test				*Mollicutes*		Total
	++	+++	Total pos.	Total neg.	Pos.	Neg.	
C. del Cóndor	1.3 -1.5	0	4 (100.0)[a]	0	1 (25.0)[d]	3 (75.0)	4
Paquisha	6 (75.0)	0	6 (75.0)[ab]	2 (25.0)	0	8 (100.0)	8
Nangaritza	30 (73.2)	3 (7.3)	33 (80.5)[b]	8 (19.5)	21 (51.2)[b]	20 (48.8)	41
Zamora	20 (62.5)	4 (12.5)	24 (75.0)[b]	8 (25.0)	11 (34.4)[c]	21 (65.6)	32
Chinchipe	1 (33.3)	0	1 (33.3)[b]	2 (66.7)	1 (33.3)[d]	2 (66.7)	3
Yacuambi	3 (60.0)	1 (20.0)	4 (80.0)[ab]	1 (20.0)	0	5 (100.0)	5
Yantzatza	104 (67.5)	25 (16.2)	129 (83.8)[b]	25 (16.2)	48 (31.2)[a]	106 (68.8)	154
Total	168 (68.0)	33 (13.4)	201 (81.4)	46 (18.6)	82 (33.2)	165 (66.8)	247

[abcd] Unequal scripts on the columns indicate significant differences (*p*< 0.05).

The bacteriological research detected *Streptococcus* spp. (27.2%), and *Staphylococcus aureus (S. aureus)* (22.6%) with the highest frequencies and no differences between them. A simultaneous bacterial infection by several bacterial agents was also observed, an indicator of the participation of some secondary pathogens, such as coliforms, and *Proteus* spp., which may act as triggers of further colonization by larger pathogens. For instance, coliforms were detected along with *S. aureus* in 10.5% of the cows (Table 2).

Table 2. Bacteriological results found in the milk from milking cow herds in the province of Zamora-Chinchipe, Ecuador

Pathogens	Positive	%
Streptococcus spp.	67	27.1[a]
Staphylococcus aureus	56	22.6[a]
S. aureus and *Coliformes*	26	10.5[b]
Coagulase-negative Staphylococcus	24	9.7[b]
Coagulase positive Staphylococcus	9	3.7[c]
Escherichia coli	7	2.8[c]
Corynebacterium spp.	6	2.4[cd]
Coagulase-negative Staphylococcus and coliforms	4	1.6[cd]
Proteus morgani	3	1.2[cd]
Proteus mirabilis	1	0.4[d]
Negative	44	18
Total	**247**	**100.0**

Pathogens like *Staphylococcus*, *Streptococcus*, and coliforms are described as the most commonly associated with mastitis (Ruegg, 2017).

The findings confirm that *S. aureus* is one of the most frequently occurring pathogens, as well as relevant in the occurrence of mastitis (Janus *et al.*, 2023; Heikkilä *et al.*, 2018; Kirkeby *et al.*, 2019; Sing *et al.*, 2023; Woudstra *et al.*, 2023). This study coincides with the occurrence of *S. aureus* and different species of *Streptococcus*, as the most frequent.

The prevalence rate (PR) of coagulase-negative *Staphylococcus* (CNS) was 9.7%, which differed very little from *S. aureus* (22.6%) (Table 3). The CNS group is also considered an emerging cause of mastitis worldwide, whose main detection has been associated with a decrease of major pathogens, such as *Streptococcus agalactiae* and *S. aureus*, though not all the species in this group have a negative impact on the increasing number of somatic cells and milk quality since they appear to inhabit the nipple skin (Cameron *et al.*, 2017).

Table 3. Comparison between the frequencies observed in different pathogens identified in the milk of cows from dairy in Zamora-Chinchipe.

Pathogen	B	SE	*P value*	PR	CI 95%	
					Minor	Major
Staphylococcus aureus	Reference category					
Coagulase-negative Staphylococcus	.624	.320	.051	1.867	.997	3.495
Coagulase positive Staphylococcus	.245	.223	.271	1.278	.826	1.976
Coliforms	1.344	.458	.003	3.833	1.561	9.414
Streptococcus spp.	-.511	.730	.484	.600	.143	2.511
Escherichia coli	1.022	.275	.000	2.778	1.621	4.761
Corynebacterium spp.	1.792	1.080	.097	6.000	.722	49.837

Note: B= Beta coefficient; SE= Standard error; PR= Prevalence rate; IC95%= Confidence interval of 95%.

In 2017, a study conducted in El Oro province, Ecuador, revealed an 11.6% prevalence of moderate clinic mastitis (CM), and 60% subclinical mastitis (SCM). The CM study found coliforms (33%), coagulase-positive Staphylococcus (25.8%), coagulase-negative Staphylococcus (20.4%) streptococcus (9.7%), *Bacillus* spp. (7.5%) and *Klebsiella* spp (3.2%). In SCM, the study found coagulase-negative Staphylococcus (55.4%), *Bacillus* spp. (22.1%), streptococcus (9.3%), and coagulase-positive staphylococcus (6.1%) (Amer *et al.*, 2018). In contrast, the Zamora-Chinchipe study revealed lower infection of SCM (10%).

Meanwhile, *Streptococcus agalactiae* (29,8%), *Streptococcus pyogenes* (11,7%), and *Corynebacterium* spp. (5.9%) were the most frequent pathogens found in dairy cattle in north Antioquia, Colombia (Ramírez *et al.*, 2018).

However, a comparison of the results from different studies shows that many other factors influencing the prevalence of mastitis should be considered since the particular set found in each herd influences the prevalence of the infection (Dahl *et al.*, 2017). Mastitis is a multifactorial disease linked to the epidemiological triad animal (host), etiological agent, and the environment (Boas da Silva *et al.*, 2023).

The relation between the diagnostic of Mollicutes and other pathogens identified in the process of mastitis (χ^2= 0.042). *S. aureus* pathogens were confirmed to be, by far, the main species co-infecting with Mollicutes, followed by *Streptococcus* spp. The other agents identified did not differ from one another (P<0.05) (Table 4).

Table 4 Bacteriological results and their relation with the diagnostic of Mollicutes in the milk from herds in Zamora-Chinchipe

Pathogen (P)	*Diagnostic of Mollicutes*		Total
	Quantity of positives (%)	Quantity of negatives	
Staphylococcus aureus	36 (43.80)[a]	46	82
Streptococcus spp.	18 (21.95)[b]	50	68
Coagulase-negative Staphylococcus	6 (7.32)[c]	23	29
Coagulase positive Staphylococcus	5 (6.10)[c]	3	8
Escherichia coli	1 (1.22)[c]	6	7
Corynebacterium spp.	1(1.22)[c]	5	6
Proteus morgani	0	3	3
Proteus mirabilis	0	1	1
Negative	15 (18.29)	28	43
Total	**82**	**165**	**247**

[abc] Unequal scripts indicate a significant difference, χ^2= 0.042 *(*P< 0.05).

The high frequency of *S. aureus* in different manifestations of mastitis not only threatens animal health in the province but also public health, with a known association with antimicrobial resistance and its possible zoonotic character (Majumder *et al.*, 2023).

S. aureus pathogenesis is a dynamic process that rests on several factors, such as the genetic composition and the host's immune response, geographical conditions, virulence factors, and the bacterium's genetic variability (Sivakumar *et al.*, 2023).

The association of the diagnostic of *Mycoplasma* spp. with other major pathogens in the local mastitis processes, and mostly, *S. aureus* (χ^2= 0.042, P<0.05) is highly significant. Though this pathogen was identified in 22.6% of the milk samples from the 247 cows studied, its involvement increased to 43.8% when co-infecting with *Mycoplasma* spp.

The potential role of *Staphylococcus* spp. is hard to predict and treat with antibiotic chronic and recurring mastitis (Mphahlele *et al.*, 2020; Janus *et al.*, 2023, Walzl *et al.*, 2023) is a relevant factor when analyzing its co-infection with Mollicutes in the present study.

Mycoplasmas have a broad resistance pattern; they are not sensitive to ß-lactamic and sulfonamide antimicrobials, and they are susceptible to drugs that interfere with proteins (tetracyclines, macrolides, lincosamides, florphenicol), or DNA synthesis (fluoroquinolones) (Maunsell *et al.*, 2011).

In turn, *Staphylococcus aureus* resistance to antimicrobials, combined with its virulence factors, insufficient host removal mechanisms, and escape from the immune response, cause the pathogen to survive long periods in the hosts (Walzl *et al.,* 2023).

S. aureus isolates in the milk from cows with mastitis have demonstrated a high incidence of virulence factors, particularly in the pathogenesis of the disease. Thus, some of these isolates can be observed in nearby herds, mainly caused by poor managing practices, the existence of common pathogens in the same area, nutrition, joint grazing of healthy and sick animals, and animal trading between herds (Sing *et al.,* 2023).

Hence, the co-infection of Millicutes and *S. aureus* may hinder the course of the infectious process in the udder, compromise their satisfactory recovery, and therefore become a non-identified factor leading to unfavorable epidemiology of the disease in herds, as well as failures in their control in many areas, such as Zamora-Chinchipe.

Mycoplasma spp. is known to act as a secondary agent that aggravates pre-existing infections, though it has also been identified as a primary agent that favors later infection by other frequent pathogens causing cattle mastitis (Deeney *et al.,* 2021). In that sense, among the priority areas of basic research on *M. bovis*, there is a need to deepen the importance of co-infection with other pathogens, about the progression of this disease made by this pathogen (Maunsell *et al.,* 2011).

Mastitis control by *M. bovis* usually fails when it relies only on detecting intra-mammary infections, and the prevention of cow-cow transmission when milking. Its recurrence due to the presence of asymptomatic carriers adds to the transmission and spreading of the pathogen through routes other than the mammaries, like aerosols, and blood, depending on other locations of the agent in the animal anatomy (Zadoks *et al.*, 2011). Besides, its survival outside animals may last long periods of time in stools, water, sand, and several bedding materials, becoming a recurring pathogen (Justice *et al.,* 2011). Therefore, the elimination of *M. bovis* cases of mastitis by sacrificing animals (Maunsell *et al.*, 2011).

It is important to point out that in the whole dairy sector, mastitis caused by *S. aureus* produces the highest losses of milk due to the broad dissemination of the pathogen, as well as moderate losses by clinical and subclinical mastitis (Heikkilä *et al*., 2018).

It is also important to identify pathogens linked to mastitis in herds to deal with the controls, and especially, the therapeutic behavior to follow. Today, the efforts to reduce the risks of antimicrobial resistance demand the determination of whether the pathogens observed hinder production or not, and even if they disappear from the mammary glands temporarily, in order to prescribe antibiotic treatments only when necessary (Ruegg, 2018). Hence, the detection of pathogens present in every herd is essential, to evaluate their sensitivity to antibiotics as part of the strategy for the control of mastitis (Hossain *et al.*, 2017).

The predominant co-infection (*Mycoplasma* spp and *S. aureus*) in cases of subclinical mastitis in the herds studied evidences the possible repercussion of mycoplasmas other than *M. bovis* on the appearance of the disease in dairy cattle herds in Zamora-Chinchipe. Accordingly, further research is necessary to elucidate the mollicute species present and their role in co-infection with other pathogens when disease controls fail.

CONCLUSIONS

The high frequency of cows co-infected with Mollicutes and *Staphylococcus aureus* (43,80%) may be the cause of the unfavorable clinical-epidemiological behavior of the disease in dairy cattle herds in Zamora-Chinchipe. This is also a red flag on the role of other species of different mycoplasmas from *M. bovis* in mastitis control due to the known resistance to antimicrobials and its increment in other major pathogens.

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AUTHOR CONTRIBUTION STATEMENT

Research conception and design: NRS, LRS, ELR, MIPA; data analysis and interpretation: NRS, LRS, ELR, APC, MIPA; redaction of the manuscript: NRS, LRS, ELR, MIPA.

CONFLICT OF INTEREST STATEMENT

The authors state there are no conflicts of interest whatsoever.