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Staphylococcus aureus in Bovine Mastitis: Prevalence and Risk Factors in Small holder Dairy Farms located in and around Hawassa, Ethiopia

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Research article

ABSTRACT

Mastitis is a widespread disease in dairy cattle that is known for its economic and public impact globally, including Ethiopia. Among others, *Staphylococcus aureus* is frequently isolated bacterial pathogen from milks of mastitis positive dairy cows. This cross-sectional study, conducted from March to August 2021 in and around Hawassa, Ethiopia, aimed to estimate the prevalence of mastitis, to identify *S. aureus* in bovine mastitis milk and explore the associated risk factors. From 29 smallholder dairy farms, 250 lactating cows were purposively selected and screened using clinical signs and the California Mastitis Test (CMT) to diagnose clinical and subclinical mastitis, respectively. A standard bacteriological study was performed on 127 mastitis-positive milk samples, and the resulting data were analyzed using STATA (version 12), with significance set at $p<0.05$. The study findings indicated a mastitis prevalence of 50.8% at the cow level (4.8% clinical, 46% subclinical) and 27.4% at the quarter level (2.9% clinical, 24.5% subclinical). In the Logistic regression model, cow's age, lactation stage, and farm cleaning frequency were significantly associated ($p < 0.05$) with mastitis. Accordingly, Highest odds of mastitis were recorded in cows ≥ 6 years old (Odds ratio [OR] =17.61, 95% confidence interval [CI] = 5.3, 58.44) and in farms less frequently cleaned (OR= 5.1, 95%CI= 1.88, 13.71). *Staphylococcus aureus* was identified in 47.2% (60/127) of milk samples, and more frequently in subclinical (47.8%) than clinical (41.6%) cases. In conclusion, our study confirmed a high prevalence of mastitis in the study area, particularly subclinical cases associated with *S. aureus*. The detection of *S. aureus* in nearly half of the mastitic milk samples suggests the potential involvement of other pathogens, warranting further research to identify additional causative agents. These findings highlight the need for routine screening of cows for timely treatment control intervention, and community awareness creation activities.

INTRODUCTION

Mastitis, characterized by inflammation of the udder and teats, is a prevalent condition in dairy cattle. It can manifest in two primary forms: clinical and subclinical mastitis (Ruegg *et al*., 2017; Taponen *et al*., 2017). The disease is known for its damage to the udder tissue, which can happen in numerous mammalian species,

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mainly in domestic dairy animals. Being under constant state of physiological stress and most productive nature, mastitis is the most frequent disease of highly producing dairy cattle and can be potentially fatal (Gutierrez-Chavez *et al*., 2019). Bovine mastitis has been reported as the most critical disease on most dairy farms associated with reduction of farm profitability due to decreased milk yield and quality, decreased reduced reproductive performance, discarded milk, high costs of treatment, death of the affected cow, and forced culling of young cows (Radostits *et al*., 2007; Julian, 2016).

Subclinical mastitis (SCM) is an inflammation of the mammary gland characterized by the absence of visible lesions in the udder or its secretions, despite the presence of pathogenic microorganisms and an elevated somatic cell count (SCC) in the milk (Smith, 1996; Radostits *et al*., 2007). While both clinical and subclinical mastitis cause significant economic losses in the dairy industry, clinical mastitis remains a prevalent issue in many dairy herds (Gezehagn *et al*., 2020). Furthermore, mastitis poses a zoonotic risk due to the potential shedding of bacteria and toxins in milk.

This complex disease is caused by diverse pathogens (mainly bacteria) that are commonly categorized epidemiologically as contagious or environmental. Contagious pathogens, primarily reside in the udder of infected cows and spread from cow to cow during milking, tend to cause chronic subclinical infections with intermittent clinical flare-ups at times of stress (Abebe *et al*., 2016). Environmental bacteria, opportunistic organisms present in the cow's surroundings, typically cause shorter-term clinical infections (Blowey and Edmondson, 2010).

Diagnosis of subclinical mastitis often relies on indirect tests, such as somatic cell count (SCC) and the California mastitis test (CMT), which measure the cellular response of the udder/cow to infection. Cows with healthy udder usually produce milk that contain SCC below 200,000 cells/mL, however, if the SCC is over 400,000 cells/mL the udder should be considered as having an intramammary infection (Idriss *et al*, 2013). During mastitis (particularly during SCM), the presence of bacteria triggers an immune response, leading to increased migration of macrophages and neutrophils from blood into the milk, and a high SCC. This is also accompanied by inflammation of the gland, damage to host defense system, and epithelial cells (Douaa *et al*., 2016). The CMT, performed by mixing proportional amount of suspected milk with a reagent, detects increased leukocyte numbers by dissolving cell walls and releasing DNA, resulting in a stringy gel formation proportional to the degree of infection (Melleneger, 2001).

Based on the existing literatures, over 140 potentially pathogenic organisms (including bacteria, fungi, algae, Mycoplasma, and Nocardia) have been recorded as potential cause of cow mastitis. However, most bovine mastitis cases involves various bacterial pathogens, classified as contagious, teat skin opportunistic, or environmental (Radostits *et al*., 2007). *Staphylococcus aureus* is a frequent etiological agent incriminated for both subclinical and chronic infections, causing substantial economic losses in dairy farming (Kubota *et al*., 2007). Due to the increasing prevalence of mastitis, and calls to investigate its causes more thoroughly, this study aimed to determine the prevalence of both clinical and subclinical mastitis in small-holder dairy farms in and around Hawassa town, Southern Ethiopia, while isolating and identifying *S. aureus* from mastitic milk and identifying the associated risk factors. Considering the number of studies already done on the isolation of *S. aureus* in Ethiopia, this study aimed to update existing information and investigate if the epidemiology of the bacteria has changed.

MATERIALS AND METHODS

Study Area

The study was carried out in and around Hawassa City (Fig. 1). Hawassa, the capital city of the Sidama Region, is located at about 275 km south of Addis Ababa. The city has a total of 157,879 inhabitants. Hawassa city, lies between 7°03'1.35"N latitude and 38°29'43.81"E longitude at an altitude of 1750 meters above sea level. Annually, the area receives an average of 800 - 1000 mm of rainfall, and an average annual temperature of 22°C and 51.8% mean relative humidity. Dry savanna and bush-type vegetation covers major part of the area. According to the central statistics authority (CSA, 2020), the region comprises about 2,413,482 cattle, 308,903 goats, 467,858 sheep, 34,709 horses, 16,376 donkeys, 1,824,841 poultry, and 44,364 beehives.

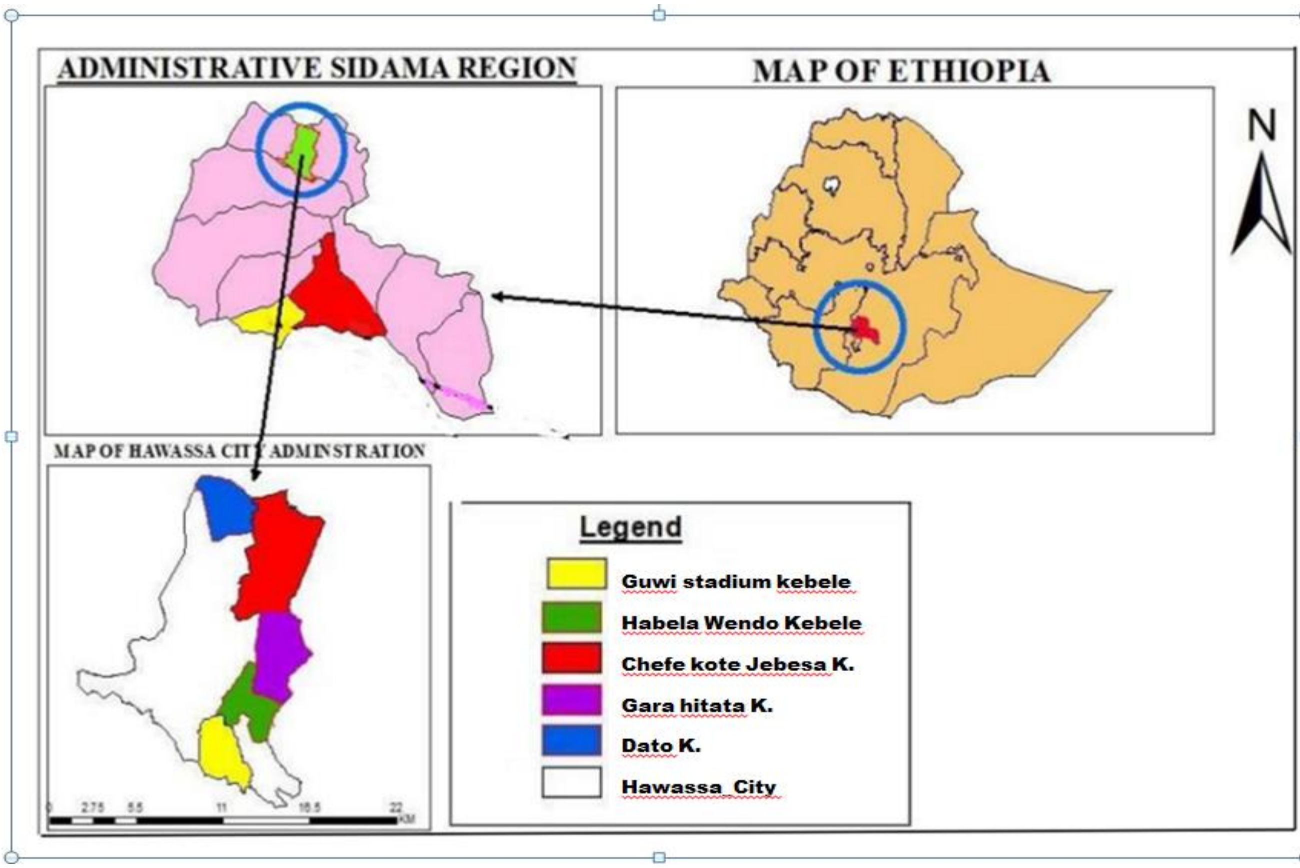

Figure 1. Study area map

Study Animals

The study was carried out on lactating cross-bred cows selected randomly from 29 small holder dairy farms and the associated risk factors were recorded on the sheet designed for it. Host related risk factors such as age, parity, lactation stage, body condition score (BCS), and average milk yield per day, were properly recorded on data recording sheet designed for this purpose. The age of the study animals (cows) were determined based on dentition as per the recommendation of Johnson (1998). Whereas, body condition score (BCS) of the cows were estimated using the standard guide developed by Sharad *et al*. (2016). For ease of data analysis and result presentation, the age classification was made into three as $\leq$3, 4 to 5 and $\geq$ 6 years. Moreover, the available records were used to collect data on the remaining host-related risk factors, including parity, lactation stage, and average milk yield per day. Once the months of lactation is known, the cows were classified into three stages of lactation: early (first 3 months), mid (4 to 6 months), and late (7 months or more).

Study Design

A prospective cross-sectional study design was used to investigate the problem from March 2021 to August 2021.

Sample Size Determination

Thrusfield *et al*. (2017) formula was used to calculate the minimum sample size required for this study. Accordingly, an expected prevalence of 81.1% (Duguma *et al*., 2014), 95% confidence interval and a significance level of 5% were used and computed to be 236. Although the minimum number of lactating dairy cows needed for the study was 236, we increased the sample size to 250.

Study Methodology

Dairy cows were purposively selected from 29 smallholder farms located within and adjacent to the Hawassa city administration to assess the prevalence and risk factors of mastitis. Each selected lactating cow underwent screening for mastitis using clinical examination and the California Mastitis Test (CMT). Cows exhibiting either clinical signs of mastitis or a positive CMT result were considered to have mastitis, and hence milk samples from these cows were collected for bacteriological culture. The primary aim of the culture was to identify the presence of *Staphylococcus aureus*. Moreover, farm visit (observation) and interviews with farm owners were conducted to gather relevant information on the putative risk factors for both clinical and subclinical mastitis, including host related risk factors (i.e. age, body condition score, parity, lactation stage, and daily milk yield), husbandry system, hygienic status of the farms, and past occurrences of mastitis within the herd.

Clinical Inspection of the Udder

To diagnose clinical mastitis, the udders of all study cows were subjected to both visual inspection and palpation. Special attention was given for indicators of acute or chronic inflammatory reactions, including: hardened (chronic) and swollen udder quarters, pain responses to udder palpation (manifested by kicking), localized heat and redness, and alterations in milk secretions such as the presence of clots or flakes, a watery consistency, or blood tinge.

California Mastitis Test

California Mastitis Test (CMT) was used to detect subclinical mastitis. From each quarter of a suspected cow, a squirt of milk was dispensed into separate wells of the CMT paddle, followed by an equal amount of CMT reagent. The mixture was gently agitated, and the resultant gel formation, if there is any, was visually scored based on its thickness. Accordingly, scores were assigned as follows: 0 (negative), T (trace), 1 (weak positive), 2 (distinct positive), and 3 (strong positive), reflecting the level of infection. Quarters with a score of 1 or greater were classified as positive for subclinical mastitis, while those with a score of 0 were classified as negative (Quinn *et al*., 2002)

Milk Sample Collection

The teats of mastitis positive cows were properly washed and disinfected with 70% alcohol, and then approximately 10 mL of milk was collected aseptically into sterile bottle from each affected quarter after discarding few squirt (the initial three streams) of milk. The bottles were properly labeled, immediately placed in a chilled icebox containing ice packs and then transport to the Veterinary Microbiology Laboratory at Hawassa University. Upon arrival, samples were stored at +4°C and held for no more than 24 hours prior to bacterial culture.

Isolation and Identification

Bacteriological culture was made following the standard microbiological techniques (Quinn et al., 2002). Briefly, a loop-full of each milk sample was streaked onto sterile blood agar base (Himedia, India) enriched with 5% sheep blood. Plates were incubated aerobically at 37°C and examined after 24–48 hours for colony growth. Colonies were initially characterized based on morphology, hemolytic pattern, and Gram staining reaction. Gram-positive colonies exhibiting a typical grape-like arrangement under microscopy were selected for further analysis.

The selected colonies were subcultured onto nutrient agar plates (Oxoid, UK) and incubated at 37°C for 24 hours. Subsequently, a catalase test was performed using 3% hydrogen peroxide (H_2O_2). Catalase-positive, Gram-positive cocci were then subcultured onto Mannitol Salt Agar (MSA) and incubated at 37°C. After 24–48 hours, MSA plates were examined for growth and color change. The presence of growth accompanied by a color shift from red to yellow on the MSA (i.e. a change in the medium's pH) was considered presumptive evidence of salt-tolerant *Staphylococcus species* (Quinn *et al*., 2002).

A tube coagulase test, following the method of Robertson *et al*. (1999), was performed. Fresh cultures of suspected staphylococci, grown in nutrient broth for 18-24 hours, were mixed with 0.5ml of 10 fold diluted sterile rabbit plasma (Sigma). This mixture was incubated at 37°C and examined every 4-24 hours for clot formation. Any degree of visible clotting was considered a positive result (Tallent *et al*., 2001). Additionally, suspected *S. aureus* cultures were inoculated onto purple agar base (PAB) media supplemented with 1% maltose and incubated at 37°C for 24 hours. *S. aureus* isolates were expected to rapidly ferment maltose, leading to the production of acidic metabolites that turn the medium and colonies yellow.

Ultimately, the isolates were considered positive for *S. aureus* if they exhibited catalase-positive and coagulase-positive results, combined with growth on MSA and PAB media with an associated yellow coloration of the media (Quinn *et al*., 2002)..

Data Analysis

Following data collection, records were entered into Microsoft Excel, coded appropriately, and then subjected to statistical analysis. Mastitis prevalence was calculated as the proportion of cows diagnosed with mastitis (encompassing both clinical and subclinical forms) relative to the total number of examined lactating cows. To explore the association between putative risk factors and mastitis prevalence, odds ratios (OR) were computed. On top of that, a logistic regression model was utilized with STATA Corp. (version 12.0) software to assess the independent contribution of each risk factor to the likelihood of mastitis. Statistical significance for all analyses was set at a 95% confidence level, with a p-value < 0.05.

RESULTS

Prevalence of Mastitis

This cross-sectional study investigated mastitis in 250 lactating cows. The results showed that 50.8% (l27/250) of the cows had some form of mastitis (either clinical or subclinical). Among these, 4.8% (l2/250) exhibited clinical mastitis, while a larger proportion, 46% (ll5/250), had subclinical mastitis. When considering individual udder quarters, the overall mastitis prevalence was 27.4% (274/l000). Clinical mastitis was identified in 2.9% (29/l000) of quarters, while subclinical mastitis affected 24.5% (245/l000) (Table l).

Table 1. Prevalence of mastitis at cow and quarter level

Forms of mastitis	Cow level (N= 250) No. (%) positive	Quarter level (N= 1000) No. (%) positive
Clinical	12 (4.8%)	29 (2.9%)
Subclinical	115 (46%)	245 (24.5%)
Total	**127 (50.8%)**	**274 (27.4%)**

Bacterial Isolation

Bacteriological analysis was performed on the l27 milk samples obtained from cows diagnosed with mastitis to determine the presence of *S. aureus*. Accordingly, *S. aureus* was isolated from 4l.6% (5/l2) of samples from cows with clinical mastitis and from 47.8% (55/ll5) of samples from cows with subclinical mastitis. The overall prevalence of *S. aureus* among all mastitis-positive samples was 47.2% (60/l27), as shown in Table 2.

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Table 2. Prevalence of S. aureus in clinical and subclinical mastitis

Form of mastitis	No. of cow examined	No. of isolated *S. aureus* Cases (%)
Clinical	12	5 (41.6)
Subclinical	115	55 (47.8)
Total	**127**	**60 (47.2)**

Risk Factors Associated with Mastitis

Multivariate logistic regression analysis revealed that age of the cows, late lactation stage, and farm hygiene frequency were significantly associated with mastitis. In contrast, milk yield, parity, and husbandry system were not identified as significant risk factors in this model (Table 3).

Table 3. Logistic regression analysis of potential risk factors for the occurrence of mastitis in the study area

Risk factors	Categories	No. of cows		Crude OR (95% CI)	Adjusted OR (95% CI)	p-value
		Examined	Positive (proportion)			
Age (years)	≤3	93	21 (22.6)	1	1	
	4-5	98	47 (47.9)	3.16(1.68, 5.92)	3.7 (1.63, 8.43)	0.002
	≥6	59	47 (79.7)	13.43(6.04, 29.85)	17.61 (5.3, 58.44)	0.000
Parity	≤2	134	43 (32.1)	1	1	
	≥3	116	72 (62.1)	3.46 (2.06, 5.83)	1.29 (0.59, 2.79)	0.512
Lactation stage (Months)	Early	85	27 (31.7)	1	1	
	Mid	89	43 (48.3)	2.01 (1.08, 3.72)	1.81 (0.9, 3.62)	0.094
	Late	76	45 (59.2)	3.11 (1.63, 5.95)	2.1 (1.0, 4.32)	0.049
Milk yield per day (lit.)	≤10	86	45 (52.3)	1	1	
	11-15	82	34 (41.5)	1.56 (0.84, 2.80)	1.26 (0.61, 2.63)	0.531
	≥16	82	36 (43.9)	1.41 (0.76, 2.58)	1.39 (0.66, 2.94)	0.384
Husbandry system	Intensive	233	102 (43.7)	1	1	
	Semi-intensive	27	13 (48.1)	1.10 (0.49, 2.45)	1.97 (0.68, 5.88)	0.213
Frequency of farm cleaning per day	≥ 4 times	36	10 (27.7)	1	1	
	3	130	53 (40.7)	2.36 (1.37, 4.15)	2.94 (1.58, 5.81)	0.002
	2	84	52 (61.9)	4.24 (1.80, 9.90)	5.1 (1.85, 13.7)	0.002

OR: odds ratio, lit.: liter, No.: number

DISCUSSION

The present study found an overall mastitis prevalence of 50.8% at the cow level and 27.4% at the quarter level. These prevalence are consistent with those reported in earlier studies by Hundera *et al.* (2005) and Abera *et al.* (2010). In most developing countries, mastitis prevalence tends to be approximately 50% in cows and 25% in quarters. Nevertheless, our findings are notably lower than those observed in several other Ethiopian studies (Abebe *et al*., 2016; Zeryehun & Abera, 2017; Elemo et al., 2017; Tegegne *et al*., 2020), which documented cow-level prevalence ranging from 62.6% to 70%. Conversely, the prevalence in this study was higher than earlier reports by Workineh *et al.* (2002), Mungube *et al*. (2004), and Kerro and Tareke (2003), which ranged from 38.2% to 40%. The prevalence difference across studies suggests that differences in husbandry practices and environmental factors play a critical role in mastitis incidence (Radostits *et al*., 2007).

The higher prevalence of sub-clinical mastitis (46%) than clinical mastitis (4.8%) encountered in the current study supports previous studies conducted in various parts of the country, which have consistently determined that sub-clinical mastitis is prevailing than clinical mastitis. In line with this, subclinical and clinical mastitis prevalence were 62.9% and 37.0% (Kerro & Tareke, 2003), 59.2% and 3.4% (Abebe *et al*., 2016), 48.6% and 22.4% (Mekbib *et al*., 2010), 36.7% and 10.0% (Abera *et al*., 2010), and 27.86% and 11.45% (Tassew *et al*., 2017) respectively. The consistently higher prevalence of subclinical mastitis in these studies, including ours, is likely due to its insidious nature and lack of overt symptoms which often delays detection and treatment (Radostits *et al*., 2007). Our study also found that mastitis prevalence was significantly higher during late lactation (59.2%), which is consistent with Almaw *et al.* (2008), Getahun *et al.* (2008), and Abera *et al.* (2012). However, this contrasts with Kerro and Tareke (2003), who reported higher rates during early lactation. Such discrepancies may stem from differences in cow age and parity (Isae & Kurtu, 2018).

The present study also showed that older cows (>6 years) had a higher prevalence of mastitis (79.7%) than younger cows (<3 years) (22.6%), supporting the findings of Kerro and Tareke (2003) and Busato *et al*. (2000). This increased risk in older cows has been attributed to anatomical changes, such as larger teats and weaker sphincter muscles, facilitating pathogen entry (Radostits *et al*., 2007).The findings of the current study regarding the increased prevalence of mastitis with parity are consistent with previous reports by Zeryehun *et al*. (2013), Abunna *et al*. (2013), Belayneh *et al*. (2014), and Dabele *et al*. (2021). The likelihood of mastitis was 3 to 13 times higher in multiparous cows compared with primiparous cows. In line with this a study conducted by Abebe *et al*. (2016) also reported odds of 24.8 for cows with four or more calvings. This association may be attributed to the fact that primiparous cows possess a more effective defense mechanism against mastitis compared to multiparous cows (Erskine, 2001). The likelihood of infection increases over time in multiparous cows, leading to a prolonged duration of infection (Radostits *et al*., 2007).

A key finding of this study was that mastitis prevalence was significantly higher (61.9%) in cows housed in facilities cleaned twice daily, compared to those cleaned four or more times daily. This suggests that the frequency of cow house cleaning is an important factor in mastitis control, particularly of the environmental ones. Notably, the study did not find a significant link between husbandry system (such as grazing, confinement) or a prior history of mastitis and current mastitis prevalence. These results should be interpreted with consideration for the context of this particular study.

The microbiological investigations identified *S. aureus* in 47.2% of mastitic cows, which corresponds with similar studies in Holeta (Mekibib *et al*., 2010) and Addis Ababa (Legesse *et al*., 2015). However, some other studies have reported both lower (Workineh *et al*., 2002; Tesfaye *et al*., 2013; Yohannis and Molla, 2013; Zeryehun *et al*., 2013) and higher (Abebe *et al*., 2016; Zenebe *et al*., 2014) prevalence of *S. aureus*. Such variations among studies are likely due to husbandry practices and environmental differences.

Staphylococcus aureus, a mastitis causing contagious pathogen, is known for its ability to establish chronic, subclinical infections, as well as acute and clinical mastitis. Consistent with Tassew *et al*. (2017), our study found that *S. aureus* was more common in subclinical mastitis cases than clinical cases. This indicates that *S. aureus* is a key causative agent in these less visible infections, as they have a tendency to establish chronic long term infections and act as a source of transmission (Radostits *et al*., 2007). The propensity of the bacteria to induce subclinical mastitis could be related to the numerous virulence factors including its capacity to produce biofilm, toxins, or various enzymes capable of damaging the udder tissue and concomitantly establish itself in the infected area (Artursson *et al*., 2016).

CONCLUSION & RECOMMENDATIONS

Mastitis remains a significant infectious disease in dairy cows, affecting the dairy industry significantly. In this study, subclinical mastitis was the most prevalent form, likely due to a greater focus on clinically visible forms by farm owners and animal health workers, which allows subclinical cases to go unnoticed. Mastitis prevalence varied significantly with cow age, lactation stage, and farm hygiene practices. These findings highlight the need for routine screening and early intervention to detect and treat subclinical mastitis. Furthermore, raising awareness about the public health risks and economic impacts associated with *S. aureus* is critical.

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