Isolation And Identification Of *Staphylococcus Aureus* From Mastitic Dairy Cows And Their Drug Resistance Patterns In Asossa District, Western, Ethiopia

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Abstract: A cross- sectional study was carried out from November 2016 to March 2017 to estimate the prevalence of mastitis caused by *S. aureus*, to assess the associated risk factors and determine the antimicrobial resistance pattern in Asossa town. From 384 lactating cows tested, 28.4 % had mastitis, of which 12.76% (49/384) and 15.62% (60/384) showed clinical and sub clinical mastitis, respectively. The quarter level prevalence was 29.68 % (456/1536); from which the clinical form was 12.8 % (196/1536) and the subclinical was 16.92 % (260/1536). Of 196 quarters with clinical cases, 26 had blind teats while 170 had active mastitis. A total of 109 (49 from clinical and 60 from subclinical cases) milk samples were collected and cultured for *S. aureus* of which 85 resulted in growth of the bacterium (37 from clinical and 48 from subclinical cases). The risk factors of mastitis like age group, stage of lactation, previous mastitis history, and pregnancy status had significant effect on (p<0.000) *S. aureus* isolation whereas, milking hygiene and parity had no effect on (p>0.05) isolates of *S. aureus*. The results of antimicrobial susceptibility test revealed that *S. aureus* was highly susceptible to Kanamycin (80.0%), Chloramphenicol (79.31%) followed by Cloxacillin (61.53%), Streptomycin (55.55%) and Trimethoprim-sulfamethoxazole (53.57%). In contrast, isolates were highly resistant to Penicillin G (92.86%), Clindamycin(78.57%), Cefoxitin (70.83%), Bacitracin (65.0%), Tetracycline (57.57%) and Gentamycin (57.14%). The most frequent multi drug resistance pattern consisting of three drugs is exhibited for kanamycin, chloramphenicol and cloxacillin with a resistance of 7 (75.0 6%) of the isolates. 42.85% of the isolates were resistant to different combinations of two or above tested antibiotics. In conclusion, this study confirms the importance of *S. aureus* as a mastitis causing bacterium and identifies risk factors associated with the disease in the study area.

1.   Introduction

Milk is a very nutritional food that is rich in carbohydrate, proteins, fats, vitamins and minerals. However, health risk to consumers can be associated with milk, due to the presence of zoonotic pathogens and antimicrobial drug residues (Bradely, 2002). The quality of milk may be lowered by a numbers of factors such as adulteration, contamination during and after milking and the presence of udder infections (Esron *et al.*, 2005). Pathogenic organisms in milk can be derived from the cow itself, the human hand or the environment (Bradely, 2002). Mastitis, inflammation of the mammary gland, is a highly prevalent problem in dairy cattle and is one of the most important threats affecting the world's dairy industry (Wallenberg *et al.*, 2002).

Staphylococcal mastitis is the commonest and economically the greatest concern wherever dairy farming is practiced. The chief reservoir of this bacterium is an infected udder. The organism is well adapted to survive in the udder and usually establishes mild sub clinical infection of long duration. Bacteria are shed into milk from infected quarters (Tsegaye, 1988).

Transmission occurs mainly at milking time through contaminated milking machines, clothes and hands of milkers or machine operators (Radostits *et al.*, 1994). Clinical signs vary with the severity of the disease and generally include pain, heat and swelling of the affected quarter or half of the gland and abnormality of milk either as clots or flakes and wateriness of the liquid phase (Miffin, 2004).

Bovine mastitis can be clinical with local (in some cases general) clinical signs and milk abnormalities or sub clinical with production losses and lowered milk quality. In present day of Ethiopia, there is a national drive to alleviate the existing food deficit by devising different agricultural strategies including improvements of the productivity of livestock sector by controlling some of the major infectious disease through regular monitoring. Mastitis, as a disease, has received little attention in Ethiopia, especially the sub clinical form which is mainly caused by *S. aureus* (Mekonnen *et al.,* 2005; Hundera *et al.*, 2005).

Efforts have only been concentrated on the treatment of clinical cases. Owing to the heavy financial implications involved and the inevitable existence of latent infection, mastitis is obviously an important factor that limits dairy production. The disease should be studied as it causes financial loss as a result of reduced milk yield, discarded milk following antibiotic therapy, veterinary expense and culling mastitic cows (Hillerton, 1987).

So far, there was no study done on to assess the epidemiology of *S. aureus* and/or MRSA in Asossa districts.

Therefore, the objectives of this study were to determine the prevalence and identify associated risk factors of bovine mastitis in Asosa town and to isolate and identify *S. aureus* from mastitic milk and to conduct *in vitro* antimicrobial susceptibility test on the isolates.

2.   Materials And Methods

Study area: The study were conducted in and around Asossa Town. Asossa is the capital city of the Benishangul-Gumuz Regional State and composed of 74 administrative peasant associations, which is geo graphically located at 8°30'and 40°27' N latitude and 34°21' and 39°1' E longitude (CSA, 2015). The altitude of Asossa ranges from 580 to over 1560 meter above sea level. The area is characterized by low land plane agro- ecology according to National Meteorological Service Agency (NMSA, 2014) with average annual rainfall of 850-1200 mm with uni-modal type of rainfall that occurs between April and October. Its mean annual temperature ranges between 18°C and 30°C. Asossa district has 36,916 cattle, 13,8677 Shoat, 57,632 poultry and 5,897 equines and 48,483 beehives (CSA 2015).

Study animals: Lactating cows were collected randomly from individual owner's farmstead (household dairycow owners).

Study design: The study design type was a cross-sectional study in which 384 lactating cows were tested for the presence of clinical and sub clinical mastitis.

Sampling Techniques and Sample Size Determination: The study sites were selected purposively as convenient. The total sample size for raw milk collection, isolation and enumeration of *S. aureus* were assigned according to statistical formula of Thrustfield (2007). A 5% absolute precision at 95% confidence interval was used during determining the sample size. Since there is no similar work in the study area, the expected prevalence will be taken as 50%. As result a total of 384 lactating cows from small house hold farmers were sampled using simple random sampling method.

Study Methodology

Data regarding the different potential risk factors (age, parity, lactation stage, housing conditions and previous history of mastitis) were collected for 384 lactating cows from household animal rearing farmers records when available and by interviewing the dairy cow owner when not. Clinical examination of the udder, screening using the California mastitis test (CMT) and bacteriological examination were also carried out.

Clinical inspection of the udder: Udders of the cows were examined by visual inspection and palpation for the presence of any lesion, pain, heat and swelling. In addition, milk from each quarter was withdrawn and checked for any change in colour and consistency.

California mastitis test (CMT): The California mastitis test was conducted to diagnose the presence of subclinical mastitis and it was carried out according to procedures given by Quinn *et al.* (1994). A squirt of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the reagent was added. A gentle circular motion was applied in a horizontal plane. Positive samples show gel formation within a few seconds. The result was scored based on the gel formation and categorized as negative if there was no gel formation, or positive if there was gel formation ranging from +1 - +3. If at least one quarter was positive by the CMT then the cow was considered positive. Therefore, a cow was considered mastitic if one or more quarters were CMT positive with or without isolation of microorganisms.

Milk sample collection: Milk samples were collected according to the National Mastitis Council NMC (1990). After a quarter had been washed with tap water and dried (in cases when there was a considerable amount of dirt to be removed) the teat end was swabbed with cotton soaked in 70% ethyl alcohol. Approximately 10 ml of milk was then collected aseptically from clinical and subclinical (CMT positive) mastitic cows into sterile universal bottles after discarding the first three milking streams. Samples from each quarter were transported on ice to microbiology laboratory of the Regional Veterinary Laboratory, Asossa, where they were immediately cultured or stored at +4°C for a maximum of 24 h until cultured on standard bacteriological media.

Bacteriological examination of milk samples: Bacteriological examination was done according to the NMC (1990), Quinn *et al.* (2002) and National Committee for Clinical Laboratory Standards (NCCLS) (1997). A loopful of milk sample was streaked on tryptose blood agar base enriched with 7% defibrinated sheep blood (Oxoid, UK) using the quadrant streaking method for each quarter. Blood agar plates were incubated aerobically at 37°C for 24 - 48 h. The plates were examined for gross colony

morphology, pigmentation and haemolytic characteristics at 24 - 48 h. Presumptive colonies of *S. aureus* were selected and sub cultured on nutrient agar (Oxoid, UK) and incubated aerobically at 37°C for 24 - 48 h. After this incubation on nutrient agar, bacteria were identified according to their Gram reaction, morphology and the catalase test. *S. aureus* were identified by the tube coagulase test (4 h), haemolysis, pigment production (golden yellow), mannitol and maltose fermentation. Samples were considered positive for *S. aureus* when at least one colony was identified as *S. aureus*.

3.   Data collection and analysis
All the collected data about age, parity, lactation stage, previous history of mastitis in the housing system were recorded. Depending on clinical inspection and CMT results cases were categorized as either positive or negative; positive cases were further categorized as clinical and subclinical mastitis. The age of the study animals was determined from birth records and categorized as young adults ($\geq$ 3 - 5 years), adults (> 6 - $\geq$ 9 years), and old (> 9 years). Parity was also categorized as few (with 1 - 2 calves), moderate (3 – 4 calves) and many ($\geq$ 5 calves). Lactation stage was classified as early ($\leq$ 3 m), medium (4 - 6 m), late (7-9 m) and dry (>9). Data

related to previous history of the mammary quarters and causes of blindness were obtained from clinical records of the Animal health clinic of study peasant associations and interviews with the owner of the house hold dairy cows. The data were recorded in Microsoft Excel spread sheet for statistical analysis. Logistic regression was used to see the association of the potential risk factors with occurrence of mastitis using Stata 10.0 statistical software. In all the analysis, the level of significance was set at 0.05.

4.   Results
4.1   Prevalence of Mastitis in dairy cows
Out of the total lactating cows examined, 109/384 (28.38%) were infected with mastitis infection. The *Staphylococcus species* responsible for the infection were *S. aureus* and *S. epidermidis*. The proportional prevalence of each species of *Staphylococcus* was 85/109(77.98%) for *S. aureus*, *24/109 (*22.01%*)* for *S. epidermidis*, were examined in bacteriological cultured media, during the study period and the proportional prevalence of *Staphylococcus* species was found to be statistically significant (P<0.000, chi2=384.00). The highest *S. aureus* distribution were observed in Amba-5 (27.08%) and lowest prevalence was seen in Megele-37 (10.20%) (table -1).

Table 1. Prevalence of lactating cow in study sites

Sites	No of examined	Positive	Prevalence %	Chi2	df	p-value
Amba-5	48	13	27.08	6.65	7	0.46
Megel-35	49	12	24.48			
Megel-37	49	5	10.20			
Amba-1	48	8	16.66			
Baro	51	12	23.52			
Amba-11	51	12	23.52			
Selega-23	41	11	26.82			
Selega-24	47	12	25.53			
Total	384	85	22.13			

4.1.1 Prevalence of Mastitis at cow level
The overall prevalence of mastitis at cow level as determined by CMT and clinical examination was 109 (28.38 %) from a total population of 384 cows; 12.76 % (49/384) were clinical whereas 15.63 % (60/384) were subclinical mastitis and 71.6% was healthy cow. The quarter level prevalence was 29.68 % (456/1536); from which 2.60 % was blind teats (figure-1).
4.1.2 Prevalence of Mastitis at quarter level
Out of 1536 quarters examined using CMT and clinical examination methods, a total quarter of 456 (29.7%) were found affected by mastitis, 42.98 %(196/456) and 260/456(57.02%) were clinical and sub clinical respectively. The quarter level prevalence

of sub clinical mastitis was 64(19.81%), 77(23.83%), 84(26.00%), and 98(30.34%) left front, left hind, right front and right hind respectively (Table-2).

Table 2. Prevalence of clinical and sub-clinical mastitis at quarter level using CMT and clinical examination

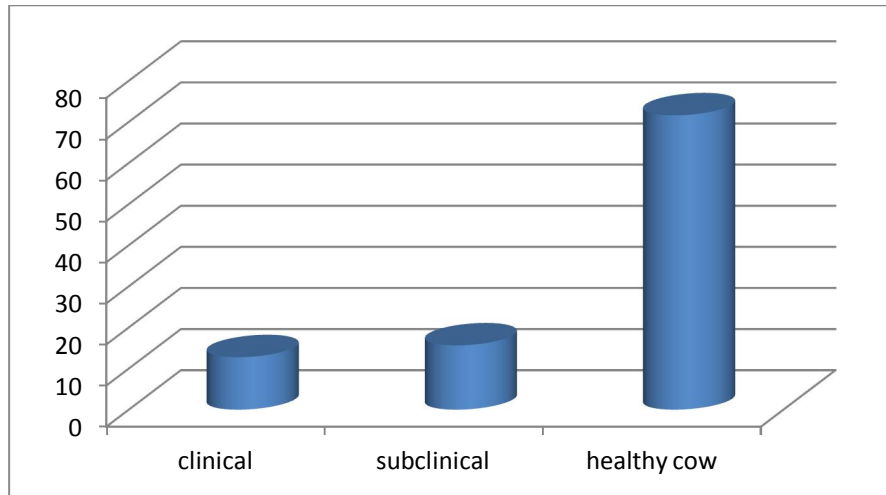| Form of mastitis	Quarter level				Total
	FL	HL	FR	HR	
Clinical	21	32	33	47	133
Sub clinical	64	77	84	98	323
Total	85	109	117	145	456

N=456

Figure 1. Prevalence of different types mastitis N=109

4.1.3 Prevalence of *S. aureus* caused sub-clinical and clinical mastitis at cow Level

The overall cow level *S. aureus* caused mastitis was 85/384 (22.14%) and the contribution of *S. aureus* to subclinical and clinical mastitis were 47/384(12.24%) and 38/384 (9.89 %) respectively (figure-2).
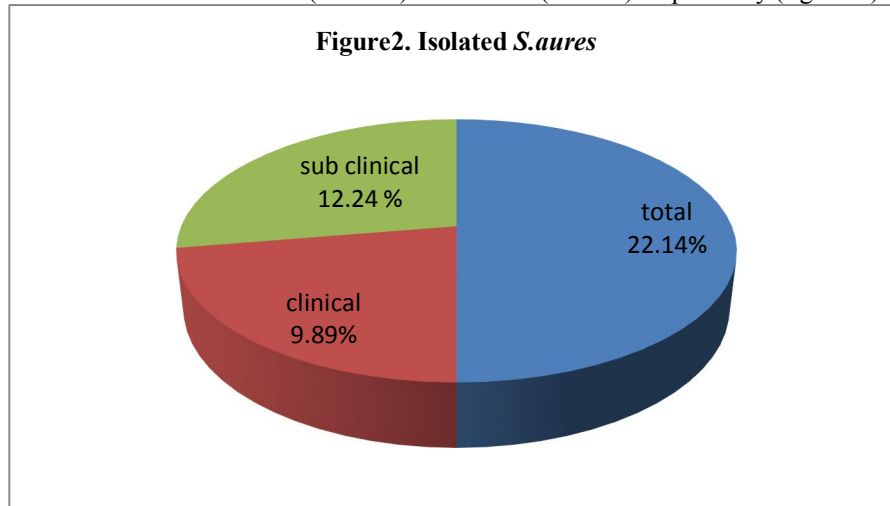

4.1.4 Prevalence of MRSA at Quarter level

MRSA was identified using Cefoxitin disk diffusion method (CLSI, 2012). The overall prevalence of Methicillin resistance *S. aureus* was 85 /384 (22.14%). From the four quarters the right hind (24.77 %) shows more prevalent and followed by right front (20.18%), left hind (17.43%), and Left front (15.59 %), respectively (Table 3).

Table 3. Prevalence of MRSA at quarter level

Prevalence of MRSA	Quarter level				Total
	FL	HL	FR	HR	
Count	17	19	22	27	85
% of total	15.59 %	17.43%	20.18%	24.77%	77.98%

N=109

4.2 Risk factors associated with Mastitis

The questionnaire survey and observation data result shows age, previous mastitis history, pregnancy status, and previous mastitis treatment history are among the potential risk factors, which are associated with mastitis disease in dairy cows farmstead (Table - 4). Accordingly, mastitis prevalence showed significant variation among different age groups (p = 0.002), previous mastitis history (p=0.000) and pregnant status (p=0.007).

Table 4. Association between risk factors *with S. aureus* caused mastitis at cow level

Factors	Categories	No examined	No (%) of positive	X^2	df	P-value
Age(years)	3-5	170	30(17.64%)	12.15	2	0.002
	6-9	200	47 (23.5%)			
	>9	14	8(57.14%)			
Parity	1-2	232	45(19.39%)	3.54	2	0.170
	3-4	104	25(24.03%)			
	$\geq$5	48	15(31.25%)			
Lactation stage(m):	$\leq$3 (early)	146	48(32.87%)	7.71	3	0.052
	4-6)(mid)	131	34(25.95%)			
	7-9(late)	78	20(25.64%)			
	>9(dry)	29	7(24.14%)			
Previous mastitis history	No	267	34(12.73%)	44.93	1	0.000
	Yes	117	51(43.58%)			
Pregnant	Non- pregnant	283	53(18.72%)	7.24	1	0.007
	Pregnant	101	32(31.68%)			
Milking hygiene	Good	118	25(21.18%)	0.089	1	0.765
	Poor	266	60(22.55%)			
CMT reaction	Reactive	151	81(53.64%)	143.33	1	0.000
	Non- reactive	233	4(1.71%)			

4.3 In-vitro antimicrobial susceptibility results

From a total of 85 isolates of *S. aureus* obtained from the study antimicrobial susceptibility tests were performed on 49 isolates. Due to the relatively small size, no separate analysis was undertaken for clinical and sub clinical isolates of *S. aureus* and were tested for antimicrobial sensitivity for 12 different types of antibiotics. The present study has demonstrated the existence of the levels of resistance of *S. aureus* to commonly used antimicrobial agents in the study area. 70.83% of the *S. aureus* was found to be resistance to Cefoxitin, which shows the prevalence of MRSA. The resistance profile of Penicillin G, Clindamycin,

Bacitracin, Tetracycline, Gentamycin and Vancomycin were 92.86%, 78.57%, 65.0%, 57.57%, 57.14% and 56.66 %, respectively (Table-7).

In this study *S. aureus* were found to be highly susceptible to Kanamycin (80%), Chloramphenicol (79.31%), Cloxacillin (61.53%), Streptomycin (55.55%) followed by Trimethoprim-sulfamethoxazole(53.57%). However these isolates were highly resistant to penicillin G(92.86%) and Clindamycin (78.57%) followed by Cefoxitin (70.83%). The antimicrobial resistance profiles are shown in Table 5.

Table 5. Resistance of *S. aureus* isolates to different antimicrobials (n = 49).

Antimicrobial	Resistance No (%)	Intermediate No (%)	Susceptible No (%)
Cefoxitin	34(70.83)	0	14(29.16)
TTC	19(57.57)	2(6.06)	12(36.36)
Cloxacillin	4(15.38)	6(23.07)	16(61.53)
Clindamycin	22(78.57)	4(14.28)	2(7.14)
Gentamycin	8(57.14)	2(14.28)	4(28.57)
Streptomycin	4(11.11)	12(33.33)	20(55.55)
Penicillin G	26(92.86)	0	2(7.14)
Chloramphenicol	2(6.89)	4(13.79)	23(79.31)
Kanamycin	2(6.66)	4(13.33)	24(80.0)
Trimethoprim-sulfamethoxazole	9(32.14)	4(14.28)	15(53.57)
Vancomycin	17(56.66)	5(16.66)	8(26.66)
Bacitracin	13(65.0)	3(15.0)	4(20.0)
Mean	14(46.66)	4(13.33)	12(40.0)

4.4 Association of MRSA with Age of Cows and Previous Treatment

Occurrence of *S. aureus* relation with age of cows were 26 (30.59%), 21(24.71%) and 14(16.47%) in young, in adult and old age group respectively. Older cows more often harbor multidrug resistant than younger cows. Adults and old age category dairy cows were found to be positive for multidrug resistant *S. aureus* of which 57.14 % and 71.43.0 % respectively (Table 6). MRSA was also found to be associated with previous treatment history of the animal. It shows that 91.76% of the isolate had previous history of treatment (Table 6). All of the isolated MRSA were from adult and old age category.

Table 6: Drug resistance pattern of *S. aureus* and age of cows

Age of cow	*S. aureus* Isolated	Resistant pattern			X^2	p-value
		One drug	Two drug	Multi drug		
Young	26	15(57.69%)	9(34.61%)	2(7.69%)	12.13	0.002
Young-adult	24	0	10(41.66)	14(58.33)		
Adult	21	0	9(42.86)	12(57.14)		
Old	14	0	4(28.57%)	10(71.43%)		
	85	15	32	38		

$X2 = 12.13$, df = 2, P= 0.0023 statistically significant (P<0.05). Multidrug resistance prevalence were =38*100/85 = 44.70%

Table 7: Association of Cefoxitin resistance pattern with previous treatment

Cefoxitin resistance	Previous mastitis treatment		
	Yes	No	Total
Positive	34	51	85
Negative	44	255	299
Total	78	306	

X^2=26.07, p=0.0001, df=1

5.   Discussion

The mastitis prevalence of 28.4 % in cows and 29.7 % in quarters reported in this study is in line with some earlier reports of 40% in cows and 19% in quarters by Kerro and Tareke (2003). This report was also in agreement with the assertion by Radostits *et al*. (2000) that, in most countries and irrespective of the cause, the prevalence of mastitis is about 50% in cows and 25% in quarters. The infection rate in cows was similar to the findings of Abdelrahim *et al*. (1990), who found a prevalence of 45.8% in Sudan. However, the present findings are lower than the prevalences report in Ethiopia (e.g. 52.8% by Hundera *et al*. (2005) around Sebeta, 53.35% by Haile (1995) in South Wollo, 53.5% by Tolossa (1987) in Kallu province, 61.11% by Tolla (1996) in South Wollo, 63% by Biru (1989) and 68.1% by Zerihun (1996) in Addis Ababa).

On the other hand, the report of Biffa (1994) in Wolaita, Ethiopia was lower (33.0%) than the present study. This variability in prevalence of mastitis between different reports could be attributed to differences in farm management practices or to differences in study methods and instruments employed by the investigators. The quarter infection rate was higher than the 19% prevalence reported by Kerro and Tareke (2003) in Southern Ethiopia; but lower than the 39% quarter infection rate reported by Abdelrahim *et al*. (1990).

The prevalence of subclinical mastitis in this study was 39.08 % which is in agreement with 38.2% prevalence reported by Workneh *et al*. (2002). In the current study the rate of sub-clinical mastitis 60/109(55.04%) was higher than that of the clinical mastitis 49/109(44.95 %) as was reported by Kerro and Tareke (2003) (62.9 versus 37.0% in Southern Ethiopia), Birru (1989) (39.5 versus 23.9%) and Hundera *et al*. (2005) (36.67 versus 16.11%) in central, Ethiopia. This variation in prevalence between subclinical and clinical mastitis may be due to the fact that, the defense mechanism of the udder reduces the severity of the disease.

The observed higher prevalence of mastitis during early lactation as compared to mid and late lactation stages was in line with the reports by Kerro and Tareke (2003) who also reported the same findings in Southern Ethiopia and this may be due to an absence of dry period therapy and birth related influences.

Radostits *et al*. (2000) suggested that, the mammary gland is more susceptible to new infection during the early and late dry period, which may be due to the absence of udder washing and teat dipping, which in turn may have increased the presence of potential pathogens on the skin of the teat. The increasing prevalence of mastitis with increasing age is in agreement with the findings by Kerro and Tareke (2003), and by Busato *et al*. (2000) who found that, the risk of clinical and subclinical mastitis increase significantly with the advancing age of the cow.

The finding of a high prevalence of mastitis in houses with muddy floors when compared with concrete floor types (p < 0.05) shows the prevalence of mastitis is strongly associated with the housing (bedding) type or condition of the farm. Similarly, prevalence of mastitis varies in different study sites, high prevalence was recorded in Amba-5(27.08%) but

low prevalence was observed in Megele-37 (10.20%) which have no significant difference(p>0.05).

A high proportion of *S. aureus* was also isolated from CMT positive cows kept in poor housing (muddy) conditions. This could be because *S. aureus* is environmentally very robust, surviving wide extremes of temperature and moisture. The organism also readily colonizes teat orifices, damaging roughened epithelium (NMC, 1990). The main source of the infection is the udder of infected cows transferred via milker's hands, utensils, towels and the environment (floor) in which the cows are kept (Radostitis *et al*., 1994).

From 384 milk samples subjected to bacteriological examination 85/109 (77.98%) isolates of *S. aureus* were isolated. This finding was in agreement with other studies (Adlan *et al*., 1980; Vaarst and Envoldsen, 1997; Kerro and Tareke, 2003; Hundera *et al.,* 2005; Mekonnen *et al*. (2005), in which *S. aureus* was the predominant isolate from clinical and subclinical mastitis. The high prevalence of *S. aureus* can most likely be attributed to the wide distribution of the organism inside mammary glands and on the skin of teats and udders (Jones *et al*., 1998). *S. aureus* has adapted to survive in the udder and establish chronic and subclinical infections. From there it is shed into the milk, which serves as a source of infection for healthy cows during the milking process (Radostitis *et al*., 1994).

Of the 1536 quarters examined, 2.6% were blind, which may be an indication of a serious mastitis problem on the respective small household dairy farm and of the absence of a culling programmed that can serve as a means to remove a source of this mammary pathogens for other cows. The antimicrobial susceptibility tests carried out in this study indicated the existence of susceptibility and resistance of *S.aureus* to some of the antimicrobials. The average susceptibility (40.0%) of *S. aureus* strains to all antimicrobials tested in this study is in agreement with the existing reports of 62.7% by Mekonnen *et al*. (2005) in Ethiopia and Myllys *et al.* (1998) in Finland.

The result of the present study shows that *S. aureus* isolates were resistant to Penicillin G (92.86%), Clindamycin (78.57%), Cefoxitin (70.83%), Bacitracin (65.0%), and TTC(57.57%), Gentamycin (57.14%) followed by Vancomycin (56.66%) and this is comparable with the higher reported resistance of 75 and 83% to ampicillin by Corrales *et al.* (1995) and Mekonnen *et al*. (2005), respectively. The resistance of *S. aureus* to Penicillin G and Cefoxitin 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 (Green and Bradely, 2004).

The present study has demonstrated the existence of alarming levels of resistance of *S. aureus* to commonly used antimicrobial agents in the study farms and the results are in accordance with reports from earlier studies in other countries (Edward *et al*., 2002; Gentilini, 2000) suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. It is therefore, very important to implement a systemic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra-mammary infections.

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