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Bovine Mastitis: *Staphylococcus aureus* isolation and identification from Small holder Dairy Farms located in and around Hawassa town, Southern Ethiopia

Nebyou Moje[1*] , Birtukan Abebaw [2]

[1]*College of Veterinary Medicine & Agriculture, Addis Ababa University, P.O.Box 34, Bishoftu, Ethiopia*
[2]*Faculty of Veterinary Medicine, Hawassa University, P.O.Box 05, Hawassa, Ethiopia*

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ABSTRACT

Mastitis is a widely distributed disease of dairy cattle in most countries, including Ethiopia. The most commonly recovered bacterial pathogen during mastitis is *Staphylococcus aureus* in dairy cows worldwide. With this, a cross-sectional study was conducted from March 2021 to August 2021 on dairy farms in and around Hawassa town to isolate and identify *Staphylococcus aureus* from bovine mastitis milk and to determine risk factors associated with the occurrence of mastitis. A total of 250 lactating cows were randomly selected for clinical and subclinical mastitis from 29 small holder dairy farms. Clinical signs and the California Mastitis Test (CMT) were used to identify clinical and subclinical mastitis, respectively. Accordingly, a standard bacteriological study targeting *S. aureus* was conducted with all (n=127) milk samples collected from clinical and subclinical mastitis cows. Data generated from these methods were analyzed using STATA Corp. Version 12. Association between the risk factors and mastitis were determined with $p<0.05$ to be a statistically significant one. During the study period, 50.8% of cows had mastitis, of which 4.8% and 46% showed clinical and subclinical mastitis, respectively. The quarter-level proportion was 27.4%; of which the clinical form was 2.9%, while the subclinical mastitis was 24.5%. Logistic regression analysis showed a significant association among cows of different age groups, lactation stages, and frequency of farm cleaning status per day with the occurrence of mastitis ($p < 0.05$). Bacterial identification targeting *S. aureus* was done, and this agent was identified in 60 (47.2%) milk samples. This pathogen was found to be higher (47.8%) in subclinical than in clinical (41.6%) mastitis. In conclusion, this study showed that mastitis was prevalent in dairy cattle of the study area, with a higher case of *S. aureus* in subclinical mastitis. However, the detection of *S. aureus* in nearly half of the milk sample collected from mastitic cows indicated the possible presence of other pathogens. Therefore, further study to recover other potential pathogens commonly causing mastitis can be a good approach.

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

**Corresponding author:*
Email: nebhawas@gmail.com +251910248878

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is reported in numerous species, mainly in domestic dairy animals. This pathology is the most frequent disease of dairy cattle and can be potentially fatal (Gutierrez-Chavez et al., 2019). Bovine mastitis has been reported as the most important disease on dairy farms because of the reduction of farm profitability, decreased milk production, discarded milk, treatment costs, and culling (Julian, 2016).

Subclinical mastitis (SCM) refers to inflammation of the mammary gland in the absence of visible gross lesions in the udder or its secretions, with the presence of pathogenic microorganisms and an increased number of somatic cells in the milk (Smith, 1996; Radostits *et al*., 2007). Even if there is a great loss related to both conditions, clinical mastitis continues to be a problem in many dairy herds (Gezehagn *et al*., 2020). Moreover, mastitis has serious zoonotic potential associated with the shedding of bacteria and their toxins in the milk. Mastitis is caused by a wide spectrum of pathogens and is epidemiologically categorized into contagious and environmental mastitis. Contagious pathogens are those for which the udders of infected cows serve as the major reservoir. They spread from cow to cow, primarily during milking, and tend to result in chronic subclinical infections with flare-ups of clinical episodes (Abebe *et al*., 2016). Environmental bacteria live in the surrounding environment of the cows and are considered opportunistic, causing clinical infections with short duration (Blowey and Edmondson, 2010).

Diagnosis of subclinical diagnosis is done with the indirect tests which usually depend on the cellular interaction between the reagent and certain protein factors in mastitis milk. The most common methods are somatic cell count (SCC) and the California mastitis test (CMT) (Rafik *et al*., 2014). During SCC, the adherence of bacteria stimulates macrophage migration and the migration of neutrophils from blood into the milk, which will lead to a high SCC, swelling of the mammary gland, damage to the host defense system, and epithelial cells (Douaa *et al*., 2016). When milk and CMT reagent are mixed in equal amounts, the CMT reagent dissolves or disrupts the outer cell wall and the nuclear cell wall of any leukocyte, which are primarily fat (detergent dissolves fat). DNA is now released from the nuclei. DNA will string or gel together to form a stringy mass. As the number of leukocytes increases in a quarter, the amount of gel formation will increase in a linear fashion (Melleneger, 2001).

Several bacterial pathogens are implicated in bovine mastitis. From an epidemiological and pathophysiological standpoint, the pathogens are regarded as contagious, teat skin opportunistic, or environmental (Radostits *et al*., 2007). *Staphylococcus aureus* is the etiological agent more commonly associated with the disease and is normally related to both subclinical and chronic infection, leading to severe economic loss to dairy farms (Kubota *et al*., 2007). According to a livestock agriculture office report, the incidence of mastitis has become popular, and some professionals also recommend digging in detail about the area, as such the study was designed to figure the prevalence value and to identify the factors associated with mastitis infection. In addition, although many studies have been conducted by different researchers on the isolation and identification of *S. aureus* from bovine mastitis milk in Ethiopia, including the present study area, it is necessary to update the information to find out if there was any change in the

epidemiology of the bacteria. Therefore, this study was aimed to estimate the prevalence of clinical and subclinical mastitis, to isolate and identify *S. aureus* from mastitic milk and to determine the associated risk factors of bovine mastitis in small-holder dairy farms in and around Hawassa town, Southern Ethiopia.

MATERIALS AND METHODS

Study Area

The study was conducted in Hawassa City and its surroundings (Fig. below). Hawassa is the capital city of the Sidama Region, which is located 275 km south of Addis Ababa with a total human population of 157,879. Geographically, it lies between 7°03'1.35"N latitude and 38°29'43.81"E longitude at an altitude of 1750 meters above sea level. The area annually receives an average of 800 - 1000 mm of rainfall, of which 67% falls in the long rainy season, which extends from June to September, with an average annual temperature of 22°C and 51.8% mean relative humidity. The area is mainly covered by dry savanna and bush-type vegetation. The total livestock population of the Sidama region (including Hawassa town) is estimated to constitute 2,413,482 cattle, 308,903 goats, 467,858 sheep, 34,709 horses, 16,376 donkeys, 1,824,841 poultry, and 44,364 beehives (CSA, 2020).

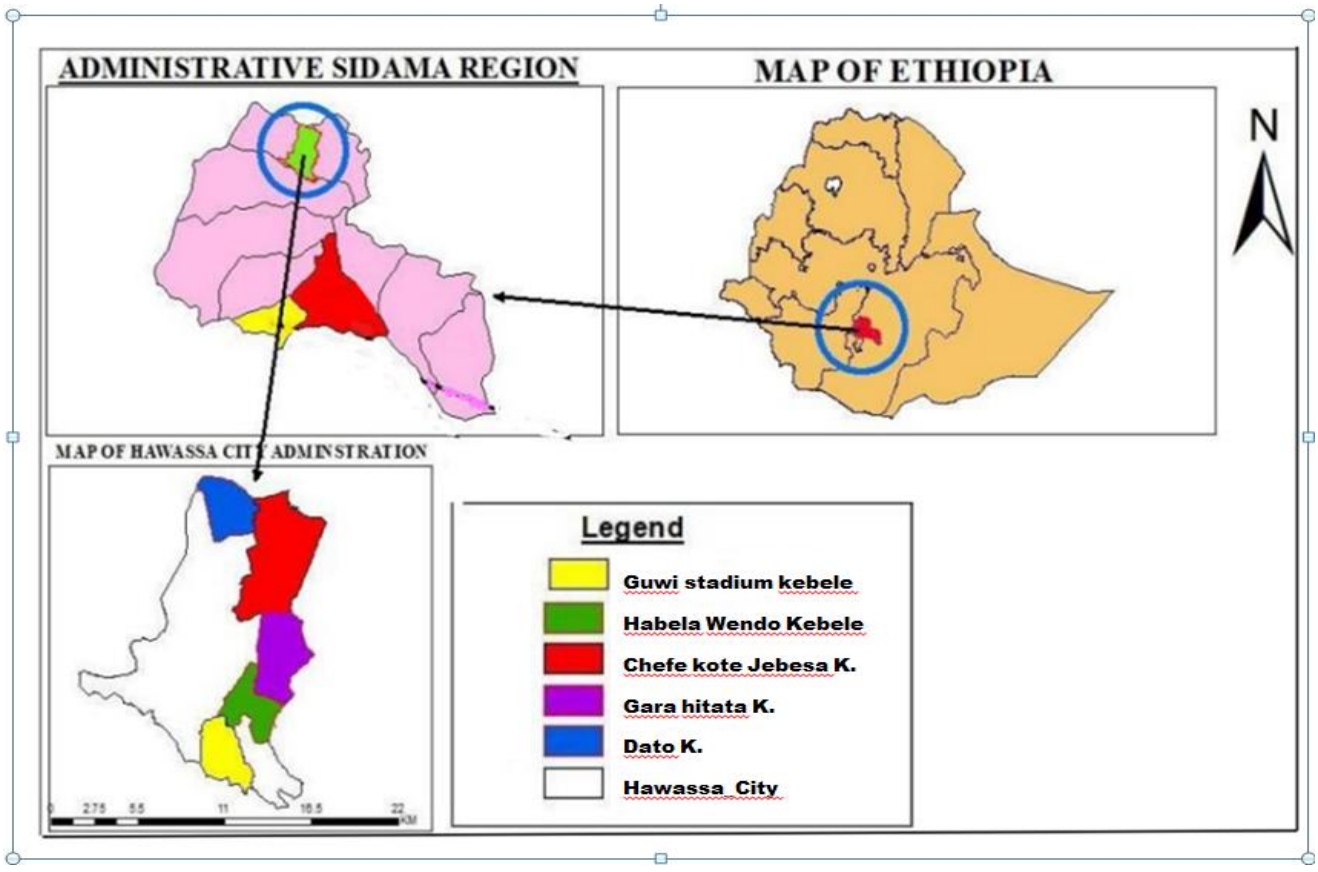

Figure. Study area map

Study Animals

The study was conducted 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. Risk factors such as age, body condition score (BCS), parity, lactation stage, and average milk yield per day, were recorded on the sheet designed for sample collection. Age and BCS were determined with observation of dentition as per Johnson (1998) while for that of body condition Sharad *et al*. (2016) classification was used. Later the age classification was made into three as $\leq$3, 4 to 5 and $\geq$ 6 years. The other information (parity, lactation stage, and average milk yield per day) was recorded from the farms recording. After identifying the months of the cow's lactation; classification was made into three as early (for the first 3 months), mid (4 to 6 months) and late (above seven months) lactation stages.

Study Design

A cross-sectional study was conducted from March 2021 to August 2021 on dairy farms in and around Hawassa to isolate and identify *Staphylococcus aureus* from clinical and subclinical mastitis cows.

Sample Size Determination

The sample size was calculated according to the formula given by Thrusfield *et al*. (2017) using expected prevalence of 81.1% (Duguma *et al*., 2014), 95% confidence interval and a significance level of 5%. The minimum number of cattle needed in the study was calculated to be 236, however, we included 250 lactating cows in this study.

Study Methodology

Dairy cows were randomly selected from 29 small holder farms located in and around Hawassa city administration. From these farms, each selected lactating cow was screened for mastitis based on clinical examinations and the California Mastitis Test. Milk samples from cows with mastitis were subjected to bacteriological examination to identify *S. aureus*. Furthermore, information regarding potential risk factors for both clinical and subclinical mastitis, such as husbandry systems, the status of farm hygiene, and previous history of mastitis, was collected from interviews with farm owners. Additionally, animal identification, including age, body condition score (BCS), parity, lactation stage, and average milk yield per day, were recorded on the sheet designed for sample collection. Age and BCS were determined with dentition (Johnson, 1998) and body condition (Sharad *et al*., 2016) observation, respectively.

Clinical Inspection of the Udder

For clinical mastitis, the udders of the study cows were examined visually and by palpation for the presence of any abnormalities, such as hard and swollen quarters, pain (kicking upon touching the udder), heat, and abnormal secretion in the mammary gland (the presence of clots or flakes in milk or watery consistency, and blood-tinged secretions).

California Mastitis Test

Cases of subclinical mastitis were diagnosed based on CMT results (i.e., observation of the nature of gel formation), which show the presence and severity of the infection. From each quarter of the udder, a squirt of milk sample was dropped into each of the strip cups on the CMT paddle, and an equal amount of CMT reagent was added to each cup and mixed gently. The test result was interpreted based on the thickness of the gel formed by the CMT reagent and milk mixture and scored as 0 (negative), T (trace), 1 (weak positive), 2 (distinct positive), and 3 (strong positive). Finally, quarters with a CMT score of 1 or above were judged as positive for subclinical mastitis; otherwise, they were considered negative (Quinn *et al*., 2002).

Milk Sample Collection

Milk samples from mastitis positive cow were collected after cleaning and disinfection with 70% alcohol before milk sampling. Approximately 10 ml of milk was taken from each quarter after discarding the first three milking streams aseptically into sterile bottles for bacteriological investigation and labeled. The sample was placed in an icebox containing ice packs and transported immediately to Hawassa University, Faculty of veterinary Medicine Microbiology laboratory. Upon arrival, the collected samples were immediately stored at +4 °C for a maximum of 24 hours until culturing.

Isolation and Identification

The bacteriological culture was performed following the standard microbiological technique (Quinn et al., 2002). A loopful of milk sample was streaked on sterile 5% sheep blood agar (Himedia, India), and the plates were incubated aerobically at 37 °C and examined after 24–48 h of incubation. The colonies were identified based on morphological characteristics, hemolytic pattern, and Gram's staining reaction. The representative colonies that were positive for Gram's staining and had a typical grape-like structure under a microscope were further sub-cultured on nutrient agar plates (Oxoid, UK) and incubated at 37 °C for 24 hrs followed by biochemical tests, and grow on mannitol salt agar (MSA) and purple agar base media.

A catalase test using 3% hydrogen peroxide (H_2O_2) was performed to identify catalase-positive and catalase-negative bacteria. The colonies that were identified as Gram positive cocci were subjected to catalase test and catalase positive isolates were sub-cultured on MSA and incubated at 37°C. Examination was made after 24–48 hrs for growth and change in the color of the medium. The presence of growth and change in pH of the media (red to yellow color) were regarded as confirmative identification of the salt-tolerant *Staphylococci*. The fermentation of mannitol by *S. aureus* causes yellow discoloration of the medium (Quinn *et al*., 2002).

The tube coagulase test was conducted according to the method of Robertson *et al*. (1999). Accordingly, 0.1 ml of fresh cultures of suspected *staphylococci* grown on Nutrient Broth for 18-24 hours was added to 0.5 ml of 1/10 diluted sterile rabbit plasma (Sigma) in the test tube. The tube was incubated at 37°C and examined every 4-24 hours to see the presence of clotting. The reaction was considered

coagulase-positive if any degree of clotting was visible (Tallent *et al*., 2001). The suspected culture was also inoculated on a purple agar base (PAB) with 1% maltose media and incubated at 37°C for 24 hrs. *S. aureus* isolates showed rapid fermentation of maltose and acid metabolites which turn the culture medium and colonies to yellow. Therefore, isolates that were considered positive for *S. aureus* showed catalase-positive, coagulase-positive, and growth with a yellowish coloration of medias (MSA and PAB) (Quinn *et al*., 2002).

Data Analysis

Collected data were entered into a Microsoft Excel spreadsheet and coded before statistical analysis. The prevalence of mastitis was calculated by dividing the number of mastitis-positive cows (clinical and subclinical) by the total number of animals examined. The degree of association between risk factors and the prevalence of mastitis was analyzed using the odds ratio (OR). Furthermore, logistic regression was used to examine the association of the potential risk factors with the occurrence of mastitis using STATA Corp. version 12.0 statistical software. In all analyses, a 95% confidence level and a p-value<0.05 were used to determine statistical significance.

RESULTS

Prevalence of Mastitis

In this cross-sectional study, from a total of 250 lactating cows examined, 127 (50.8%) were found to be affected with both clinical and subclinical mastitis infection. Of these, 4.8% (12/250) and 46% (115/250) showed clinical and subclinical mastitis, respectively. The quarter-level overall prevalence of mastitis was 27.4% (274/1000), while quarter-level clinical and subclinical mastitis were 2.9% (29/1000) and 24.5% (245/1000), respectively. There was a higher prevalence of subclinical mastitis than clinical mastitis, both at the cow and quarter levels (Table 1).

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

Out of 127 milk samples collected from mastitis positive cows and subjected for bacteriological examination of *S.aureus*. *S. aureus* was isolated from 41.6% (5/12) of the clinical cases and 47.8% (55/115) of the subclinical cases, respectively. The overall prevalence of *S. aureus* was 47.2% (60/127), as indicated in Table 2.

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 analyses showed risk factors such as parity, milk yield, and husbandry system were found to be not significantly associated with the occurrence of mastitis. However, cows age, late lactation stage and farm hygiene frequency were significantly associated with the occurrence of mastitis (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

In the present study, the overall prevalence of mastitis was 50.8% and 27.4% at cow and quarter level, respectively. This result was in line with that of Abera *et al*. (2010) who reported prevalence of 46.7% at cow level and 29% at the quarter level in Adama town and Hundera *et al*. (2005) who reported 52.8% at cow level around Sebeta. In most countries, irrespective of the cause, the prevalence of

mastitis is about 50% in cows and 25% in quarters. However, the present findings are lower than the previous reports of Abebe *et al*. (2016), Zeryehun and Abera (2017), Elemo *et al.* (2017), and Tegegne *et al.* (2020) who reported in Hawassa, South Ethiopia (62.6%); Eastern Harrarghe Zone, Eastern Ethiopia (64.3%); in Asella, Southern Eastern Ethiopia (65.36%); Addis Ababa, central Ethiopia (70%), respectively. However, the finding was higher than the previous reports of Workineh *et al*. (2002) Mungube *et al*. (2004) and Kerro and Tareke (2003) who reported 38.2%, 39.8% and 40% in Adami Tulu (Central Ethiopia), in and around Addis Ababa and Southern Ethiopia; respectively. This variability in the prevalence of mastitis, irrespective of the cause, between different reports could be attributed to differences in farm management practices, or environmental conditions in different parts of the country (Radostits *et al*., 2007).

In the present study the prevalence of subclinical mastitis (46%) is higher than clinical mastitis (4.8%). This finding supports previous studies conducted in various regions of the country, which have consistently concluded that subclinical mastitis is more prevalent than clinical mastitis. For instance, Kerro and Tareke (2003) reported a prevalence of 62.9% and 37.0%, Abebe *et al*. (2016) found a prevalence of 59.2% and 3.4% in southern Ethiopia, Mekbib *et al*. (2010) reported a prevalence of 48.6% and 22.4% in Holeta, Abera *et al*. (2010) observed a prevalence of 36.7% and 10.0% in Adama, and Tassew *et al.* (2017) found a prevalence of 27.86% and 11.45% in and around Assosa town of subclinical and clinical mastitis, respectively. The higher prevalence of subclinical mastitis compared to clinical mastitis can be attributed to the challenges in detecting subclinical cases, as they lack visible symptoms that prompt animal owners to seek treatment. In contrast, clinical cases are more easily detectable due to their observable signs, leading to higher treatment-seeking behavior (Radostits *et al*., 2007).

In the present study, it was observed that the prevalence of mastitis was significantly higher during the late stage of lactation, accounting for 59.2% of cases. This finding is consistent with previous studies conducted by Almaw *et al*. (2008), Getahun *et al.* (2008), and Abera *et al*. (2012), which also reported a higher prevalence of mastitis during this stage. However, this finding contradicts the results of a study by Kerro and Tareke (2003), where a higher prevalence of mastitis was reported during the early stage of lactation. These discrepancies regarding the effect of lactation stages among different studies could potentially be attributed to variations in the age and parity of the sampled cows, as suggested by Isae and Kurtu (2018).

The present study revealed that the prevalence of mastitis was higher in older cows (>6 years of age) (79.7%) than in younger (<3 years) cows (22.6%).In support to this, the findings by Kerro and Tareke (2003), and Busato *et al*. (2000), who found that the risk of clinical and subclinical mastitis increases significantly with the advancing age of the cow. It has been well documented that older cows have larger teats and more relaxed sphincter muscles, which increase the accessibility of infectious agents in the cows' udder (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). 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).

According to the current findings, there was a higher prevalence of mastitis (61.9%) observed in cows housed in facilities that were cleaned twice per day (less frequently cleaned), compared to cows housed in facilities cleaned four times or more per day (more frequently cleaned). This suggests that the frequency of cleaning the cow houses had an impact on the prevalence of mastitis, with less frequent cleaning associated with a higher prevalence. Interestingly, the study did not find a significant association between the husbandry system and the previous history of mastitis with the prevalence of mastitis in the cows. This implies that factors such as the type of husbandry system (e.g., grazing, confinement) and the presence of a previous history of mastitis did not have a notable effect on the prevalence of mastitis in the specific study. It is important to consider that these findings are specific to the study conducted and may not be universally applicable.

Based on the microbiological analysis conducted in this study, it was found that the overall prevalence of *S. aureus* isolates in cows with both clinical and subclinical mastitis was 47.2%. This finding aligns with the previous studies conducted by Mekibib et al. (2010) (47%) in Holeta town and Legesse *et al*. (2015) (48.3%) in Addis Ababa city. However, it is worth noting that the present prevalence was higher than that reported in other studies (Workineh *et al*., 2002; Tesfaye *et al*., 2013; Zeryehun *et al*., 2013;Yohannis and Molla, 2013). On the other hand, there were studies, namely Abebe *et al*. (2016) and Zenebe *et al*. (2014) that reported a higher prevalence of *S. aureus* isolates compared to the present study. The variations in the occurrence of *S. aureus* among different reports could be attributed to differences in the farm management practices and environmental factors.

In this study, *S.aureus* was more frequently identified in subclinical mastitis than in clinical cases. This is almost similar to previous studies that proved *S.aureus* is the principal causative agent of subclinical mastitis (Tassew *et al*., 2017). This difference might be explained by the already documented evidence that *Staphylococcus* species are adapted to survive in the udder and usually establish chronic subclinical infection of long duration, from which it is shed through milk, serving as a source of infection for other healthy cows and transmitted during the milking process (Radostits *et al*., 2007).

CONCLUSION & RECOMMENDATIONS

Mastitis is one of the most important infectious diseases of dairy cows. The subclinical form is the most prevalent type of mastitis in the study farms, which might indicate that dairy farm owners give more attention to clinical mastitis than to subclinical mastitis, which receives very little attention. The prevalence of mastitis was found to be significantly different among age groups, lactation stages of cows and cleaning frequency of the farm (Hygiene). Based on the above conclusions, regular screening for early

detection and treatment of subclinical mastitis together with creating awareness in the community of the risk of *S. aureus* as a public health concern should be in place.

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