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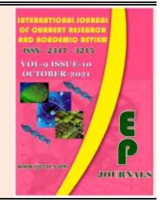

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Characterization of Drug Resistance Patterns of *Staphylococcus aureus* Isolated from Cases of Subclinical Mastitis in the Research Dairy Farm, Central Ethiopia

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Abstract

Mastitis is a disease of production which is caused by a wide range of pathogens that can induce inflammation of the mammary tissues. A cross sectional study was conducted to detect and profile antimicrobial resistance pattern of Staphylococcus aurous in cases of subclinical mastitis. A total of 94 lactating cows were examined using California mastitis test (CMT) and 58.5% were positive for mastitis infection, 25.45% of cultured milk samples contained S. aurous. The antibiotic sensitivity test showed that the highest susceptibility profile was recorded for Tetracycline (92.86%) and gentamycin (92.85%) whereas the highest resistance profile was recorded against sulfamethoxazole trimethoprim (71.42%) and penicillin G (57.15%). On the other hand, 35.72.0% and 28.57% of the isolates, respectively, carried multiple antibiotic resistance phenotypes against TE*PG*ER*SMT and TE*PG* ER*SMT*GE. The findings of this study indicated that antibiotics were imprudently used in the dairy farm which resulted in the emergence of antimicrobial resistant strains. In conclusion, further studies are required to elucidate search of alternative antimicrobials and their effect on the induction and spread of resistant genes as well as standardization of therapeutic uses of antimicrobials, which ensures the right choice and use of drugs locally in the dairy farms.

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Keywords

Antibiotics, Antimicrobials, Mastitis, Resistance.

Introduction

Antibiotics, microbiologically produced compounds, are used in animals to treat or prevent various infectious diseases. In dairy production, these drugs are used, at low doses, over extended periods, to improve feed utilization and as growth promoters (Gustafson, 1991). However, the major classes of antibiotics that are used to treat human infections either belong to the same general classes or have the same mode of action as those used in animals (Joshi, 2002). Antibiotics remain in the milk after their use in lactating cows. Their presence in milk and tissues reflects failure to observe withdrawal periods, inappropriate use of drugs and incorrect dosages, and poor absorption in the gut of treated animals. Mackie *et al.,* (2006), reported that 25% - 75% of the antibiotics administered to feedlot animals could be removed unaltered in feces.

The major concern arising from antibiotic usage is drug resistant bacteria that is transferred to humans through consumption of milk or other animal products and by environmental contact (Codex Alimentarius Commission, CAC, 2010). Emergence of antimicrobial resistant strains of animal origin and its effect on health is the major public health concerns associated with frequent use of drugs (Smith *et al.*, 2005) that may also lead to treatment failure (FDA 2008). Other heath impact includes the risk of allergic reactions by penicillin-sensitized persons and inhibition of bacterial starter cultures used in dairy production. High proportion of milk in the market may contain several pathogenic strains that are resistant against drugs commonly used for treatments.

As the pathogens have become more resistant to the indiscriminate usages of antibiotics, including over – and under - dosages, incidences of disease have increased, therapeutic efficiency has fallen, and therapeutic doses have increased which require alternatives to reduce these shortfalls (Eickhoff, 1992).

In particular, bacterial resistance to aminoglycosides, mostly streptomycin, neomycin and kanamycin, is very common (Prescott and Baggot, 1993). *Staphylococcus aurous* and *E. coli* as well as *L. monocytogenes* may easily develop antimicrobial resistance (Peles *et al.*, 2007).

In Ethiopia, information and practices regarding multidrug resistance profiles of *S. aurous* isolates from mastitic cows is lacking (Tassew *et al.*, 2016).

Information and practices on withdrawal periods in lactating dairy cows has not been extensively studied in the study farms and by this account antibiotic resistance is a growing phenomenon that has emerged as one of the prominent public health concerns. Therefore, the objectives of this study were:

To isolate and characterize antimicrobial resistance phenotypes in *S. aurou*s isolated from cases of subclinical mastitis in the dairy farm

Materials and Methods

Study Area

Holeta Agricultural Research Center (HARC) is located at 34Km west of Addis Ababa at an elevation of 2400 m.a.s.l in the central high land of Ethiopia. The area is characterized by mild subtropical weather, with average minimum and maximum annual temperature of 6.3 and 22.1°C, respectively. The animals were under intensive and semi intensive management systems. The area also experience bimodal rainfall pattern with a long rainy season extends from June to September while the short rainy season extends from March to April.

Milking cows

All milking cows in the dairy farm were screened for mastitis using pen side California Mastitis Test (CMT). Briefly, the udder was washed with soap and dried with clean towels. The teats were similarly cleaned and disinfected with 70% alcohol. Then, after collecting the milk by the application of CMT paddle, equal volumes of milk and CMT reagent were mixed in the CMT paddle and gently shaken to see gel formation. Accordingly, subclinical mastitis infected cows were sampled for isolation and antimicrobial susceptibility profiles.

Isolation and identification of *Staphylococcus aurous*

Bacteriological culture was performed following the standard microbiological techniques. The collected milk sample was cultured on 5% sheep blood agar, incubated aerobically at 37 °C for 24-48 h and examined for growth of bacterial colonies. Colonies were identified based on colony morphology and hemolytic pattern on blood agar.

Typical colonies were then sub-cultured on Mannitol salt agar plate and incubated at 37 °C for another 24 h. The isolated colonies from nutrient agar were subjected to primary and secondary biochemical tests for identification of *Staphylococcus aurous* according to (Quinn *et al.*, 2002).

Phenotypic characterization of Antimicrobial patterns of S. *aurous*

The in vitro susceptibility and resistance of the isolated organism from Mac-conkey and Mannitol salt agar against different antibiotics was measured by the kriby-Bauer disk method (Marjan *et al.*, 2014). The *S. aurous* isolates were subjected to antimicrobial susceptibility testing by kriby-Bauer disk diffusion method (CLSI, 2008). The commonly used antimicrobials were Gentamycin (10µg), Erythromycin (15µg), Tetracycline (30 µg), Amoxicillin (25 µg), Chloramphenicol (30 µg), Trimethoprim-Sulfamethoxazole (25µg), Penicillin G (10 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg). The isolates were inoculated into tryptone soya broth (TSB) and incubated at 37 °C for 24 h. After adjusting the turbidity of the suspension to visually match that of 0.5 McFarland standards, a sterile cotton swab was dipped into the suspension and evenly spread over the entire surfaces of readily prepared Muller Hinton Agar (MHA) plates to obtain uniform inoculums. Then antimicrobial discs were applied over the surface of the inoculated plates by means of sterile needle within a distance of 5 mm.

After 24 h of incubation at 37 °C under aerobic condition, the diameters of the zones of complete inhibition on the plates were measured to the nearest whole millimeter using zone inhibition scale. Inhibition zone diameters for individual antibiotics were interpreted and classified according to procedures established by CLSI (2008) as susceptible, intermediate or resistant.

In this study, intermediate inhibition zones (results) were regarded as resistant (Huber *et al.,* 2011). Multiple antibiotic resistant (MAR) phenotypes were determined if the isolate is resistance to three and more antimicrobials (Rota *et al.,* 1996).

Multiple Antibiotic Resistance Index

The MAR Index of an isolate is defined as the number of antibiotics to which the isolate was subjected and showed resistance patterns. Bacterial isolates that had more than 0.2% MDR index is originated from a niche of high antibiotic use (Tambekar, 2006).

Data Analysis

The data generated from laboratory investigations were recorded and coded using Microsoft Excel spreadsheet and analyzed using SPSS (version 20.0). Drug sensitivity and resistance was determined by measuring the diameter of the growth inhibition zone around the antibiotic discs. The presence of antibiotic resistance was determined by dividing the number of resistant isolates out of the total isolates subjected to the particular drugs.

Results and Discussion

Subclinical mastitis infection and detection of *S. aurous*

Overall, 94 lactating dairy cows were examined using CMT and the prevalence of subclinical mastitis infection in the farm was 58.5% (N=55). Out of 390 teats examined, 31% (N=122) of the teats were infected and positive for subclinical mastitis (Figure 1).

Out of 94 lactating dairy cows tested and diagnosed for subclinical mastitis, 58.8% were positive and 22.45% of mastitic milk harbored *S. aurous (*Table 1).

Phenotypic characterization of drug resistance S. *aurous*

Multi-drug resistance *S. aurous* isolates were identified and determined by the resistance patterns of each isolates to the used antibiotics. The pattern was susceptible (S), resistance (R) and intermediate (I) as formulated in the table (Table 2).

The MDR Index analysis of *S. aurous* revealed that isolates four and seven had 60% MDR index values whereas isolates five, seven, eight, nine, ten and thirteen had 40% MDR index values (Table 3).

Antimicrobials sensitivity test was conducted for all 14 isolates of *S. aurous.* Overall, from the total isolates tested, 75.86% were susceptible, 3.44% intermediate and 41.37% were resistant to various antimicrobial discs.

Of the antibiotics used, the highest percentage of susceptibility profile was recorded to Tetracycline (92.86%) followed Gentamycin (92.85%) and Erythromycin (64.28%), Meanwhile, the highest percentage of resistance profile was recorded against sulfamethoxazole trimethoprim (71.42%) and penicillin G (57.15%) (Table 4).

Multiple drug resistance phenotypes were profiled for 85.71% of *S. aurous* isolates. Of these, 21.42% was resistance against TE*PG*ER and 28.57% was resistance against TE*PG*ER*SMT. Among all MAR phenotypes of *S. aurous* isolates, 35.72% were resistant against five antimicrobials (Table 5).

The high incidence of mastitis begets an extensive use of antibiotics for its treatment and control (Owens *et al.*, 1991). In the present study, the overall prevalence of subclinical mastitis infection in the dairy farm was 58.5%. This result was lower than the study of (Mekibib *et al.*, 2010) who reported 71% prevalence in exotic dairy cows in Holeta areas.

The finding was higher than reports of (Addisu *et al.*, 2015) and (Abunna *et al.*, 2013) who reported the prevalence of subclinical mastitis in and around Batu town was 35% and 36.7% respectively. This discrepancies is due to variation could differences in breed and management practices, strain variability and susceptibility of the host. Quarter level subclinical mastitis infection was about 31% and this result was higher than the findings of (Ketema, 2015)) who reported the prevalence of 19.9% in infected quarters.

Table.1 Subclinical Mastitis diagnosis and *S. aurous* detection

No of screened cows (N=94) and status (%)		***S. aurous* detected (%)**
CMT positive (%)	55 (58.5)	14 (25.45)
CMT negative (%)	39 (41.5)	0
Overall infection	**55 (58.5)**	**14 (25.45)**

Table.2 Formulation of Multi-drug resistance (MDR) profiles of *S. aurous*

S/no	**Antibiotics used and antimicrobial sensitivity test results**				
	Tetracycline **(30μg)**	Penicillin G **(10ug)**	Erythromycin **(15μg)**	Gentamycin **(10μg**	Sulphamethazole trimethoprim **(25μg)**
1	S	R	S	S	S
2	S	S	I	S	R
3	S	R	R	S	S
4	R	S	S	R	R
5	S	R	R	S	S
6	S	S	S	S	R
7	S	R	R	S	R
8	R	S	S	S	R
9	S	R	S	S	R
10	S	R	S	S	R
11	S	R	S	S	R
12	S	S	S	S	R
13	S	R	R	S	I
14	S	S	S	S	R

Table.3 Multiple Antibiotic Resistance (MAR) Index

Isolates	**No. of antibiotics to which the isolate was resistant**	**MAR Index**
1	1	0.2
2	1	0.2
3	2	0.4
4	3	0.6
5	2	0.4
6	1	0.2
7	3	0.6
8	2	0.4
9	2	0.4
10	2	0.4
11	2	0.2
12	1	0.2
13	2	0.4
14	1	0.2

Table.4 Antimicrobial resistance profiles of *S. aurous* isolates from mastitic milk

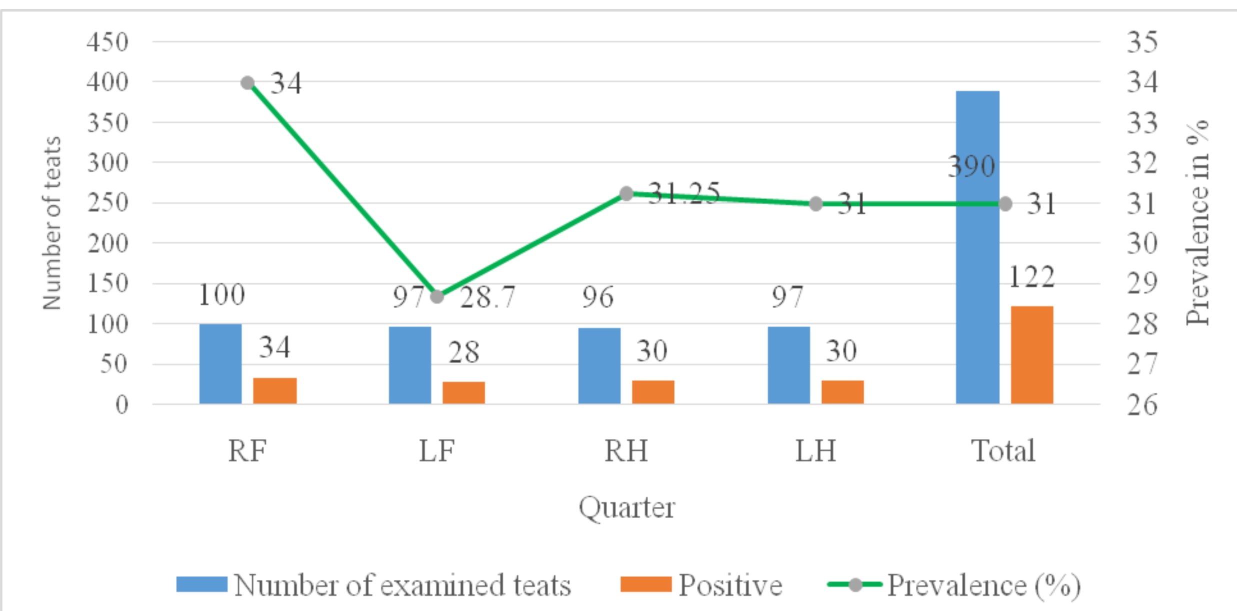
Antimicrobials	No of *S. aurous* isolated	Susceptible (%)	Intermediate (%)	Resistant (%)
Tetracycline (30µg)	14	13 (92.86)	0 (0)	1 (7.14)
Pencilline G (10µg)	14	6 (42.85)	0 (0)	8 (57.15)
Erythromycin (15µg)	14	9 (64.28)	1 (7.12)	4 (28.57)
sulfamethoxazole trimethoprim (25µg)	14	3 (21.42)	1 (7.12)	10 (71.42)
Gentamycin (10µg)	14	13 (92.85)	0(0)	1 (7.15)
Total		**75.86**	**3.44**	**41.37**

Table.5 Multi-drug resistance profiles of *S. aurous* isolated from cases of subclinical mastitis

(N=14)

MDR pattern	Phenotypic resistance (Phenotypes)	No. of isolates	Percentage (%)
III	TE *PG* ER	3	21.42
IV	TE*PG*ER*SMT	4	28.57
V	TE*PG*ER*SMT*GE	5	35.72
Total		**12**	**85.71**

Fig.1 On station prevalence of sub clinical mastitis infection



This could be due to vulnerability of hind quarters to contamination and the amount of milk produced enforces the opening of teat canal and allows the entry of pathogens into udder environmental. The reported prevalence (22.45%) of *S. aurous*, from screened positive cows was lower than the 26.78% reported from Bishoftu, Ethiopia (Berhe *et al.*, 2017) and 75% report from Bangladesh (Begum *et al.*, 2007).

The variation could be due acquisition of mobile genes from surrounding resistant strains of bacteria that may occur through insertion, transformation or other mechanisms and degree of udder inflammation.

The high susceptibility of *S. aurous* isolates to Tetracycline (92.86%) followed by gentamycin (92.85%) and erythromycin (64.28%) was in concordance with previous reports (Elemo *et al.*, (2017) recorded 95.3% susceptibility to gentamycin. On the other hand, *S. aurous* isolates exhibited the highest resistance against sulfamethoxazole trimethoprim (71.42%), Penicillin G (57.15%). Erythromycin (28.57%) followed by Tetracycline (7.14%). Elemo *et al.*, (2017) previously reported higher resistance against penicillin G (87.3%) and Tetracycline (82.2%) in and around Asella Township. The finding was also lower than the reports of Abebe *et al.*, (2013) who reported 94% and 73.8% resistance against penicillin G and tetracycline in and around Addis Ababa, Ethiopia. In another study (Alekish *et al.*, 2013) reported 87.4% and 84.5% of the *S. aurous* isolates were resistant against sulphamethoxazole and penicillin G respectively. The variation in drug susceptibility could be attributed to frequent use of drugs in the study areas. It could also be attributed to the production of beta lactamase enzyme by *S. aurous* that inactivates penicillin and related antibiotics. The beta-lactams are the drugs of choice for intramammary infections and inappropriate use of these drugs resulted in the emergence of resistant bacteria. Jaims *et al.*, (2002) noted that the emergence of resistant strains is associated with repeated and indiscriminate use of drugs and production of beta-lactamase by *S. aurous*. Consistent with the other reports from within the country (Gebreyohanes 2008) and abroad (Adesiyum *et al.*, 2007; Solomakos *et al.*, 2009) reported several strains of *S. aurous* isolates from milk and milk products showed multidrug resistance properties. The asymptomatic, healthy, animal carriers of MDR strains can serve as a potential threat for transmission to human (Oliver *et al.*, 2005) since these MDR *S. aurous* strains have acquired resistance to commonly used antibiotics, leading to treatment failures (Kumar *et al.*, 2010). The current result also showed that *S. aurous* isolates had 60% of MDR Index values. This could indicate that strains that had more than 0.2 MDR index originated and spread from a niche of high antibiotics use (Tambekar, 2006).

Recommendations

The study revealed that subclinical mastitis (58.5%) and multidrug resistant strains of *S. aurous* (25.45) was prevalent in dairy farms. The result showed that 75.86% of *aurous* were susceptible, 3.44% intermediate and 41.37% were resistant to various antimicrobial discs. The highest percentage of susceptibility profile was recorded to Tetracycline (92.86%) and Gentamycin (92.85%) whereas the highest percentage of resistance profile was recorded against sulfamethoxazole trimethoprim (71.42%) and penicillin G (57.15%). About 21.42% of *S. aurous* isolates were resistance against multiple drugs TE*PG*ER and 28.57% against TE*PG*ER*SMT. Excessive use of antimicrobials for prevention and treatment of diseases in dairy farms and inappropriate use dry cow therapy could contribute the development of drug resistant strains. Further studies are required to elucidate search of alternative antimicrobials and their effect on the induction and spread of resistant genes as well as standardization of therapeutic uses of antimicrobials, which ensures the right choice and use of drugs locally in the dairy farms.

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