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Full Length Research Paper

Prevalence, risk factors and multidrug resistance profile of *Staphylococcus aureus* isolated from bovine mastitis in selected dairy farms in and around Asella town, Arsi Zone, South Eastern Ethiopia

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A cross-sectional study was carried out from November 2012 - May 2013 to estimate the prevalence of multi drug resistance *Staphylococcus aureus* from bovine mastitis and to assess its associated risk factors in selected dairy farms in and around Asella town, Arsi Zone, Ethiopia. A total of 384 fresh raw milk samples from dairy cows were examined for mastitis. Milk samples collected from 251 mastitic lactating cows were subjected to bacteriological examinations for isolation and identification of *S. aureus*. *S. aureus* isolates were identified from 44.62% (112/251) mastitic milk samples. Higher prevalence rates of *S. aureus* isolates were recorded in subclinical mastitis (45.78%, 103/225) compared to clinical cases (34.62%, 9/26), however, no statistically significant difference (p > 0.05) in prevalence of *S. aureus* between subclinical mastitis and clinical cases. Multivariate logistic regression analysis of the effect of different risk factors on the prevalence of *S. aureus* revealed that; cross breed (OR = 2.501, 95%CI: 1.173 - 5.334), late stage of lactation (OR = 4.260, 95%CI: 1.9309.402), previous mastitis record (OR = 2.553, 95%CI: 1.3324.894), large sized herd (OR = 15.824, 95%CI: 6.36839.320) and poor udder hygiene (OR = 2.040, 95%CI: 1.184,3.514) were more likely to be infected with antimicrobial resistance *S. aureus*. All the isolates of *S. aureus* were subjected to antimicrobial susceptibility testing. The highest rate of susceptibility was to chloramphenicol (97.5%) followed by gentamycin (95.3%), vancomycin (92.7%), and clindamycin (90.9%). Whereas, the highest rate of resistance among the isolates was against penicillin G (87.3%) followed by tetracycline (82.2%), trimethoprim-sulfamethoxazole (69.1%), oxacillin (56.4%), ampicillin (55.1%) and cefoxitin (58.1%). The results of the present study reveal that 65.18% of the isolates were found to be multiple antibiotic resistance phenotypes. Regular antimicrobial sensitivity testing and best practices for achieving hygienic milking should be established.

Key words: Milk, bovine mastitis, multidrug resistance, prevalence, risk factors, *Staphylococcus aureus, Assella.*

INTRODUCTION

Staphylococcus aureus is a truly diverse pathogen which is capable of causing a wide variety of illnesses in both humans and animals (Jensen and Lyon, 2009). It is a common cause of mastitis in dairy cows (Virgin et al., 2009; Sharma et al., 2015); a primary reason for antibiotic use on farms (Groot and van't Hooft, 2016). Aside from causing bovine mastitis, *S. aureus* may be implicated in mastitis in humans, wound infections, toxic shock syndrome, bacteremia, scalded skin syndrome, osteomyelitis and meningitis, among other syndromes (Kloos et al., 1995; Lowy, 1998). *S. aureus* is also a major cause of non-fatal food poisoning due to the production of highly stable extra cellular enterotoxins, which are powerful emetics (Genigeorgis, 1989; Leloir et al., 2003).

The emergence of pathogenic microorganisms resistant to commonly used antibiotics is a worldwide concern of the 21st century. Antibiotic resistance seems to be increasing, and multiple antibiotic resistant strains have started to emerge (Otter and French, 2010). Infections with antibiotic resistant bacteria have been known to be associated with frequent treatment failure and increased severity of the disease (Finch and Hunter, 2006; Swartz, 2004; Angulo et al., 2004). One of the most important bacteria in this regard is *S. aureus*, in particular its methicillin-resistant strains (Cosgrove et al., 2003; von Eiffet al., 2001). Both human and non-human antimicrobial usage may result in increased occurrence of bacterial resistance (Anderson et al., 2003). The problem with *S. aureus* became more complicated when it was found that it quickly developed resistance and was capable of producing many antibiotic resistant strains (Farzana et al., 2004; Cookson and Phillips, 1998). Development of resistance has been attributed to the indiscriminate and extensive use of antibiotics for therapeutic or as growth promoters in food animal production (Normanno, 2005). Isolates of *S. aureus* are frequently resistant to β-lactam antibiotics (Deurenberg and Stobberingh, 2008). The resistance to β-lactam antibiotics to *S. aureus* is mediated by the mecA gene which resides on a staphylococcal chromosomal cassette (SCCmec) that encodes a modified penicillin-binding protein (PBP), the PBP2a or 2', which shows reduced affinity to penicillins, such as methicillin and oxacillin and for all other β-lactam antibiotics (Kwon et al., 2006; Pantosti et al., 2007).

The description "methicillin-resistant *S. aureus* (MRSA)" was first used in 1961, based on the discovery of a human *S. aureus* infection in the United Kingdom that was resistant to methicillin (Fitzgerald et al., 2001). Since that time, MRSA has emerged as a significant problem worldwide, and the term has evolved to include resistance to additional ß-lactam antibiotics. Currently, the term MRSA is often used to describe multi-drug resistant *S. aureus* (Leclercq, 2002; Garipcin and Seker (2015).

The first case of MRSA in veterinary species was identified in the milk of a cow with mastitis (probably of human origin) in 1972 (Duquette and Nuttall, 2004; O'Mahony et al., 2005). *S. aureus* is a significant cause of mastitis in cows and small ruminants (Vanderhaeghen et al., 2010; Unal et al., 2012). In dairy cows, *S. aureus* is a common bacterial cause of mastitis and MRSA is known to cause mastitis as well. Antimicrobial resistance has been detected in *S. aureus* isolates collected from bovine intramammary infection (IMI) at frequencies which vary widely by compound and region sampled. A seven-year survey of Michigan dairy herds found that 49.6% of the *S. aureus* isolates tested were resistant to penicillin and ampicillin, but that resistance to other compounds such as tetracycline, pirlimycin, erythromycin and oxacillin was low, ranging from 8.5% down to less than 1% (Erskine et al., 2002). *S. aureus*-related bovine mastitis is a common reason for therapeutic and/or prophylactic use of antibiotics on dairy farms (Vanderhaeghen et al., 2010; Kumar et al., 2010).

Methicillin resistant *S. aureus* is emerging as a zoonotic and veterinary bacterial pathogen of public health importance (Smith et al., 2009; Springer et al., 2009). It is a zoonosis that has garnered the attention of scientists and the public in recent years. Several reports suggest that animals may serve as reservoirs for Methicillin Resistant *S. aureus (*MRSA) infection of humans (Loeffler and Lloyd, 2010). The advent of antibiotic-resistant staphylococci poses additional potential food safety and occupational health concerns. Resistance genes of *S. aureus* can disseminate from animals to humans by direct contact or through the food chain (Kluytmans, 2010; Loeffler and Lloyd, 2010; Cuny et al., 2015).

The indiscriminate and intensive use of antibiotics in veterinary medicine might be associated risk factors attributed to the increasing occurrence of antibiotic resistant strains of *S. aureus* in cows with mastitis (Hawkey, 2003). Current management practices employed for milk production and individual cow factors might be other contributing factors associated with the dissemination of antibiotic-resistant bacterial strains (Acar and Moulin, 2006). The *staphylococci* have adapted to survive in the udder; they usually establish chronic, subclinical, infection and are shed in the milk which serves as a source of infection for other health cows during the milking process (Radostits et al., 2007). The

main source of infection for *S. aureus* mastitis is the udder of infected cows which is transferred via milker's hands, utensils, towels and the environment (floor) in which the cows are kept (Radostits et al., 2007).

Antibiotics on dairy operations are used to treat highly prevalent infections, such as subclinical mastitis, and as a preventive measure during dry cow therapy (Zadoks et al., 2002). Monitoring the emergence of resistant pathogens in animal reservoirs is important particularly for those with zoonotic potential (Normanno et al., 2005). There is a lot of interest in recent years in bovine *S. aureus* resistance to multiple antibiotics because of comparison with MRSA (multiple/methicillin resistant *S. aureus* infections) in humans, including nosocomial infections in hospitals and nursing homes all over the world (Van Loo et al., 2007).

A number of reports indicated that multi drug resistant *S. aureus* is the predominant organisms isolated from bovine mastitis. Several studies have been conducted worldwide (Levy, 1998; Green and Bradley, 2004; Kumar et al., 2010; Wang et al., 2013) to investigate the prevalence of *S. aureus* in milk.

Recently, there have been several studies conducted on multidrug resistance profile of *S. aureus* isolated from bovine mastitis in various parts of Ethiopia (Sori et al., 2011; Daka et al., 2012; Abebe et al., 2013; Tassew et al., 2016). However, prevalence, risk factors and multidrug resistance profile of *S. aureus* isolated from bovine mastitis has been insufficiently investigated in the study area. Moreover, to date there is no published data on its status, magnitude and distribution in Arsi zone in general and in and around Asella town in particular. Therefore, the objective of this study was: (i) to isolate and identify *S. aureus* and establish its prevalence; (ii) to determine multi drug resistance profile of *S. aureus* isolates from mastitic cows' milk, and (iii) to assess potential risk factors associated with the prevalence of *S. aureus*.

MATERIALS AND METHODS

Study area

The study was conducted in and around Asella town of Arsi zone, Oromia Regional State, South Eastern Ethiopia. Asella is located at a distance of 175km south east of Addis Ababa at 7°57'N and 39°7'E with an altitude of 502 to 4130 m above sea level. The annual rainfall of the study area ranges from 200 to 400 mm with mean annual temperature of 22.5°C. Agricultural production system of the study area is mixed crop and livestock farming. Dairy farming using improved breeds is a common practice in and around Asella town. The study area is known by the abundance of dairy farms that constituted the known milk sheds (Land O'Lakes, 2010).

Study animals

The study animals were lactating dairy cows in and around Asella town. The breeds of animals were local zebu and zebu crossbred with Holstein-Friesian. The status of multidrug resistance profile of *S. aureus* isolated from bovine mastitis in and around Asella district was unknown since no study had been conducted in the two districts before. The animals included in the study consisted of 384 lactating dairy cows, 86 (22.4%) indigenous zebu and 298 (77.8%) Holstien-zebu crosses (proportional allocation), selected by simple randomly from dairy farms in the study area. The farms were categorized in to large (> 10 dairy cattle), medium (5-10 dairy cattle) and small (<5 dairy cattle) according to the guideline of ILRI (1996).

Study design

A cross sectional type of study supported by laboratory tests was carried out to determine multi drug resistance profile of *S. aureus* isolates from mastitic cows' milk, to isolate and identify methicillin resistant *S. aureus* (MRSA) and to assess potential risk factors associated with the disease from October 2012 to May 2013 on dairy cows in and around Assella, Arsi Zone, South Eastern Ethiopia. Relevant individual animal biodata and farm level information were collected using a semi-structured questionnaire.

Sampling method and determination of sample size

The sampling was undertaken using a two level approach, choosing primarily individual farms with mastitis history and then sampling randomly individual cows from each farm. Greater proportions of cows (62%) were sampled from smallholder farms (small herd size) while the remaining (38%) were from medium and large herd size in and around the study district.

Since there was no reasonable research done in this area so far; the sample size was calculated by the formula recommended by Thrusfield (2007), with 95% confidence interval, at 5% desired absolute precision and expected prevalence of 50%. Hence, the total numbers of sample required for this study was 384 lactating dairy cows. Proportionality of incorporating cows in the sample was applied as per the population size of each herd.

Questionnaire survey of risk factors

Data was collected using a semi-structured questionnaire. The questionnaire was administered with the primary objective of elucidating the multifactorial background of mastitis. Data collected include intrinsic factors such as age, breed, parity, stage of lactation, previous history of mastitis and body condition. Extrinsic factors such as dry cow therapy, herd size, udder hygiene and floor type were also recorded.

Sample collection and bacteriological examination *Collection of milk samples*

Milk samples were collected according to the National Mastitis Council, NMC (2004). Firstly, the quarters were thoroughly washed with clean water and wiped dry. Teats were then disinfected with 70% ethyl alcohol. Approximately 10 ml of raw milk was then collected aseptically from clinical and subclinical (CMT positive) mastitic cows into sterile universal bottles after discarding the first three milking streams. The samples were transported under cold chain to Asella regional diagnostic laboratory. The samples were then stored in ice at 4°C until cultured on standard bacteriological media (Quinn et al., 2004).

Clinical examination and 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 according to previously established method (Quinn et al., 2004). It is used to determine the prevalence of sub-clinical mastitis and also as screening test for selection of samples to be cultured for the cows under study. A small milk sample (approximately ½ teaspoon) from each quarter was collected in to a plastic paddle that has 4 shallow cups marked A, B, C and D. An equal amount of California Mastitis Test reagent was added to the milk. The paddle was rotated to mix the contents. The CMT result was interpreted as negative (0), trace (T), weak positive (+1), Distinct positive (+2) and strong positive (+3) as per the recommendation given by Quinn et al. (2004).

Bacterial isolation and identification

The collected raw milk samples were cultured on 5% sheep blood and Mackonkey agar (Oxoid, UK) and the plates were incubated aerobically at 37°C and examined after 24 h of incubation for growth of bacterial colonies. The colonies were conditionally isolated based on Gram's stain reaction, cellular morphology, colony morphology, pigmentation and hemolytic feature on blood agar and other environment from which the bacterium were isolated. The representative colonies were sub cultured on nutrient agar (Oxoid, UK) and incubated at 37°C for 24 h (to obtain pure isolates for further identification). The isolated colonies from nutrient agar were subjected to catalase test, slide or tube coagulase test. Manitol salt agar, Purple Agar Base media plate with 1% of maltose and Voges Proskauer tests were done on the coagulase positive Staphylococci to identify *S. aureus* (Quinn et al., 2004; NMC, 2004). All of the bacterial isolates were cryopreserved in brain heart infusion broth (BHI, Becton, Dickinson and Company, Sparks, MD, USA) with 20% glycerol at -20°C for further analyses.

Antimicrobial susceptibility testing

The *S. aureus* isolates were subjected to antimicrobial susceptibility testing by Kirby-Bauer disc diffusion method (Carter and Chengappa, 1991; Quinn et al., 2004; CLSI, 2008). Antimicrobials of animal and human health significance were taken into consideration. Antimicrobial agents from various classes were employed. The following antibiotics (Oxoid, Hampshire, England) were used for testing: Ampicillin (10 μg), vancomycin (30 μg), gentamycin (10 μg), erythromycin (15 μg), clindamycin (10 μg), tetracycline (30 μg), oxacillin (1 ug), amoxacillin (25 μg), chloramphenicol (30 μg), trimethoprim-sulfamethoxazole (25 μg), cefoxitin (30 μg), and penicillin G (10 μg). In brief, the isolates were inoculated in tryptone soya broth (TSB) and incubated at 37°C for 24 h. The turbidity of the suspension was adjusted to obtain turbidity visually comparable with that of 0.5 McFarland standards. Muller-Hinton Agar (MHA) plate was prepared and a sterile cotton swab was dipped into the suspension and swabbed on the surfaces of Muller-Hinton Agar plate. Then, the antibiotic discs were placed on the agar plate using sterile forceps and pressed gently to ensure the complete contact with the agar surface. The plates were read 24 h after incubation at 37°C under aerobic condition. The inhibition zones of antimicrobial discs were recorded in millimeters, interpreted and classified according to procedures established by CLSI (2008) as susceptible, intermediate or resistance. Intermediate results were regarded as resistant (Huber et al., 2011). Multiple antibiotic resistant (MAR) phenotypes were documented for isolates revealing resistance to three and more antimicrobials (Rota et al., 1996).

Data analysis

All data from laboratory investigations and questionnaire survey were entered into computer using Microsoft Excel and transferred to STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA) for analysis. Prevalence was calculated as a percentage value. The association between the explanatory and response variables was analyzed using the Chi-square test ($\chi 2$). Multivariate logistic regression analyses were used to analyze the effects of different potential risk factors on the prevalence of *S. aureus* mastitis. The independent or explanatory variables considered in the model were those that showed statistical significance (<0.2). Odds ratio (OR) was used to evaluate the degree of association between putative risk factors with prevalence of *S. aureus* mastitis. The 95% confidence interval and a p-value < 0.05 was considered statistically significant.

RESULTS

A total of 384 lactating dairy cows with either clinical or subclinical (CMT positive) mastitis were examined for the involvement of *S. aureus*. The overall prevalence of mastitis was 65.36% (251/384). Out of 251 mastitis positive cows, 6.78% (26/384) and 58.59% (225/251) were found to be clinical and subclinical mastitis, respectively. *S. aureus* was isolated from 34.62% (9/26) and 45.78% (103/225) of the clinical and sub-clinical cases, respectively. The overall prevalence of *S. aureus* was 44.62% (112/251). There was no statistically significant difference ($p > 0.05$) in prevalence of *S. aureus* between clinical and subclinical mastitis as indicated in Table 1.

A Chi-square analysis revealed that prevalence of *S. aureus* isolates was significantly associated with the age groups, breed and parity ($p<0.05$); stage of lactation ($P<0.001$), mastitis record ($P<0.001$), herd size ($p<0.001$), udder hygiene ($P<0.01$) and floor type ($p<0.05$) (Table 2).

Multivariate logistic regression analysis of the effect of different risk factors on the prevalence of *S. aureus* is presented in Table 3. Hence, multivariate analysis revealed that; cross breed (OR = 2.501, 95%CI: 1.173, 5.334), late stage of lactation (OR = 4.260, 95%CI: 1.930, 9.402), previous mastitis record (OR = 2.553, 95%CI: 1.332, 4.894), large sized herd (OR = 15.824, 95%CI: 6.368, 39.320) and poor udder hygiene (OR = 2.040, 95%CI: 1.184, 3.514) were more likely to be infected with *S. aureus.*

All the isolates of *S. aureus* were tested for antimicrobial susceptibility as illustrated in Table 4. Of the entire antibiotics used in this study, the highest rate of susceptibility was to chloramphenicol (97.5%) followed by gentamycin (95.3%), vancomycin (92.7%), and clindamycin (90.9%). Whereas, the highest rate of resistance among the isolates was against penicillin G (87.3%) followed by tetracycline (82.2%), trimethoprim-sulfamethoxazole (69.1%), oxacillin (56.4%), ampicillin (55.1%), and cefoxitin (58.1%). From the total isolates tested, 55.5% were susceptible, 4.7% intermediate and 39.8% resistance to antimicrobials discs.

Multiple antibiotic resistance phenotypes were determined for the *S. aureus* isolates as depicted in Table 5.

Table 1. *Staphylococcus aureus* isolated by the mastitis type.

Form of mastitis	No. positive samples	*S. aureus* (%)	P value
Clinical	26	9 (34.62)	0.527
Subclinical	225	103 (45.75)	
Overall	251	112 (44.62)	

Table 2. Chi-square analysis of intrinsic and managemental factors with prevalence of *S. aureus*.

Factor	Category	No. examined	No. positive	Prevalence (%)	P value
Age (years)	≤ 5	108	22	20.37	0.018
	> 5	276	90	32.61	
Breed	Local	86	14	16.28	0.003
	Cross	298	98	32.89	
Parity	Primiparous	55	9	16.36	0.024
	Multiparous	329	103	31.31	
Stage of lactation	Early (< 3months)	75	13	17.33	0.000
	Mid (3-5 months)	203	43	21.18	
	Late (> 5 months)	106	56	52.83	
Mastitis record	No	312	74	23.72	0.000
	Yes	72	38	52.78	
Herd size	Small	238	41	17.23	0.000
	Medium	104	39	37.50	
	Large	42	32	76.19	
Udder hygiene	Poor	156	57	36.53	0.009
	Good	228	55	24.12	
Floor type	Soil	258	84	32.55	0.036
	Concrete	126	28	22.22	

Table 3. Multivariate logistic regression analysis of associated risk factors with prevalence of *S. aureus* isolates.

Factor	Category	Positive (%)	COR (95%CI)	AOR (95%CI)	P value
Age (years)	≤ 5	22 (20.37)	1	1	0.127
	> 5	90 (32.61)	1.891 (1.112, 3.219)	1.848 (0.839, 4.071)	
Breed	Local	14 (16.28)	1	1	0.018
	Cross	98 (32.89)	2.520 (1.354, 4.691)	2.501 (1.173, 5.334)	
Parity	Primiparous	9 (16.36)	1	1	0.237
	Multiparous	103 (31.31)	2.329 (1.099, 4.938)	1.794 (0.682, 4.721)	
Stage of lactation	Early (< 3 months)	13 (17.33)	1	1	0.000
	Mid (3-5 months)	43 (21.18)	1.282 (0.645, 2.546)	1.013 (0.471, 2.182)	
	Late (> 5 months)	56 (52.83)	5.342 (2.628, 10.855)	4.260 (1.930, 9.402)	
Mastitis record	No	74 (23.72)	1	1	0.005
	Yes	38 (52.78)	3.595 (2.113, 6.114)	2.553 (1.332, 4.894)	
Herd size	Small	41 (17.23)	1	1	0.000
	Medium	39 (37.50)	2.883 (1.713, 4.851)	2.786 (1.568, 4.951)	
	Large	32 (76.19)	15.376 (7.008, 33.735)	15.824 (6.368, 39.320)	
Udder hygiene	Good	55 (24.12)	1	1	0.01
	Poor	57 (36.53)	1.811 (1.160, 2.827)	2.040 (1.184, 3.514)	
Floor type	Concrete	28 (22.22)	1	1	0.655
	Soil	84 (32.55)	1.690 (1.031, 2.770)	1.174 (0.581, 2.370)	

COR, Crude odds ratio; AOR, Adjusted odds ratio; CI, Confidence interval; 1, Reference.

Table 4. Antimicrobial resistance profiles of *S. aureus* isolated from mastitic milk (N = 112).

Antibiotics tested	Susceptible (%)	Intermediate (%)	Resistance (%)
Ampicillin	36.4	8.5	55.1
Vancomycin	92.7	-	7.3
Gentamycin	95.3	4.7	-
Erythromycin	69.5	12.7	17.8
Clindamycin	90.9	7.3	1.8
Tetracycline	16.8	-	83.2
Oxacillin	43.6	-	56.4
Amoxicillin	40.2	6.5	53.3
Chloramphenicol	97.5	2.5	-
Streptomycin	65.9	6.4	27.7
Trimethoprim-sulfamethoxazole	21.6	9.3	69.1
Cefoxitin	40.7	1.2	58.1
Penicillin G	10.9	1.8	87.3

Table 5. The predominant MAR phenotypes for *S. aureus* isolated from mastitic milk (N=112).

MDR pattern	Phenotypes	Number observed	Percentage
Three	PG-TE-STR	4	3.57
	PG-TE-Ox	21	18.75
Four	PG-TE-TMX-Ox	7	6.25
	PG-TE-CXT-Ox	6	5.36
Five	PG-TE-TMX-CXT-Ox	8	7.14
Six	PG-TE-TMX-CXT-AP-Ox	17	15.18
	PG-TE-TMX-CXT-AML-Ox	5	4.46
Seven	PG-TE-TMX-CXT-AP-Ox-E	2	1.79
Eight	PG-TE-TMX-CXT-AP-STR-Ox-VA	2	1.79
	PG-TE-TMX-CXT-AML-STR-Ox-DA	1	0.9

The predominant multiple antibiotic resistance phenotypes for the isolates in the study area were PG-TE-Ox and PG-TE-TMX-CXT-AP-Ox in 18.75 and 15.18% of the isolates, respectively. It is thus evident that MAR *S. aureus* were isolated from mastitic milk sample. Out of the total *S. aureus* isolates recovered from the study district, 65.18% of the isolates develop multiple antibiotic resistance phenotypes. Among all MAR phenotypes of *S. aureus* isolates, 52.05% of them were resistant to three or four antibiotics and 41.10% were resistant to five or six antimicrobials. Furthermore, 6.85% of them were resistance to seven or eight antibiotics.

Percentages of the phenotypes were calculated by dividing the number of a particular MAR phenotypes by the total number of isolates identified in the study area. PG, penicillin G; TE, tetracycline; Ox, oxacillin; AP, ampicillin; AML, amoxicillin; TMX, trimethoprim-sulfamethoxazole; CXT, cefoxitin; VA, vancomycin; E, erythromycin and DA, clindamycin.

DISCUSSION

The bacteriological examination done for this study using different techniques revealed an overall prevalence of *S. aureus* in bovine mastitic milk was 44.62%. This is comparable with previous findings of Workineh et al. (2002), Kerro and Tareke (2003), Mekibib et al. (2010) and Abo-shama (2014) who reported 39.2% *S. aureus* isolates at Addis Ababa, 40.3% at Southern Ethiopia and 47% from dairy farms of Holeta town, Ethiopia and 40% at Sohag governorate, Egypt, respectively. However, the present finding is higher than the reports of Abebe et al. (2013) who reported 15.5% at Addis Ababa. Furthermore, other studies undertaken in various corners of the country at different times disclosed lower prevalence rates than the present study (Sisay et al., 2012; Sori et al., 2011). On the contrary, higher prevalence rates than the current result was recorded in recent times from Ethiopia (Alemu, 2010; Gebremichael et al., 2013). The differences in the

prevalence rates of *S. aureus* in mastitic cows from various findings could be due to variability in farm management practices, breeds of targeted cows, level of production and variations in the study methods and materials employed by the investigators. Based on the observations made during the collection of samples, improper hygiene and poor farm management practices contributed to the high prevalence rates of *S. aureus* in the milk. *S. aureus* is a contagious pathogen which can spread from one animal to another or personnel by contact with cows during unhygienic milking procedures (Rowe, 1999).

Prevalence of *S. aureus* isolates was higher in subclinical mastitis, 45.78% (103/225) compared to clinical mastitis, 34.62% (9/26), however, with no statistical significant difference (P > 0.05) in prevalence of *S. aureus* between the two forms of mastitis. The predominance of *S. aureus* in subclinical mastitis than the clinical cases is similar with previous studies that proved *S. aureus* is the principal causative agent of subclinical mastitis (Radostits et al., 2007; Andrew et al., 2004; Garedew et al., 2015). Moreover, *S. aureus* has acclimatized to dwell in the teat resulting in chronic and subclinical diseases. From there it could release into the milk, which serves as a source of infection for healthy cows during the milking process (Radostits et al., 2007).

Current result revealed that prevalence of *S. aureus* isolates was significantly varied with the age categories and parity. Significant association of age and parity with mastitis was reported by other authors (Moges et al., 2011; Zeryehun et al., 2013). Cows with many calves (>7) have about 13 times greater risk (62.9%) of developing an udder infection than those with fewer (3) calves (11.3%) (Biffa et al., 2005). The increased prevalence of *S. aureus* in older animals in this study can be related to increased susceptibility of pathogenic organisms in udder relaxed sphincter muscles of teats. According to Erskine et al. (2002) primiparous cows have more effective defense mechanism than multiparous cows.

Higher prevalence rates of *S. aureus* were recorded in cross breed than local zebu. In multivariate logistic regression analysis, cross breed were identified as risk factors (OR = 2.501; p = 0.018). According to Radostits et al. (2007), this could be correlated with variations in anatomical and physiological features of the mammary gland, as well as high milk yielding of the cows. Furthermore, increase in milk yield from genetic selection might be related with genetic vulnerability to mastitis.

The results of the current study disclosed that incidence of *S. aureus* was significantly varied with stage of lactation. Late stage of lactation had shown to have a significant effect (p<0.001, OR=4.260, 95% CI: 1.930, 9.402) on the prevalence of *S. aureus* when compared to early stage. This finding was in harmony with the report of Abera et al. (2010) from Adama town. The current result might be due to the fact that chronic mastitis, most often subclinical, is more frequent later during the lactation. *S. aureus* is a predominant cause of subclinical mastitis (Radostits et al., 2007; Garedew et al., 2015).

Cows with previous history of mastitis had higher *S. aureus* prevalence (P<0.001) compared to cows with no previous history of mastitis. Multivariate logistic regression analysis also showed significant effect of previous mastitis record (OR=2.553, 95%CI=1.332, 4.894, p<0.01) with prevalence of *S. aureus*. The present investigation was in agreement with the report of Abera et al. (2012). *S. aureus* is the most prevalent bacteria isolated from mastitis (Rall et al., 2013). The current result implies that treatment of cows for mastitis may not be effective in eradicating the pathogens and the disease may be carried over from previous lactations to the next. Moreover, investigation by Firaol et al. (2013) recorded antimicrobial resistant agents among pathogens which cause mastitis in Ethiopia.

The highest prevalence of *S. aureus* (76.19%) was observed in a large size herd categories followed by medium and small scale. Increase in prevalence with increased herd size was observed with highly significant association with prevalence of *S. aureus* (p<0.000). Significantly higher risk was observed in large sized herds (OR=15.824, 95%CI =6.368, 39.320) than corresponding herds. *S. aureus* have 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 health cows during the milking process. It is generally observed that large herds are characterized by increased stocking density and increased risk of exposure to infection (Radostits et al., 2007).

Prevalence of *S. aureus* was significantly associated (p<0.01) with poor udder/teat hygiene. Poor udder hygiene (OR = 2.040, 95%CI: 1.184, 3.514) were more likely to be infected with *S. aureus.* The present observation was in line with the report of Mulugeta and Wassie (2013). Sanitary milking habits are important to avoid the spreading of bacteria or their proliferation. In this study, owners who did not wash teats before and after milking found to have high prevalence of *S. aureus* mastitis than owners who used to. Radostits et al. (2007) documented that udder preparation both before and after milking influence the spread of mastitis pathogens. The predominant source of the infection is the udder of infected cows transmitted through milker's hands, utensils, towels and the environment (floor) in which the cows are kept (Radostits et al., 2007).

The present study disclosed that cows kept in houses with soil floor had higher prevalence rates of *S. aureus* than those managed on concrete floor. Houses with soil floor increased the risk of *S. aureus* infection. The association between soil floor and high prevalence of *S. aureus* revealed in our finding was in agreement with the result of Abera et al. (2010). This could be attributed to the favorable environment created for survival and multiplication of mastitis bacterial pathogens. Cows that

were kept in dirty and muddy shelters favor the proliferation and spread of mastitis pathogens. In vitro antimicrobial susceptibility patterns of S. aureus isolates revealed that highest rate of susceptibility among the isolates was recorded against chloramphenicol (97.5%) followed by gentamycin (95.3%), vancomycin (92.7%) and clindamycin (90.9%). Gizat (2004) also reported good efficacy of chloramphenicol (100%). Gebreyohanes (2008) reported susceptibility to chloramphenicol (84%). A result finding reported from South Africa showed that all of the isolates of S. aureus (100%) from two commercial farms were susceptible to vancomycin (Ateba et al., 2010). This antibiotic is no longer used for treatment of animal diseases in many countries (Pace and Yang, 2006), which might be contributed for the the current findings recorded. De Neeling et al. (2007) reported that the tested livestock - associated MRSA isolates were highly susceptible to most classes of antimicrobial drugs, except β-lactams and tetracyclines, the latter of which has been attributed to its high usage in animal husbandry. On the other hand, S. aureus isolates of mastitic milk were most resistance among others to penicillin G (87.3%) followed by tetracycline (82.2%), trimethoprimsulfamethoxazole (69.1%), cefoxitin (58.1%), oxacillin (56.4%), ampicillin (55.1%). The current investigation was in harmony with the report of Abebe et al. (2013) who recorded resistance of S. aureus to penicillin G and tetracycline found to be 94 and 73.8%, respectively, around Addis Ababa. Moreover, the present report was comparable with the result of Daka et al. (2012) who found resistance of S. aureus isolates to penicillin G (67.9%); ampicillin (67.9%); oxacillin (60.3%) and amoxicillin (30.8%). Gianneechini et al. (2002) recorded high resistance of S. aureus isolates against trimethoprim/sulphamethoxazole (90%, 100%), oxytetracycline (85%, 98%) and penicillin (87%, 94%). Alekish et al. (2013) reported 87.4% of the isolates were resistant against trimethoprim/sulphamethoxazole, 84.5% against penicillin, and 77.7% against oxytetracycline. The variability in susceptibility result could partly arise on how frequent a drug was in use in the study area. The betalactams, tetracycline, sulfonamides and some aminoglycosides have become the first line of antimicrobial agents used for treatment of bovine mastitis in Ethiopia. S. aureus are frequently resistant to other antibiotic agents in clinical use, including β-lactams, fluoroquinolones, aminoglycosides, rifampin, and mupirocin (Carbon, 2000). The resistance of S. aureus to penicillin G may be attributed to the production of beta lactamase enzyme that inactivates penicillin and closely related antibiotics. Resistance to penicillin G is used as a marker to assess the susceptibility of S. aureus isolates against other beta-lactam antibiotics (Waage et al., 2002; Pace and Yang, 2006). This correlates with the present findings. In the study area, the beta-lactams were the drugs of choice for therapy of intramammary infections, such that frequent and often inadequate use of these medications has probably contributed to emergence of resistant bacteria in the herds. Similar finding was reported by Jaims et al. (2002) that the emergence of antimicrobial resistant strain is nearly always as a result of repeated therapeutic and/or indiscriminate use of them. Probably around 50% of mastitis caused by S. aureus strains produce beta-lactamase and there is evidence that these strains are more difficult to cure with all antibiotics (Levy, 1998; Green and Bradley, 2004). It was observed that large percentages of cefoxitin (58.1%) resistant S. aureus were isolated from the study area. Disc diffusion testing using cefoxitin disc is far superior to most of the currently recommended phenotypic methods like oxacillin disc diffusion and is now an accepted method for the detection of MRSA by many reference groups including CLSI (Skov et al., 2003). Therefore, one can easily conclude that these are Methicillin resistant S. aureus (MRSA). CLSI recommends usage of cefoxitin instead of oxacillin when using the disk diffusion method to determine resistance against methicillin for S. aureus (CLSI, 2008). Significant proportion of S. aureus isolates were resistant to cefoxitin (58.1%), implying they were methicillin resistant S. aureus (MRSA). Resistance to cefoxitin is a marker for methicillin resistance S. aureus (Broekema et al., 2009). Methicillin resistant S. aureus is the term applied for any strain of S. aureus that has showed resistance to βlactam antibiotics (de Medeiros et al., 2011; Swenson et al., 2009; Batabyal et al., 2012 and Zutic et al., 2012). The antimicrobial susceptibility tests revealed that the isolates had the characteristics of general multidrug resistance pattern (penicillin, tetracycline, trimethoprim/ sulfamethoxazole, cefoxitin, oxacillin, ampicillin andamoxicillin). This is comparable with the report of Gebreyohanes (2008) and Huber et al. (2009). This could be attributed to the erratic and extensive use of antibacterial drugs without prior antimicrobial susceptibility testing. Such antimicrobial resistant organisms can pose serious health related hazards to animals as well as human beings. In recent times, an increasing antimicrobial resistance rate has been reported in S. aureus from bovine mastitis (Saini et al., 2012; Wang et al., 2013; Sharma et al., 2015).

Conclusions

The present study revealed that multidrug resistant S. aureus isolates is prevalent in dairy farms in the study area. Breed, stage of lactation, previous mastitis record, herd size and udder hygiene were found to be risk factors significantly related to S. aureus prevalence. It was observed that S. aureus isolates were highly sensitive to chloramphenicol (97.5%) followed by gentamycin (95.3%), vancomycin (92.7%) and clindamycin (90.9%). Whereas, the highest rate of resistance among the

isolates was against penicillin G (87.3%) followed by tetracycline (82.2%), trimethoprim-sulfamethoxazole (69.1%), oxacillin (56.4%), ampicillin (55.1%), and cefoxitin (58.1%). *S. aureus* from mastitic cows showed multidrug resistance to a great extent to commonly used antibiotics ensuring that the right use of antibiotics of choice is very important in line of treatment and control of the infections caused by *S. aureus*. Moreover, it is suggested that advanced molecular methods should be employed to characterize these isolates for the presence of antibiotic resistance determinants which may provide data to support conclusions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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