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ISOLATION, IDENTIFICATION AND ANTIMICROBIAL RESISTANCE OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM MASTITIS CASES OF LACTATING DAIRY COWS FOUND IN SULULTA AND HOLLETA TOWNS, OROMIA, ETHIOPIA

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ABSTRACT

A cross-sectional study was carried out from January 2021 to August 2021 on lactating dairy cows suffering from mastitis cases to isolate and identify *Staphylococcus aureus* and to assess their antimicrobial susceptibility patterns. The lactating cows (n=300) from Sululta and Holleta towns, Oromia Special Zones, Oromia National Regional State, Ethiopia, were enrolled in the study. All cows were screened for mastitis using California Mastitis Test (CMT). Two-thirds of cows (66.7%) had mastitis, of which 185 (61.7%) and 15 (5%) showed subclinical and clinical mastitis, respectively. CMT positive milk samples were collected from CMT positive dairy cows in both towns and examined using standard microbiological techniques, including culture, colony characterization, primary and secondary biochemical tests, and species-level identification of *Staphylococcus aureus*. The BiOLOG identification system conducted the species-level identification for *Staphylococcus aureus*. Overall, 15 samples were positive for *Staphylococcus aureus* by BiOLOG. Finally, the antimicrobial profiles of the 15 *Staphylococcus aureus* confirmed isolates were assessed using 10 different antimicrobials. Out of these, 93.3% of isolates were highly resistant to Penicillin G, 80% of isolates were equally resistant to Cefoxitin and Oxacillin, and 73.3 and 60% of isolates were found to be resistant to Amoxicillin and Tetracycline, respectively. However, all 15 isolates of *Staphylococcus aureus* were highly susceptible (100%) to five antimicrobials, namely Ciprofloxacin, Chloramphenicol, Sulphamethoxazole+Trimethoprim, Erythromycin, and Gentamicin. In conclusion, this study determined the importance of *Staphylococcus aureus* as one of the mastitis-causing bacteria of the dairy industry and investigated its antimicrobial resistance pattern. Therefore, the existence of *Staphylococcus aureus* in raw milk imposes a great risk to public health. Should awareness-raising training be arranged for dairy farmers and field veterinarians to use the antimicrobial agents against susceptible *Staphylococcus aureus*. Implementation of appropriate biosecurity measures and further research are needed to investigate why certain *Staphylococcus aureus* strains developed resistance to antimicrobial agents.

***Keywords*:** Antimicrobial resistance, BiOLOG, CMT, Holleta, Mastitis, *Staphylococcus aureus*, Sululta

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1. INTRODUCTION

In the last two decades' animals has been the source of most infectious diseases (nearly ¾ of infectious diseases) which were transmitting to humans. *Staphylococcus aureus* is an opportunistic pathogen which is known as the major bacterial microorganism leading to severe diseases in the dairy industry. In the past few years, food poisoning caused by staphylococcus species has been reported as the third cause of food-borne diseases globally (Girmay et al. 2020; Du et al. 2022).

The bacterium *Staphylococcus aureus* produces a variety of toxins like various Staphylococcal entero-toxins and proteins. There are five major Staphylococcal entero-toxins and Staphylococcal like proteins, namely Staphylococcal entero-toxin A, Staphylococcal entero-toxin B, Staphylococcal entero-toxin C, Staphylococcal entero-toxin D and Staphylococcal entero-toxin E which has been known to lead to almost all cases of Staphylococcal food poisoning (Angelidis et al. 2020; Alebie et al. 2021).

Milk and milk products are highly vulnerable to be contaminated with a lot of microorganisms mainly during handling and processing and also due to unclean environment that cause severe concerns to the public health due to the very high nutritive value and complex chemical composition of milk. When milk is exposed to variations in temperature or is stored at temperatures from 37°C and 42°C, there will be production of heat resistant staphylococcal entero-toxins which are very harmful for the public health (Thaker et al. 2013).

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Mastitis causes a serious problem which leads to decrease in milk production and stays one of the major economically important diseases for the dairy sector in the world regardless of the animal species (Girma et al. 2012; Cobirka et al. 2020; Liu et al. 2020; Bude and Mengesha 2021; Hasan et al. 2022). Staphylococcal mastitis is one of the frequently appearing and the main economic threat wherever dairy farming is practiced with the most important place of infection for this bacterium being an infected udder (Qayyum et al. 2016b; Bi et al. 2020). The pathogen is well adapted to exist in the udder and teats and most of the time leads to less severe sub clinical infection for extended periods. The organism is released into milk from infected quarter. Transmission happens mainly during milking period via contaminated milking machines, clothes and hands of millers' or machine operators. Mastitis in bovine can be subclinical which can be indicated by losses in milk production and low quality of milk or clinical with local clinical signs and milk abnormalities (Abera et al. 2010; Hussain et al. 2013; Schmenger and Krömker 2020; Hasan et al. 2022).

Ethiopia owns an enormous livestock population which is the backbone of the agricultural sector. The country has the largest livestock resource of any African country with an approximate number of 52.13 million of which dairy cows accounting for around 7.2 million. Only the clinical form of mastitis has been getting all the treatment efforts but very little attention has been given to subclinical mastitis in Ethiopia, which is mostly caused by *Staphylococcus aureus* (Mekonnen et al. 2017; Bude and Mengesha 2021).

In spite of having high number of dairy cows, still in Ethiopia the availability of milk and milk products is limited due to various factors with the prevalence of mastitis being dominant. Over the past years lot of researchers have conducted. Different studies conducted to determine the presence of mastitis in Ethiopian dairy cows reported a prevalence rate in the range between 23.2 and 81.1% (Abebe et al. 2016; Belay et al. 2022).

Staphylococcus aureus has the unique ability to be resistant to many antimicrobial agents due to its main virulence factor, the coagulase protein. Methicillin resistance is common in other coagulase negative species of staphylococcus (Abbas et al. 2014). Since the existence of *Staphylococcus aureus* in milk could be a way of infection to humans, it is necessary to practice an antimicrobial sensitivity test before the use of the antimicrobial agents in prevention as well as treatment of infections in the mammary gland. The development of resistance of the bacteria to antimicrobial agents makes mastitis more difficult to control. Antimicrobial resistance has become a huge public health issue worldwide (Anueyiagu and Isiyaku 2015; Canisso et al. 2021).

Therefore, the objectives of this study were to isolate and identify *Staphylococcus aureus* from masititic milk and to perform antimicrobial susceptibility test for the isolates.

2. MATERIALS AND METHODS

2.1. Study Area

The study was carried out in Sululta and Holleta districts of Oromiya regional state. Sululta town is one of the special zones of Oromia National Regional State which is found 26 km northeast of Addis Ababa (Fig. 1). The area is located at 9° 11°N latitude and 38° 45° E longitude with an average elevation of 2765 meters (range 2851-3700 meters) above sea level. The area receives mean annual rain fall of 1140 mm. The total cattle population in the area is 224,600 with 15% being crossbreed (Regasa et al. 2019). Holleta is also one of the special zones of Oromia National Regional State, with an approximate location of 09° 03′–19.43"N latitude and 38° 30′–25.43" E longitude. The study area is characterized by having a mild subtropical weather; with an average annual temperature of 14.5°C. The area also goes through two types of rainfall pattern with an average annual rainfall of 1067 mm (Abunna et al. 2017).

2.2. Study Population

The study animals included 300 lactating crossbred and exotic cows from 39 different dairy farms and small-scale individual owners in Sululta and Holleta towns.

2.3. Study Design

A cross sectional study was conducted from January 2021 to August 2021 to detect mastitis cases from lactating dairy cows in different farms of Holleta and Sululta towns and to isolate and identify *Staphylococcus aureus*, which is the major causative agent for bovine mastitis and to investigate its antimicrobial resistance pattern.

2.4. Study Methodology

The study started by recording information about the study animals (including age, parity, lactation stage, previous history of mastitis and the management practices) from the records of the farm and by taking history from the farm owners. Clinical inspection and palpation of the udder was performed to detect any sign of inflammation and visualization of the milk for any change in color and/or consistency to determine the presence of clinical mastitis. The suspected samples were aseptically collected and subjected to California Mastitis Test (CMT) test.

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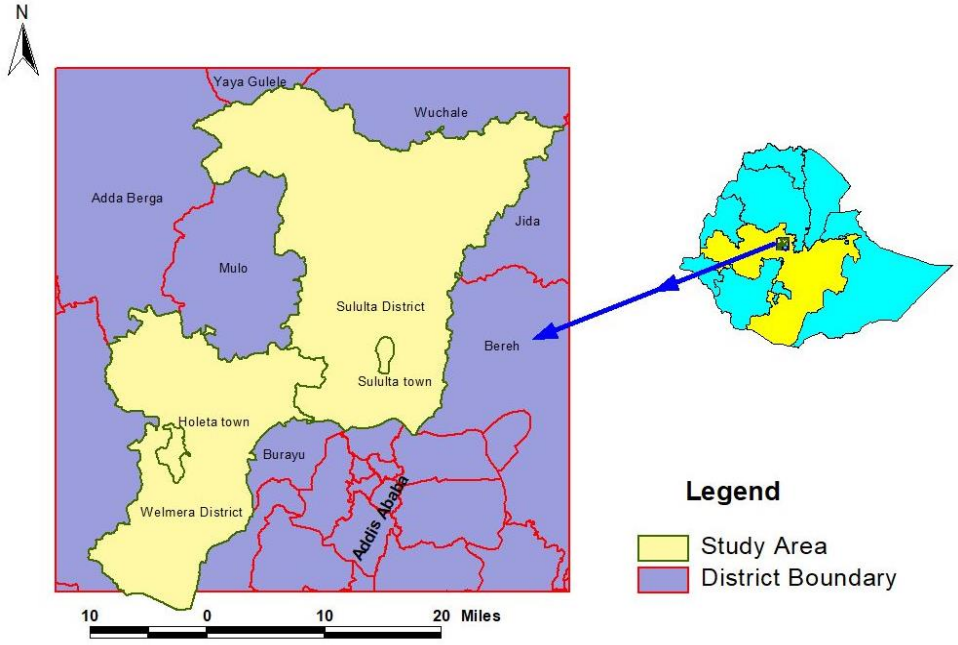

Fig. 1: Map of Ethiopia (right side) showing study area on the left side (yellow color).

Ethiopia Region, District shapefile source EMA, CSA 2021 GIS software ArcGIS 10.2 ESRI USA

2.4.1. Sample Collection and Transportation

Sampling was carried out daily for 10 consecutive days where a total of 300 milk samples were collected aseptically from lactating cows in different dairy farms of Sululta and Holleta towns. The collected milk samples were screened on site by CMT test.

2.4.2. California Mastitis Test (CMT)

The CMT was carried out to assess the occurrence of subclinical mastitis (Annex 1) by putting a squirt of milk from each udder of the cow into the corresponding cups of the CMT paddles and then an equal amount of the CMT reagent was added and moved gently in a horizontal plane where within seconds formation of gel was observed for positive samples (Regassa et al. 2013). The CMT test was primarily conducted for screening purpose of samples so that positive cases could be sampled for further research. The milk of CMT positive cows was re-sampled in a sterile screw capped tubes and kept in an ice box having ice packs and transported immediately to NAHDIC Microbiology laboratory and kept at -20°C refrigerator for further research.

2.4.3. Bacterial Isolation and Identification

Bacterial isolation and identification were conducted under the protocols of NAHDIC. Since the primary and main step of any bacteriological isolation starts with preparation of media, the study started by preparing different selective, differential and indicator media that were needed for the research. Mannitol Salt Agar (MSA) media, Maltose Purple Agar (MPA) media and also Nutrient agar media, blood agar media, DNase Media, BiOLOG Media and Muller-Hinton agar Media were prepared whenever they were needed to do colony characterization and to perform further biochemical, confirmatory and susceptibility tests.

2.4.4. Biolog MicroStation with GENIII Microplate and Software Identification system

BiOLOG tests were performed for species level identification of *Staphylococcus aureus* for samples that resulted positive for both catalase and coagulase tests. The BiOLOG test was carried out to provide clear indication and certainty of species level identification of *Staphylococcus aureus* (Wragg et al. 2014). It was performed by sub-culturing pure colonies of the organism from nutrient and blood agars on its own specific BiOLOG media and incubated at 37°C for 24h and then a pure colony from the BiOLOG media was inoculated with Biolog inoculators into the BiOLOG fluids at 100 absorptions measured by a BiOLOG turbidimeter and then the bacteria inoculated fluid was also measured again to make sure it was in the acceptable range for the growth of the bacteria which was between 92 to 98%.

After that 100µL of the bacteria inoculated fluid or cell suspension was dispensed into the BiOLOG reservoirs and then drawn to each of the specific BiOLOG GEN III 96 well micro- plates by using a BiOLOG multi-channel micropipette and a 25ml sterile, non-pyrogenic, RNase/DNase free BiOLOG reagent reservoir and then incubated at

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37°C for 20h. After 20h of incubation the micro-plates were read in the Micro-station semi-automated reader and interpreted by the GEN III data base, version 5.2.1. Computer software which correctly identifies the isolates up to species level and notifies those samples that needed further incubation so that they could be re-incubated and re-read after 3-6h. Fifteen isolates were identified as *Staphylococcus aureus*. Those bacteria that could not been identified within 26h were perceived as having no identification (Wragg et al. 2014). Overall, fifteen isolates were confirmed as *Staphylococcus aureus* by BiOLOG tests and were preserved in a sterile peptone water with glycerol broth to conduct antimicrobial susceptibility test.

2.5. Antimicrobial Susceptibility Test: Disc Diffusion (Bauer-Kirby) Susceptibility Test

Antimicrobial Susceptibility test of the *Staphylococcus aureus* isolates was analyzed for 10 different antimicrobials namely Penicillin G (10units), Amoxicillin (30μg), Tetracycline (30μg), Oxacillin (1μg), Ciprofloxacin (10μg), Chloramphenicol (30μg), Cefoxitin (30μg), Sulphamethoxazole + Trimethoprim (25μg), Erythromycin (15μg) and Gentamicin (10μg) (Annex 3). The antimicrobials were selected based on their availability at the current market. The test was carried out by using the agar disc diffusion method, first 5ml of 0.85 saline water was dispensed in a test tube labeled for 15 isolates and the colonies from a nutrient media was then taken by a swab and put in the saline water and each suspension with the organism was mixed well and measured for a turbidity of 0.5 and cultured thoroughly on Muller- Hinton Agar (MHA) media of 4mm depth and 90mm diameter. After that the ten different antimicrobial discs listed above were taken from their corresponding containers by using forceps and diffused in the respective MHA Media of the fifteen isolates and incubated at 37°C for 18h. After incubating for 18h, measurement of the diameter of the clear zones of inhibition (ZOIs) around and including the antimicrobial discs of each isolate were conducted and interpreted to categorize as susceptible, intermediate and resistant according to the performance standards given by Clinical and Laboratory Standards Institute (CLSI 2020).

2.6. Data Analysis

Data were entered to Microsoft excel sheet, coded and analyzed by using a computer software called SPSS (Statistical Package for Social Sciences) Version 20 (Nie et al. 1975). Descriptive statistics such as proportion or percentage were used to summarize the data and calculate status of Staphylococcus species at farm.

3. RESULTS

3.1. California Mastitis Test (CMT)

Out of the 300 lactating dairy cows examined during the study 200 (66.7%) cows were found to be CMT test positive. Of the CMT test positive, 185 (61.7%) and 15 (5%) showed subclinical and clinical mastitis, respectively.

3.2. Bacterial Growth

According to this study, all the 200 CMT test positives were subjected to culturing in different media and the results revealed that 124/200 (62%) and 66/124 (53.2%) isolates showed growth on MSA and MPA (Table 1), respectively or were positive for Staphylococcus.

3.3. Tube Coagulase Test

Twenty-eight samples out of 66 (42.2%) isolates were found to be positive for tube coagulase test and based on the results from the coagulase test further identification was done to identify the bacteria.

3.4. BiOLOG Identification

A total of 15 (7.5%) isolates were confirmed as *Staphylococcus aureus* by the BiOLOG identification system and these isolates were analyzed for 10 different antimicrobials to assesses their antibiotic profiles and categorize them as susceptible, intermediate or resistant.

Table 1: Number of samples that showed growth on MSA and MPA and that were positive for catalase and coagulase tests

Study Area	Growth on		Positive for test		Suspected for
	MSA	MPA	Catalase test	Coagulase	*Staphylococcus aureus*
Sululta (100)	63	55	45	28	28
Holleta (100)	41	11	7	0	0
Total (200)	124	66	52	28	28

MSA: Mannitol salt agar, MPA: Maltose purple agar. A total of 124 and 66 samples showed growth on MSA and MPA Medias and 52 and 28 isolates were positive for catalase and coagulase tests, respectively.

3.5. Antimicrobial Resistance (AMR) Test

A total of 15 isolates were subjected to the ten antibacterial agents for AMR test. The AMR test results showed

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that *S. aureus* is 100% susceptible to five antimicrobial agents and showed different percentage of resistance to five antimicrobials (Table 2). As shown in Table 2, all fifteen isolates of *Staphylococcus aureus* were found to be highly susceptible to five antimicrobials namely Ciprofloxacin (100%), Chloramphenicol (100%), Sulphamethoxazole + Trimethoprim (100%), Erythromycin (100%) and Gentamicin (100%). However, 14 isolates were also found to be highly resistant to Penicillin G (93.3%) followed by 12 isolates being equally resistant to Cefoxitin and Oxacillin at (80%) and also11 and 9 isolates were found to be resistant to Amoxicillin (73.3%) and Tetracycline (60%), respectively. The isolates that showed resistance to different antibacterial agents are summarized in Table 3 below.

Table 2: Susceptibility and Resistance of *Staphylococcus aureus* isolates to 10 different antimicrobials

Antimicrobials	Susceptible		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Penicillin G	1	6.70	0	0	14	93.3
Cefoxitin	3	20.00	0	0	12	80
Amoxicillin	4	26.7	0	0	11	73.3
Tetracycline	6	40	0	0	9	60
Oxacillin	3	20	0	0	12	80
Ciprofloxacin	15	100	0	0	0	0
Chloramphenicol	15	100	0	0	0	0
Sulphamethoxazole + Trimethoprim	15	100	0	0	0	0
Erythromycin	15	100	0	0	0	0
Gentamicin	15	100	0	0	0	0

Out of 200 samples conducted for *Staphylococcus aureus* isolation and identification 15 pathogenic *Staphylococcus aureus* were identified and tested for antimicrobial susceptibility tests. The percentage of susceptibility, intermediate and resistance were done by dividing the total number of (susceptibility, intermediate and resistance) to total isolates (15) then we found the percentages.

The *Staphylococcus aureus* isolates that were resistant to multiple antimicrobial agents (Table 3) can be interpreted as there are 6 (40%) isolates that were resistant to 5 antimicrobials, 11 (73.3%) isolates that were resistant to 4 antimicrobials followed by 12 (80%) and 13 (86.7%) isolates being resistant to 3 and 2 antimicrobials, respectively. In general, none of the isolates were fully resistant or susceptible to all antimicrobials which were indicated by 0 percentages (Table 3).

3.6. Risk Analysis

In this study the occurrence of a number of risk factors leads to variation in the prevalence of mastitis between Sululta and Holleta towns and even among the different farms in each town. A significant difference was observed between the results of Sululta and Holleta towns. Accordingly, prevalence of the disease also revealed significant differences in each town between cows of the different age groups, herd size, body conditions and stages of lactation ($P<0.001$) however, no significant relationship was observed between the prevalence of mastitis and parity ($P=0.485$) as well as previous history of the case in the study areas.

Table 3: *Staphylococcus aureus* isolates that showed resistance to multiple antimicrobial agents

Resistant to Antimicrobials	Number of isolates	Percentage
1	14	93.30
2	13	86.70
3	12	80
4	11	73.30
5	6	40
6-10	0	0

4. DISCUSSION

A through research was conducted for isolation and identification of one of the most economically important pathogens of the dairy industry, *Staphylococcus aureus* (Khan et al. 2013; Qayyum et al. 2016a; Birhanu et al. 2017). The pathogen was detected by CMT from 200 mastitic milk samples and a total of 15 isolates were confirmed as *Staphylococcus aureus* by the BiOLOG identification system.

Regardless of the source of infection most countries have an average occurrence rate of 50% for subclinical mastitis infection. The results of the present study revealed that there was a 66.7% prevalence rate of mastitis, of which 61.7% and 5% accounting for Subclinical and clinical mastitis, respectively and the report of this study on subclinical mastitis is more or less in line with the reports of Abebe et al. (2016) in Hawassa who found a prevalence of 59.2%. However, the rate of occurrence of subclinical mastitis in this study was higher than the findings of most reports including a prevalence of 36.7% (Abera et al. 2010) in Adama, 38.0% (Bedane et al. 2012) in Borena zone, 16.1 and 28.26% in Bishoftu (Birhanu et al. 2017; Bude and Mengesha 2021, respectively), 43.75% (Anueyiagu and Isiyaku 2015) in Nigeria and 57.7% (Bamayi and Aniesona 2013) in Maiduguri. Tolosa et al. (2013) reported that 62% of the cows and 51% of the quarters have subclinical mastitis in Jimma, Ethiopia. Based on the clinical examination and CMT, 39.67% overall prevalence of mastitis on cow level was recorded in four districts of west Wollega, western Oromia, Ethiopia. Of them, 16.70% and 22.70% were clinical and subclinical mastitis, respectively (Kitila et al. 2021).

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Since bovine mastitis is a complex disease a lot of risk factors including agro ecologies, herd size, management practices (housing, milking) and the genetic makeup of the study animals in the different dairy farms of both towns might contribute to the significant differences observed between the results of the samples obtained from Sululta and Holleta towns and also between the results of the present study and the findings from previous scholars.

According to the results of this study, the occurrence of mastitis and herd size was directly related because there was a high prevalence of mastitis in farms with large number of cows when compared with the ones with small number of cows. The probability of having a cow positive for CMT test was higher in bigger farms with intensive management which contributes to poor management practices (like closure of the animals, dirty environments, contaminated materials, increased humidity and poor ventilation) than in smaller the farms.

The World Health Organization has stated that there's a great risk of increasing resistance to antimicrobials whenever one is in use against bacterial diseases Cheng et al. (2019). The current study revealed the resistance of *Staphylococcus aureus* to Penicillin-G (93.3%), Oxacillin (80%), Cefoxitin (80%), Amoxicillin (73.3%) and Tetracycline (60%) whereas was highly susceptible to some antibiotics like, Ciprofloxacin (100%), Chloramphenicol (100%), Sulphamethoxazole + Trimethoprim (100%), Erythromycin (100%) and Gentamicin (100%) (Table 3). This study is almost in agreement with the resistance findings of Tessema and Tsegaye (2017) who reported 95.55%, 77.19% and 63.41% for Penicillin, Cefoxitin and Tetracycline, respectively. Also, the resistance pattern of penicillin and tetracycline agreed with reports of Brînda et al. (2010) in Timisoara, Romania who reported 93.1 and 60%, respectively. However, the resistance of *Staphylococcus aureus* isolates in the present study was found to be higher than the reports of Jamali et al. (2014) in Malaysia who reported penicillin G (86%) but this study was lower in case of tetracycline (76.7%).

The resistance of *Staphylococcus aureus* to penicillin and amoxicillin may be associated with the production of an enzyme called β-lactamase that inactivates penicillin and closely related antibiotics. It is thought that about 50% of the *Staphylococcus aureus* strains that lead to mastitis produce the enzyme β-lactamase (Kateete et al. 2013).

Out of the total isolates (15) onto which drug susceptibility test was performed about 12 showed resistances to multiple antimicrobials or they were resistant to three or more antibiotics. The present study has revealed that the presence of increasing levels of resistance of *S. aureus* to frequently used antimicrobial agents in the study farms and the results are in line with reports from existing studies suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. It is therefore, very necessary to practice a systemic application of an in vitro antibiotic sensitivity test before the use of antimicrobials in both prevention and treatment of intra-mammary infections.

The highest antibiotic resistance observed in the current study might be due to high antimicrobial use in dairy farms and individual cows, to treat various diseases affecting the dairy sector. The development of resistance to antimicrobials among the bacteria causes a problem of concern. The success of recent treatments and ability to control infectious diseases in both animals and humans may become difficult.

5. Conclusion and Recommendations

Staphylococcus aureus is a highly prevalent and the most economically important pathogen of the dairy industry that adapts well to the mammary glands of dairy cows especially on the udders and teats causing severe subclinical and chronic infections. Out of the 300 lactating cows examined during the study 185 cows were found to be infected with subclinical mastitis which could be a sign of high mastitis problem in both Sululta and Holleta towns. However, the study also showed that the prevalence of *Staphylococcus aureus* as a causative agent for bovine mastitis in Sululta town is higher than in Holleta town. and in general both town have low prevalence of *Staphylococcus aureus* compared to other bovine mastitis causing organisms and bacterial species like other Staphylococcus species including *Staphylococcus intermidus* and *Staphylococcus epidermids, Macrococcus brunensis, Bacillus kribbensis, Paenibacillus lactis, Streptococcus oralis* and *Streptococcus downei* were ranked respectively to occur frequently during the process of species level identification of the suspected isolates by BiOLOG system. The prevalence of *Staphylococcus aureus* was low as confirmed by the study when in fact there were a high number of CMT positive samples because of the presence of other pathogens in the milk samples as detected by the BiOLOG identification system. The antimicrobial susceptibility tests conducted in the research revealed the presence of antibiotic resistant strains of *Staphylococcus aureus* especially to Penicillin G (93.3%), Cefoxitin (80%) and Oxacillin (80%). Finally, we can conclude that the existence of *Staphylococcus aureus* in raw milk and their resistance to commonly used antimicrobials impose a great risk to the public health. Therefore, relying on the conclusion given above the following recommendations are forwarded:

- Awareness rising trainings should be given to dairy farm owners and care givers on the predisposing factors to mastitis and on proper prevention and management strategies.
- Trainings should also be given to farm owners on the proper usage of antimicrobials and their withdrawal period.
- Implementation of proper bio-security measures as well as surveillance strategies is needed.

- Initiation of further research on molecular characterization of antimicrobial resistant *Staphylococcus aureus* strains is recommended.

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