Staphylococcus aureus isolates from cow's milk in dairy farms at Shinshicho town, Kembata Tembaro Zone, Southern Ethiopia: Prevalence, risk factors, and antimicrobial susceptibility profile.

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Abstract

Bovine mastitis is an economically important and highly prevalent infectious disease in dairy herds worldwide. *Staphylococcus aureus* is a common microorganism causing infectious mastitis. A cross-sectional study was conducted between December 2018 and September 2019 in Shinshicho town, Kembata Tembaro Zone, Southern Ethiopia, to estimate the prevalence, and assess associated risk factors and antimicrobial susceptibility profile of *S. aureus* isolates from cow's milk in dairy farms at Shinshicho town. Lactating dairy cows were screened for mastitis based on clinical examinations and the California mastitis test (CMT) followed by laboratory identification of *S. aureus*. All the *S. aureus* isolates were subjected to antimicrobial susceptibility tests using a disk diffusion test. Multivariable logistic regression analysis of the effect of different risk factors on the prevalence of mastitis was performed. From a total of 384 lactating cows examined and tested, 41.7 % (n=160) were found positive for mastitis. Out of the occurrences of mastitis, 5% (n=19) and 36.7% (n=141) were clinical and subclinical respectively. *Staphylococcus aureus* was isolated from 36.84% (n=7) and 39.01% (n=55) of the clinical and subclinical mastitis respectively with a total isolation frequency of 38.75 % (n=62). According to the results of this study, greater herd sizes (OR=2.91, 95% CI: 1.62-5.21), higher parity cows (OR=3.91, 95% CI: 1.73-8.82), late lactation stage (OR=3.36, 95% CI: 1.27-8.91), and muddy floor (OR=2.37, 95% CL: 1.31-4.27) are risk variables linked to the occurrence of *S. aureus* mastitis. In addition, *S. aureus* has total

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resistance to ampicillin, amoxicillin, penicillin-G, and Polymyxin. Similarly, 53.2% of the isolates proved resistant to three or more of the antibiotics used. Therefore, regular antimicrobial susceptibility testing should be performed to select potent modified antibiotics, and the effects and dynamics of genetic determinants of antibiotics should also be studied using molecular methods.

Keywords: Antimicrobial susceptibility; Milk; Prevalence; Risk factors; Southern Ethiopia; *S. aureus*.

Introduction

Ethiopia has the largest livestock population in Africa, estimated at 70 million cattle (CSA, 2020/21). Cows represent the biggest portion of the country's cattle population with around 21.4 % of the total cattle heads being milking cows (CSA, 2020/21). However, milk production often does not meet national requirements due to the low genetic potential for milk production of indigenous breeds, the extensive and low inputs of animal husbandry practices under which they are reared, and widespread livestock diseases (Ahmed *et al*., 2004). Mastitis is one of the factors contributing to reduced milk production (Biffa *et al*., 2005). Several etiological agents cause mastitis of which *S. aureus* is a major pathogen that also poses food safety and antimicrobial resistance threats (Kumar *et al.,* 2011).

Staphylococcus aureus is the most predominant cause of mastitis in dairy cows in Ethiopia (Seid *et al*., 2015). The Bacterial contamination of milk from an infected udder may render it unsuitable for human consumption by causing food poisoning or providing a vehicle for the spread of zoonotic diseases to humans (Bitew *et al*., 2010). In Ethiopia, *Staphylococcus* species isolated from animal-origin food in central and southeastern parts of the country indicate the presence of an alarming level of resistance isolates (Beyene *et al*., 2016; Tessema *et al*., 2016). Infection with antimicrobial-resistant bacteria has been known to be associated with frequent treatment failure and increased severity of the disease (Finch and Hunter, 2006). Resistant bacteria from food animals may be passed through the food chain to humans resulting in resistant infections (Anderson *et al*., 2003).

Recently, the same studies were conducted on isolation, identification, and drug resistance patterns of *S. aureus* in lactating dairy cows milk in various parts of Ethiopia, such as in Kombolcha town (Tassew *et al*., 2016), Wolaita Sodo (Derib *et al*., 2017), Assosa Town (Tassew *et al*., 2017), and Asella Town

(Seyoum *et al.*, 2018). However, the information on the prevalence, risk factors, and antimicrobial susceptibility profile of *S. aureus* isolates from mastitic lactating cow's milk in Shinshicho town is lacking. Therefore, the objectives of this study were:

- To isolate, characterize and estimate the prevalence of *S. aureus* in mastitic lactating cow's milk
- To assess different potential risk factors associated with the prevalence of *S. aureus* and to investigate the antimicrobial susceptibility profiles of the isolates.

Materials and methods

Description of the study area

The study was conducted in Shinshicho town, Kembata Tembaro Zone, Southern Nation Nationalities, and Peoples Regional State, Ethiopia (Figure 1). It is located at a distance of 293 km South of Addis Ababa at 7°12′N and37°46′E with an altitude of 1875 meters above sea level (Fig.1). The annual mean rainfall ranges from1200 to 1500 milmiters with an average minimum and maximum annual temperature of 18°C and 31°C respectively. It has two administrative Kebeles with an estimated human population of 55,563 of which 25025 males and 30538 were females (STAFEDO, 2018). The land coverage of the study area is 1543 hectares, and the agricultural production system is mixed farming. In the study area, dairy farms typically employed intensive system to manage their improved crossbreed animals. They are often provided with a supplementary diet in addition to the natural pasture and agricultural by-products. The livestock population of the town is estimated to be 11739 cattle, 3035 sheep, 3870 goats, 39100 poultry, and 1450 equines (STAFEDO, 2018).
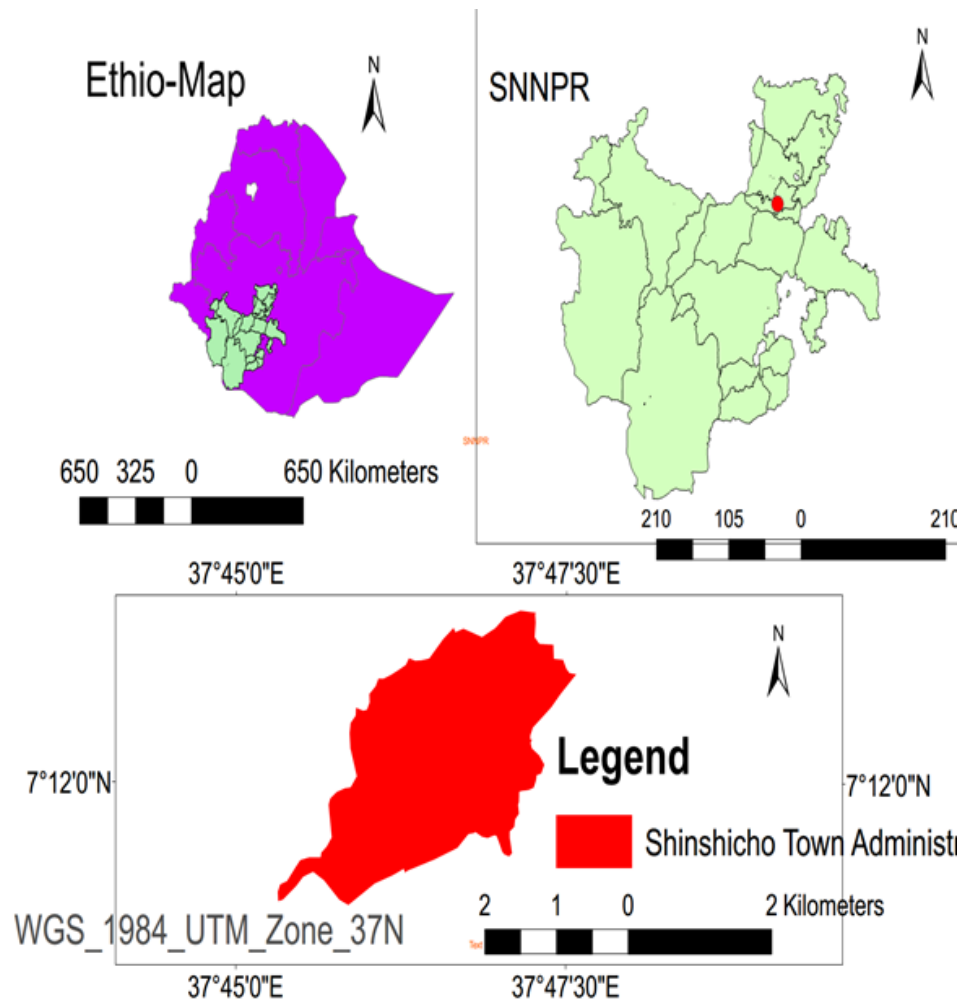
Figure 1. Location map of the study area. Source: (Ethiopia GIS Data Base 1994)

Study design

A cross-sectional study design was conducted between December 2018 and September 2019 in Shinshicho town.

Study animals

The study animals were lactating dairy cows in Shinshicho town (Holstein-Friesian, Jersey, and Zebu cross breeds) in the selected smallholder dairy farms kept under intensive management system. The animals were kept indoor and received concentrated feed in addition to hay and crop residues (such as corn stalks, wheat/barley straw, and other leftovers from grain threshing. The age of the study animals was determined based on birth records, and dentation characteristics and categorized as young (< 4 years), adult (4 to 8 years), and old (>8 years) as described by Torell *et al.* (2003). Parity numbers were categorized as low (1 and 2 calves), moderate (3 and 4 calves), and many (>4 calves). The lactation stage was grouped as early (≤2 months), medium (>2 to 6 months), and late (>6 months). The floor system of the dairy house was classified as cemented and soil or mud types (Tassew *et al.*, 2016). The hygienic condition of milking is arranged as good and poor depending upon washing and drying the udder of lactating cows before milking. Previous treatment for mammary gland infection was rated yes or no (Abera *et al.*, 2010).

Sample size determination and sampling procedures

The sample size of the study was determined as described by Thrusfield (2018), with a 95% confidence interval and 5% desired absolute precision. Since there was no previous study conducted in the area, the expected prevalence of 50% was assumed and this corresponds to the required minimum sample size by the formula given as follows.

$$n = \frac{1.96^2 pexp\,(1\text{-}pexp)}{d^2}$$

Where: -n=required sample size;
Pexp =expected prevalence;
d2 = desired absolute precision

Therefore, the calculated sample size was 384 lactating dairy cows.
According to Mekonnen *et al.* (2018), the sampling frame of the study area indicated that the farms were smallholder dairy farms having an average of two to three lactating cows each. About 250 smallholder dairy farms with total dairy cows of 674 (199 Holstein-Friesian, 255 Jersey, and 220 Zebu-cross breed) were registered in the Shinshicho town administrative agricultural office. Thus, 146 smallholder dairy farms having a minimum of two and a maximum of five lactating cows were purposely selected based on the availability of lactating dairy cows and the willingness of the owners to participate in the study. Then

focusing on the sampling frame in the smallholder dairy farm, each breed of lactating cow was sampled by simple random sampling techniques using a lottery method. Accordingly, 152 Holstein-Friesian, 117 Jersey, and 115 Zebu-cross cows were sampled.

Questionnaire survey

A semi-structured questionnaire was developed and the data based on the study objective was recorded. Data regarding the different potential risk factors were assessed for each of the 384 lactating cows based on observation and by interviewing the farm owners. The cow's level factors such as breed (Holstein Friesian, Jersey, or Zebu-cross), age (young, adult, or old), parity (few, moderate, or many), lactation stage (early, medium, or late), and pregnancy (pregnant or non-pregnant) were recorded. The farm-level factors such as herd size (≤5 animals or >5 animals), floor types (soil or concreted); previous mastitis infection (present or absent), dry cow therapy (yes or no), and use of the towel (yes or no) were recorded. The hygiene level of milking (good or poor) was assessed by evaluating the hygiene of the udder, which was scored separately according to the methodology outlined by Schreiner and Ruegg (2002). Udder and milk abnormalities (injuries, swelling, milk clots, and abnormal secretion) were also recorded.

Clinical examination and California Mastitis Test (CMT)

Each selected lactating cow was screened for mastitis based on clinical examinations and the California Mastitis Test. Clinical examination of the udder was based on the method previously indicated (Radostits *et al*., 2007). The clinical findings considered include abnormalities of the secretion, abnormalities of the udder and teat, and systemic reaction. The California Mastitis Test was performed to detect subclinical mastitis according to the procedures described by the National Mastitis Council (NMC, 2004). Subclinical mastitis was screened by taking 2 mL of milk sample from each quarter placed into a plastic paddle by adding an equal amount of the CMT reagent to the milk. The paddle was rotated to mix the contents. Positive samples showed gel formation within a few seconds. The CMT result was scored as negative if there was no gel formation and recorded as zero (0) or positive if there was gel formation ranging from trace (T), weakly positive (+1), distinct positive (+2) and strongly positive (+3) as per the recommendation given by NMC (2004). Hence, the cow was considered subclinical mastitis positive if one or more quarters were CMT positive with or without isolation of the microorganisms (Quinn *et al*., 1994).

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Milk sample collection

The milk samples were taken from mastitis-positive cows that were not treated with antibiotics recently or currently, and collected according to the earlier protocol (Quinn *et al.*, 2004). Briefly: quarters were thoroughly washed with tap water and wiped dry. The teat ends were then cleaned with cotton soaked with 75% ethyl alcohol and 8-10ml of milk was collected aseptically into a sterile test tube after discarding the first three streams of milk. Finally, milk samples were transported in an icebox with icepacks to the Wolaita Sodo Regional Veterinary Laboratory for bacteriological examination. The milk samples were immediately inoculated in bacteriological media or kept at 4°C overnight until cultured in standard bacteriological media (Quinn *et al.*, 2004).

Bacteriological examination of milk samples

The milk samples were bacteriologically examined according to the procedures employed by Quinn *et al.* (2004). Mastitic positive quarters samples were inoculated separately onto sterile blood agar plates (Himedia, India) enriched with 5% heparinized sheep blood and incubated at 37°C for 24-48 hours under aerobic culture conditions. The plates were examined for the presence of *Staphylococcus* colonies. The identification of bacteria in primary culture as *Staphylococcus* was done according to the procedures described in Quinn *et al.*, 2004. When growth was not observed after incubation for 24 to 48 hours, the milk sample was re-inoculated with enriched tryptone soya broth (Himedia, India) to amplify the bacterial growth. The plates were examined for growth, colony morphology features such as golden, and yellow color, and β- hemolysis in blood agar within 24-48 hours. A presumptive colony of *Staphylococcus* was selected and subcultured on nutrient agar (Himedia, India) and incubated aerobically at 37°C for 24-48 hours to get a pure culture. After this incubation on nutrient agar, bacteria were identified according to their Gram reaction; morphology, catalase test, and oxidase test (Quinn *et al.,* 2002). *Staphylococcus aureus* was identified by the coagulase test, hemolysis, pigment production (golden yellow), Mannitol, and maltose fermentation (Quinn *et al.*, 2004). Samples were considered positive for *S. aureus* when at least one colony was identified as *S. aureus*.

Antimicrobial susceptibility testing

The *S. aureus* isolates were subjected to antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method on Mueller Hinton agar following

the procedures described by Quinn *et al.* (2011) and CLSI (2016). Antimicrobials of animal health significance were taken into consideration. The following antibiotics discs (Himedia, India) with their corresponding concentration were used for testing: Chloramphenicol (30µg), Gentamicin (10µg), Kanamycin (30µg), Streptomycin (10µg), Cephalothin (30µg), Polymyxin-B (PB 300units), Ampicillin (10µg), Amoxicillin (20µg), Penicillin-G (10 units) and Tetracycline (30µg). In brief: the isolate was inoculated in tryptone soya broth (TSB) and incubated at 37°C for 24 hours. The turbidity of the suspension was adjusted to obtain turbidity visually comparable with that of 0.5 McFarland standards. Muller-Hinton Agar plate (Oxoid, England) was prepared and a sterile cotton swab was dipped into the suspension and swabbed on the surface of the Muller-Hinton agar plate. Then, the antibiotic discs were placed on the agar plate using sterile forceps and pressed gently to ensure complete contact with the agar surface. The plates were read 24 hours after incubation at 37°C under aerobic conditions. The diameter of the zone of complete inhibition was measured in millimeters, interpreted, and classified according to procedures established by CLSI (2016) as susceptible, intermediate, or resistant. Moreover, isolates showing resistance to three or more antimicrobial subclasses were considered multiple drug-resistant (Rota *et al*., 1996).

Data management and statistical analysis

The data was recorded in the Microsoft Excel Spreadsheet 2010 and coded before statistical analysis. All the statistical analysis was performed using Stata 14 statistical software (Stata Corp, 4905 Lake Way Drive, and College Station, Texas 77845 USA). The prevalence of *S. aureus* was calculated as the proportion of *S. aureus*-positive cows (clinical and subclinical) divided into the total number of animals examined. A cow was denoted as positive for mastitis if at least a single teat with clinical mastitis or a CMT-positive result was detected. Accordingly, descriptive statistics such as percentages and frequency distribution were used to describe/present bacterial isolates and antimicrobial susceptibility which was expressed as a percent of susceptible and resistant. In addition, the proportions of bacteria resistant to three or more antimicrobial subclasses were calculated. The association between the dependent variable, *S. aureus* (0=negative and 1=positive), and categorical independent variables were assessed using multivariable logistic regression analysis. The independent variables evaluated were breed, age, parity, lactation stages, previous mastitis treatment, herd size, floor type, milking hygiene, previous infection, and use of a towel for udder cleaning and drying. All independent variables

with p-value <0.25 in the initial univariable logistic regression analysis were checked for multicollinearity using Goodman and Kruskal gamma statistics and those variables whose gamma value ranging between −0.6 and +0.6 were considered in a multivariable logistic regression analysis (Dohoo *et al.*, 2009). The final model was built on a backward elimination procedure of the log-likelihood ratio. Values were considered significant at p< 0.05.

Results

Prevalence of *S. aureus*

A total of 384 lactating cows were examined, and the prevalence of mastitis was 41.7% (160/384). Among them, 19 cases (5%) were clinical mastitis, and 141 cases (36.7%) were subclinical mastitis. Milk samples from 160 CMT-positive cows were subjected to microbiological examination, and *S. aureus* was isolated from 7 (36.84%), and 55 (39.01%) of the clinical and subclinical cases, respectively. The overall prevalence of *S. aureus* in this study was recorded to be 62 (38.75%). There was a statistically significant difference (p<0.05) in the prevalence of *S. aureus* between clinical and subclinical mastitis (Table 1).

Table1. Prevalence of *Staphylococcus aureus* **in sub-clinical and clinical mastitis**

Forms of mastitis	No. of animal's positive	No.of *S. aureus* isolated	Prevalence	χ^2	P-values
Subclinical	141	55	39.01		
Clinical	19	7	36.84	10300.57	≤ 0.01
Total	160	62	38.75		

Risk factors associated with the prevalence of *S. aureus* isolates

Among the risk factors examined in initial univariable logistic regression, age was withdrawn from further analysis in consideration of collinearity with parity but parity as a risk factor was retained, due to its important implications regarding mastitis based on biological plausibility. Also, the milking hygiene was eliminated from further analysis because of collinearity with floor types of husbandry systems. Likewise, previous mastitis was removed from further analysis due to collinearity with herd sizes, and the use of towels was excluded from further analysis because of collinearity with dry cow's therapy. Therefore, the variables considered for further analysis with multivariable logistic regression were parity, lactation stages, previous infection treatment, herd size, floor

type, and dry cow therapy. Thus, the results revealed that infection with *S. aureus* was more likely to occur in cows with higher parities than cows with few parities (OR=3.91, 95% CI: 1.73-8.82), and cows in the late lactation stage were more likely to be positive to *S. aureus* than cows in early lactation stage (OR=3.36, 95% CI: 1.27-8.91) (Table 2).

Table 2. Multivariate logistic regression analysis of cow's level associated risk factors with the prevalence of *S. aureus* isolates

Risk factors	Category	No. of exa mined	Prevalence of *S. aureus* (%)	Crude odds ratio (95%CI)	Adjusted odds ratio (95%CI)	P-values
Parity in calves	Few (1 and 2)	45	13 (28.9)	1	1	
	Moderate (3&4)	76	29 (38.2)	1.59 (0.79-3.20)	1.88 (0.90-3.94)	0.10
	Many (>4)	39	20 (51.3)	3.25 (1.50-7.04)	3.91(1.73-8.82)	0.00
lactation stage in months	Early (1 & 2)	26	8 (30.8)	1	1	
	Medium (3- 6)	103	40 (38.8)	2.53 (1,14-5.61)	2.65 (1.17-6.02)	0.02
	Late (>6)	31	14 (45.2)	3.00 (1.14-7.87)	3.36 (1.27-8.91)	0.01
previous infection treatment	Yes	79	11 (13.9)	1	1	
	No	81	51 (62.9)	1.56 (0.77-3.14)	1.90 (0.90-4.02)	0.09

1=Reference, CI=Confidence interval

The dairy farms with a larger herd size of more than 5 animals were more likely to be positive for *S. aureus* than small herd sizes less than or equal to 5 animals (OR=2.91, 95% CI: 1.62-5.21), and soil or muddy floor housing were more likely to be positive than cemented floor housing (OR=2.37, 95% CI: 1.31-4.27) (Table 3).

Table3. Multivariate logistic regression analysis of farms level associated risk factors with *S. aureus* isolates

Risk factors	Category	No. of Examined	Prevalence of *S. aureus* (%)	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)	P-values
Herd size	≤5animals	98	24(24.5)	1	1	
	>5animals	62	38(61.3)	2.81(1.61-4.91)	2.91(1.62-5.21)	0.00
Floor types	Concreted	104	34(32.7)	1	1	
	Soil	56	28(50)	2.45(1.40-4.28)	2.37(1.31-4.27)	0.00
Dry cow therapy	Yes	85	11(13)	1	1	
	No	75	51 (68)	0.26(0.13-0.52)	0.27(0.13-0.53)	0.00

1=Reference, CI=Confidence interval

Antimicrobial susceptibility testing

A total of 62 isolates of *S. aureus* were tested against ten antimicrobial agents following CLSI guidelines. From 62 isolates of *S. aureus* showed 95.2% susceptibility to Chloramphenicol, similarly susceptiblities to Gentamicin (91.9%), Cephalothin (90.3%), Kanamycin (88.7%) and Streptomycin (80.6%) were also recorded. However, 100% of the isolates were resistant to Penicillin-G, Polymyxin, Amoxicillin, and Ampicillin while resistance ot Tetracycline was 80.6% (Table 4).

Table4. Antimicrobial susceptibility patterns of *S. aureus* isolates (n = 62).

Antimicrobial agents	Inhibition zone diameter by millimeters																											
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	>29
AMX	0	0	33	6	7	4	5	3	1	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AMP	0	50	6	0	4	0	0	0	2	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0
CEP	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2	1	2	4	6	17	5	8	6	8	2	0	0	0
CHR	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	5	4	13	5	16	9	7	0	0	0
GEN	0	0	0	0	0	0	0	0	1	0	0	1	6	0	4	5	7	16	6	5	3	6	5	0	0	0	0	0
KAN	0	0	0	0	0	0	0	0	1	1	0	2	1	0	1	0	1	7	10	19	6	13	0	0	0	0	0	0
PG	0	62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PLY	0	62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STM	0	0	0	2	0	0	1	2	0	1	2	1	2	1	3	4	22	11	1	9	0	0	0	0	0	0	0	0
TTC	0	0	15	11	18	6	0	0	0	0	0	0	0	0	2	1	0	0	2	3	4	0	0	0	0	0	0	0

The black color shaded represents zone diameters in the intermediate and breakpoints range; the gray and light color shaded represent zone diameters in the resistant and susceptibility ranges respectively. AMX=Amoxicillin, AMP= Ampicillin, CEP= Cephalothin, CHR=Chloramphenicol, GEN= Gentamicin, KAN = Kanamycin PG=Penicillin, PLY=Polymyxin, STM=Streptomycin and TTC=Tetracycline.

Multiple antibiotic-resistant phenotypes of *S. aureus* isolates

Multiple antibiotic-resistant phenotypes were determined for the *S. aureus* isolates as depicted in Table 5. Out of the total *S. aureus* isolates recovered from the study area, 53.2 % of the isolates developed multiple antibiotic-resistant phenotypes. Among all multiple antibiotic-resistant phenotypes of *S. aureus* isolates, 24.2% of them were resistant to five or six antibiotics, and 6.4% of the isolates were resistant to seven or eight antibiotics. The percentages of the resistant phenotype were calculated by dividing the number of particular antimicrobial-resistant phenotypes by the total number of isolates identified in the study area.

Table 5. The predominant MAR phenotypes for *S. aureus* isolated from mastitic milk (N=62)

Phenotype	Number observed	Percentage
AMP-PG-PLY	3	4.8
AMX-PG-PLY	2	3.2
AMP-AMX-PG-PLY	6	9.7
AMX-PG-PLY-TTC	3	4.8
AMP-AMX-PG-PLY-TTC	9	14.5
AMP-AMX-KAN-PG-PLY	2	3.2
AMP-AMX-PG-PLY-STM-TTC	3	4.8
AMP-AMX-CHR-PG-PLY-TTC	1	1.6
AMP-AMX-CEP-PG-PLY-STM-TTC	2	3.2
AMP-AMX-KANA-PG-PLY-STM-TTC	1	1.6
AMP-AMX-CEP-GEN-KAN-PG-PLY-TTC	1	1.6

AMP=Ampicillin, AMX=Amoxicillin, PG=Penicillin, PLY=Polymyxin, CEP=Cephalothin, CHR=Chloramphenicol, GEN=Gentamicin, KAN=Kanamycin, TTC=Tetracycline and STM=Streptomycin.

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Discussion

The present study showed a 38.75% overall prevalence of *S. aureus* in the mastitic cows in the study area, which is in agreement with the 39.1% prevalence reported from Asella, Oromia Regional state, Ethiopia (Bedada *et al.*, 2009), but lower when compared with the reports of Abera *et al.*, 2010; Kemal *et al.*, 2017; and Seyoum *et al.*, 2018 who reported 42.1% in Adama town and 44.62% and 46.5% *S. aureus* isolates in the town of Asella. The variable prevalence of *S. aureus* in cows with mastitis across different reports may be attributed to differences in farm management practices, environmental conditions, and awareness of disease transmission. *S. aureus* is a contagious pathogen that spreads from infected cows to healthy ones during unhygienic milking procedures and contact with animals (Radostits *et al.*, 2007).

Multivariable logistic regression analysis revealed that the prevalence of *S. aureus* isolates was significantly different among higher parity groups. A significant association of higher parity with the prevalence of *S. aureus* isolates was reported by other authors (Seyoum *et al.*, 2018) in and around Asella town Ethiopia. In the present study, cows with higher parity had 3.91 times more risk of infction with *S.aureus* as compared to the cows with low parity. This might be due to the increased opportunity of infection with time and the prolonged duration of infection (Schukken *et al.*, 2011). Higher parity, or the number of times a cow has calved, is inversely associated with cure rates, possibly due to the mammary gland's large volume of specialized secretory cells and supporting tissues, which hinder antibiotic penetration and infection clearance. (Macias and Hinck, 2012).

The current result revealed that cows in late lactation stages were 3.36 times more likely infected as compared with early stages. This result was found consistent with the previous reports of Nibret *et al.* (2012), and Garedew *et al.* (2015) in Hawassa and Addis Ababa towns, respectively. Nevertheless, the report from the Southern part of Ethiopia indicates a higher prevalence in the early stage of lactation which disagrees with this study's results (Kerro and Tareke, 2003; Biffa *et al.,* 2005). The current result might be because chronic mastitis, most often subclinical, is more frequent later during lactation. *S. aureus* is a predominant cause of subclinical mastitis (Radostits *et al.*, 2007).

A significantly higher risk (p<0.05) was observed in the herd size of more than 5 animals (OR=2.91, 95% CI: 1.62, 5.21), than in small herd sizes of less than or equal to 5 animals. This result agreed with the finding of Kemal *et al*. (2017) who reported higher risk in the larger herd size from Asella town, Ethiopia. This is due to *S. aureus* having adapted to survive in the udder; known by their contagious nature and are shed in the milk which serves as a source of infection for other healthy cows during the milking process. It is generally observed that larger herds are characterized by increased stocking density and increased risk of exposure to infection (Radostits *et al*., 2007).

The present study revealed that dairy farms with muddy floors were 2.37 times more likely to be infected with *S. aureus* than cemented floor housing systems. The association between soil floor, and the high prevalence of *S. aureus* revealed in this finding agreed with the result of Kemal *et al*. (2017) who reported from Asella town, Arisi Zone, Eastern Ethiopia. The association can be attributed to poor sanitation practices and the housing of cows in dirty and muddy common barns with bedding materials that promote the survival and transmission of mastitis pathogens.

In vitro, antimicrobial susceptibility tests of *S. aureus* isolates revealed that the highest rate of susceptibility among the isolates was recorded against Chloramphenicol, Gentamicin, Cephalothin, Kanamycin, and Streptomycin. The results agree with the finding of Abera *et al*.(2010) who reported susceptibility to *S. aureus* to Chloramphenicol, Gentamicin, Kanamycin, and Streptomycin, Thaker *et al*. (2013) who reported the isolates sensitive to Cephalothin, and Gentamicin, Kemal *et al*. (2017) who reported the isolates susceptible to Chloramphenicol and Gentamicin, and Beyene (2016) who reported the *S. aureus* susceptible to Chloramphenicol Streptomycin and Gentamicin.

On the other hand, *S. aureus* isolates showed the highest resistance to Penicillin-G (100%), Polymyxin-B (100%), Amoxicillin (100%), Ampicillin (100%), and Tetracycline (80.6%). The current investigation was in agreement with the report of Tsige (2018) who reported the resistance of *S. aureus* to Penicillin (100%), Ampicillin (100%), Amoxicillin-clavulanic (82.7%), and Tetracycline (60.49%) in Arsi Negelle, Ethiopia. Moreover, the present report was comparable with the result of Lencho (2015) in Ambo and Gudar town recording 100% for both Penicillin and Ampicillin followed by 90% for Amoxicillin. This is supported by the findings of Tassew *et al*. (2016) from Kombolcha who reported *S. aureus* resistant to penicillin (100%), amoxicillin (100%), and tetracycline

(77.4%). Similarly, *S. aureus* resistance to penicillin (100%), and amoxicillin (100%) were reported from Bangladesh (Jahan *et al.*, 2015). The variability in susceptibility results could partly arise from how frequently a drug was in use for dairy cow treatments in the study area.

The resistance of *S. aureus* isolates to penicillin-G may be attributed to the production of beta-lactamase enzyme that inactivates penicillin and closely related antibiotics (Huber *et al.*, 2011). Resistant to penicillin-G is used as a marker to assess the susceptibility of *S.aureus* isolates against other ß -lactam antibiotics (Waage *et al.*, 2002; Pace *et al.*, 2006). In the study area, penicillin was the drug of choice for the therapy of intramammary infections, such that frequent and often inadequate use of these medications has probably contributed to the emergence of resistant bacteria in the herds. A similar finding was reported by Kemal *et al.* (2017) that the resistance of *S. aureus* to penicillin and ampicillin may be attributed to the production of beta-lactamase, an enzyme that inactivates penicillin and closely related antibiotics. It is believed that around 50% of mastitis-causing *S. aureus* strains produce beta-lactamase and there is evidence that these strains are more difficult to cure with all antibiotics (Green and Bradley, 2004).

The current study has demonstrated the existence of alarming levels of resistance to *S. aureus* to commonly used antimicrobials (including penicillin-G, amoxicillin, and tetracycline) in the study farms. Reports from Argentina suggested a possible development of resistance from prolonged and indiscriminate usage of the same antimicrobial (Gentilini, 2000; Edward *et al.*, 2002). Therefore, it is important to implement a regular application of an in-vitro antimicrobial susceptibility test before the use of antimicrobials in both therapeutic and prophylactic Staphylococcal mastitis infections. Antibiotic-resistant *S. aureus* isolates pose a challenge to both animal and public health (Kashoma *et al.*, 2015)

Multiple antibiotic-resistant in this study was relatively high. Thus, 53.2 % of the isolates develop multiple antibiotic-resistant phenotypes. This result was in line with the findings of Mekuria *et al.* (2013) who reported from Addis Ababa, 50.6%, and Kashoma *et al.* (2015) who reported 43.2% from Tanzania. This result was higher than the finding of Mohammed (2015) who reported 26.09% of multiple drug-resistant *S. aureus* isolated from cow milk in Tanzania. This might be due to the variation in the type and frequency of use of these antibiotics for the treatment and prevention of prevailing bacterial diseases

including mastitis. Multiple antibiotic-resistant *S. aureus* strains have been isolated from milk obtained from dairy animals in many parts of the world (Waage *et al.*, 2002; Shitandi and Sternesjo, 2004; Ateba et *al.*, 2010). The prevalence of antibiotic resistance in *S. aureus* usually varies between isolates from different sample stations and even between isolates from different herds and/or flocks of the same farm (Pace and Guang, 2006). Besides, *S. aureus* has developed multidrug resistance in many regions of the world and the usage of antibiotics correlates with the emergence of antibiotic-resistant traits (Pesavento *et al.*, 2007).

Conclusions

The present study revealed that multiple antibiotics-resistant *S. aureus* isolates are prevalent in dairy farms in the study area. Parity, lactation stages, herd size, and floor type were risk factors significantly related to *S. aureus* prevalence. It was observed that *S. aureus* isolates were highly sensitive to Chloramphenicol, Gentamicin, Cephalothin, Kanamycin, and Streptomycin. Whereas, the highest rate of resistance among the isolates was against Penicillin-G, Amoxicillin, Ampicillin, and Tetracycline. *Staphylococcus aureus* from mastitic cows showed multiple antibiotic resistance to a great extent to commonly used antibiotics, ensuring that the right use of antibiotics of choice is very important in the line of treatment and control of the infections caused by *S. aureus*. Hence, regular antimicrobial sensitivity tests to select effective and alteration of antibiotics must be carried out, and the impacts and dynamics of genetic antibiotic determinants should also be investigated using molecular methods.

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Disclosure

The authors report there is no conflict of interest.

Author contributions

Conceptualization, A.M., F.B., Y.A. T.T.A.M., F.B., Y.A. T.T.; analysis, A.M., F.B.; investigation and Lab analysis, A.M.; writing—original draft preparation, A.M.; writing—review and editing, A.M., F.B., Y.A. T.T. All authors have read and agreed to the published version of the manuscript.

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