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Antibiotic resistance of *Staphylococcus aureus* isolates from milk produced by smallholder dairy farmers in Mbeya Region, Tanzania

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Abstract

Aim: The study determined and evaluated the prevalence and antibiotic resistance of *Staphylococcus aureus* isolated from milk collected along the milk value chain from farm herds, milk collection center, and milk shops in Mbeya rural and Mbozi districts, Tanzania.

Materials and Methods: A total of 150 milk samples were collected; 96 from farmers' herds, 18 from milk collection centers, and 36 from milk shops. The samples were cultured in Mannitol salt agar for pathogen isolation and biochemical tests performed for confirmation of *S. aureus*. Kirby–Bauer disk diffusion method was employed for antibiotic resistance testing.

Results: One hundred and forty samples yielded *Staphylococcus* species; these were from farmer's herd (92), milk collection center (18), and milk shops (30), respectively. Biochemical tests showed that 21 (15%) were positive for *S. aureus*. The corresponding prevalence rates from the value chain nodes were 14.1%, 16.7%, and 16.7%, respectively. Resistance to penicillin was frequently observed (57.1%) and vancomycin was effective to all *S. aureus* isolates tested. Resistance along the sampling points showed a significant positive correlation ($r=0.82$, $p<0.0001$; $r=0.65$, $p<0.003$; and $r=0.61$, $p<0.01$) between farmers, milk collection points, and milk shops, respectively. More than half (57.1%) of the isolates exhibited resistance to three or more of the antibiotics used in this study. *S. aureus* isolates were shown to have a multiple antimicrobial resistance patterns, particularly with respect to penicillin, ampicillin, erythromycin, and tetracycline.

Conclusion: The level of staphylococcal isolates and the antibiotic resistance of *S. aureus* found in this study is an indication of subclinical mastitis, poor hygiene, and inappropriate use of antibiotics; therefore, education of farmers on subclinical mastitis control and proper use of antibiotics would be of benefits in these areas.

Keywords: milk contamination, milk products, multiple antibiotic resistant.

Introduction

Staphylococcus aureus is a facultative anaerobic Gram-positive bacterium. The majority of *S. aureus* strains are catalase-positive which constitute the pathogenic species. It is one of the most important causes of food poisoning worldwide [1]. Counts above 10^3 CFU/ml increase the risk of production of staphylococcal toxins that are resistant to boiling and pasteurization processes [2]. Food contamination with *S. aureus* may occur directly from infected food-producing animals or may result from poor hygiene during production processes, or the retail and storage of food. The pathogen is also significantly involved in nosocomial and community-acquired infections in humans. Furthermore, *S. aureus* is a major causative pathogen of clinical or subclinical mastitis of dairy animals [3,4]. Mastitis infections caused by *S. aureus* are estimated to be present in up to 90% of dairy farms and are responsible for 35% of the economic losses in the dairy industry [5].

Antibiotics have been widely used in the treatment of infection caused by *S. aureus* in farm animals [6] and their use has been linked to the spread of resistant bacteria to humans through the consumption of animal food products [7]. In addition, the extensive misuse of antibiotics in all settings has created strong selection pressure, which has resulted in the survival and persistence of resistant strains [8]. This poses a challenge to veterinarians, health professionals, and dairy cattle producers due to its negative impact on the response to antimicrobial therapy [9]. Studies carried out in different countries reported increased antimicrobial resistance among *S. aureus* isolates [10].

Several guidelines are available for the appropriate use of drugs in animals; however, little is being done in developing countries, particularly Tanzania,

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in terms of quality control and surveillance of antimicrobial resistance. Uncontrolled sale and use of veterinary drugs among livestock keepers are a common practice in developing countries and can result in increased resistant strains in the dairy industry. There is great concern about the use of veterinary drugs by livestock farmers who have no knowledge in treating cattle without first determining their infection status. Although antibiotics have been widely used for the treatment of bacterial infection in the dairy industry in Tanzania, there is limited literature on antibiotic resistance profile of *S. aureus* isolated from milk.

This study aimed to determine the antibiotic-resistant profile of *S. aureu*s isolated from raw milk in the Mbeya and Mbozi districts of Tanzania. The result of this study will provide baseline information on the resistance profile of antibiotic and guide for the selection of effective antibiotic in the study area.

Materials and Methods

Ethical approval and informed consent

The permission to carry out this study was granted by the Executive Directors of Mbeya and Mbozi Districts Councils. The Vice Chancellor of Sokoine University of Agriculture (SUA) issued a research permit letter on behalf of the Tanzania Commission for Science and Technology (COSTECH). Verbal consent was obtained from each of the respondent after explaining the purpose and importance of the study prior to start of data collection.

Study area

The study was done in Mbeya and Mbozi districts, Tanzania (Figure-1). The details of the study area, design, sample size estimation, sampling procedure, isolation of *S. aureus* from milk samples, and bacteria isolation procedures can be found in Massawe *et al.* [11]. The study was conducted during the wet season from March 2015 to June 2015.

Antibiotic susceptibility testing

Determination of antibiotic susceptibility was performed on 21 positive *S. aureus* isolates using Kirby–Bauer disk diffusion method as described by Clinical Laboratory Standards Institute guidelines [12] on Mueller-Hinton agar (Becton, France). Three to four fresh overnight colonies were dissolved in 10 ml of 0.9% saline; the density was pre-calibrated to 0.5 McFarland turbidity. The antimicrobial discs used in this study were from Oxoid Ltd., England and included penicillin (10 IU), ampicillin (10 µg), sulfamethoxazole (300 µg), vancomycin (30 µg), cloxacillin (10 µg), ciprofloxacin (15 µg), enrofloxacin, tetracycline (30 µg), chloramphenicol, and erythromycin (15 µg). The ATCC 25923 was used as reference strain as an

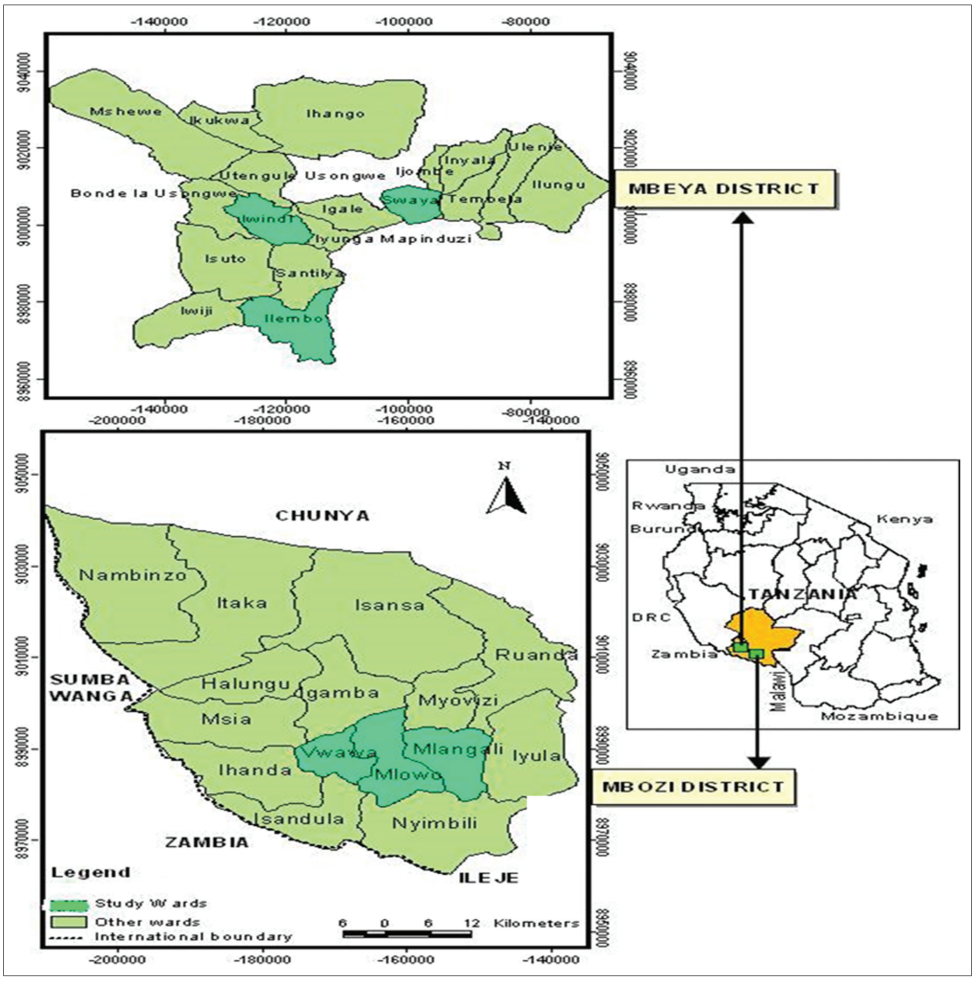

Figure-1: A map showing the study area in Mbeya region, Tanzania.

internal positive control, whereas sterile water was used as a negative control. The inhibition zone diameters were measured by a ruler and values obtained were interpreted using [12] and given the appropriate status; susceptible (s), intermediate (i), or resistance (R). Multiple antibiotic-resistant (MAR) phenotypes were recorded for isolates showing resistance to more than three antibiotics.

Statistical analysis

Analysis of antibiotic testing was performed by category agreement, whereby the zone of inhibition was divided into three categories (i.e., susceptible, intermediate, and resistant). Statistical significance of the differences in resistance was evaluated using the statistical analysis system [13]. Univariate analysis was performed to verify if the zone of inhibition was affected by a single antibiotic. Pearson correlation was conducted to assess the correlation of antimicrobial resistance between the sampling points. Furthermore, comparison of the zone of inhibition between the samples was done and p-value of p≤0.05 was considered a statistically significant difference.

Results

Of 140 milk samples analyzed (92, 18, and 30 from farmers herds, milk collection points, and milk shops, respectively), 21 (15%) samples were positive for *S. aureus*. Of the 21 samples 13 (14.1%), 3 (16.7%), and 5 (16.7%) were from farmers herds, milk collection points, and milk shops, respectively (Figure-2).

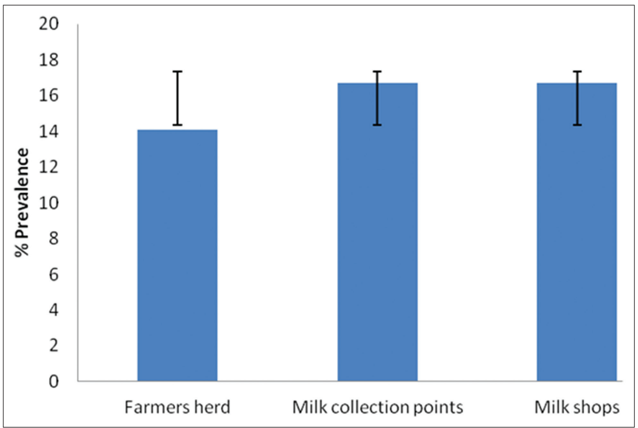

Figure-2: Prevalence of *Staphylococcus aureus* in different collection sites from cows' milk obtained from smallholder farms in Mbeya and Mbozi districts, Tanzania.

Antimicrobial resistance

The number of isolates tested for antimicrobial resistance was 21, 13 (61.9%) from Mbozi and 8 (38.1%) from Mbeya district. *S. aureus* was most resistant to penicillin (57.2%) followed by erythromycin (28.6%), chloramphenicol (19%), and tetracycline (19%) (Table-1). About 33.3% of isolates were resistant to all antibiotics tested. All isolates were sensitive to vancomycin (Table-1). 12 isolates showed intermediate responses to penicillin, ampicillin, erythromycin, enrofloxacin, cloxacillin, and tetracycline. Resistance along the sampling points showed significant positive correlation (r=0.82, p<0.0001; r=0.65, p<0.003; and r=0.61, p<0.01) between farmers, milk collection points, and milk shops, respectively (Table-2).

Multiple antibiotic resistance (MAR)

Twelve of 21 (57.1%) *S. aureus* isolates were resistant to three or more antibiotic (Table-3). Penicillin, ampicillin, erythromycin, and tetracycline were frequently observed among MAR patterns. Penicillin was observed in nine patterns, followed by ampicillin, erythromycin, and tetracycline (Table-4).

Antibiotic susceptibility

Regarding susceptibility of *S. aureus* to the antibiotic tested, the results showed that all *S. aureus* isolates were susceptible to vancomycin (Figure-3). Moreover, *S. aureus* isolates showed higher susceptibility to cloxacillin (95.2%), ciprofloxacin (95.2%), sulfamethoxazole (95.2%), enrofloxacin (85.7%), and chloramphenicol (81%).

Discussion

The prevalence of *S. aureus* in the milk showed an increased rate from farm level to milk collection points. Whereas similar prevalence was observed between milk collection points and milk shops, suggesting that good hygienic practices along each step

Table-1: Antibiotic drug resistance for *Staphylococcus aureus* isolates from cows' milk obtained from smallholder farms in Mbeya and Mbozi districts, Tanzania (n=21).

Antibiotics	Number of resistant isolates (%)	Number of intermediate isolates (%)	Number of susceptible isolates (%)
Ampicillin	3 (14.3)	2 (9.5)	16 (76.2)
Chloramphenicol	4 (19)	0 (0)	17 (81)
Enrofloxacin	1 (4.8)	2 (9.5)	18 (85.7)
Cloxacillin	0 (0)	1 (4.8)	20 (95.2)
Erythromycin	6 (28.6)	2 (9.5)	13 (61.9)
Ciprofloxacin	1 (4.8)	0 (0)	20 (95.2)
Penicillin	12 (57.2)	4 (19)	5 (23.8)
Sulfamethoxazole	1 (4.8)	0 (0)	20 (95.2)
Tetracycline	4 (19)	1 (4.8)	16 (76.2)
Vancomycin	0 (0)	0 (0)	21 (100)

The percentage presented in this table was obtained by dividing the number of a particular phenotype (resistance, intermediate, and susceptible) by the total number of *Staphylococcus aureus* isolates tested of the production chain are of great important and necessary to eliminate the contaminations of *S. aureus* in the chain. The prevalence of *S. aureus* in the present study is comparable with the study conducted in Ethiopia [14] reported the prevalence of 13.8%. Lower prevalence of 6.6% and 10.8% were reported in India [15] and Brazil [16], respectively. Conversely, a higher prevalence of 40%, 74.5%, and 100% has been reported in Morocco [17], India [18], and South Africa [19], respectively. The observed prevalence can be due to the presence of subclinically infected cows (mastitis) and negligence of hygienic conditions such as improper milking procedures, milk handling technique, and improper storage which increase *S. aureus* in the milk. The previous studies revealed that *S. aureus* infection originating from dairy products was of a public health concern worldwide [20,21] and among the reasons mentioned were improper food handling, unsanitary production environment, storage, transportation, and personnel cleanliness. Another reason could be subclinical mastitis because this pathogen has been frequently isolated from the cows with subclinical mastitis [3].

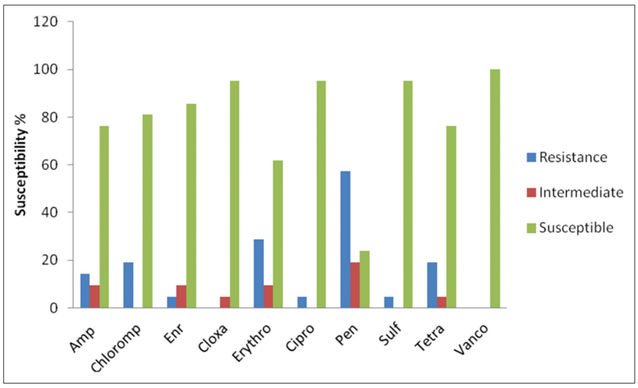

Figure-3: Susceptibility of *Staphylococcus aureus* from cows' milk obtained from smallholder dairy farms in Mbeya and Mbozi districts in Tanzania to different antibiotics NB. Erythro - Erythromycin, Pen - Penicillin, Tetra - Tetracycline, Amp - Ampicillin, Enr - Enrofloxacin, Chloramp - Chloramphenicol and Sulf - Sulfamethoxazole.

Table-2: Pearson correlation for the prevalence of antimicrobial resistance for *Staphylococcus aureus* isolates from the milk collected along the value chain (n=10).

Sample source	Farmers	MCP	MSHOP
Farmers	1		
MCP	0.8231***	1	
MSHOP	0.6555**	0.6121*	1

MCP=Milk collection points, MISHOP=Milk shops. ***Significance p<0.0001, **p<0.003, *p<0.01

The prevalence of *S. aureus* in milk collection points and milk shops was 16.7%. This finding is lower than that reported in Ethiopia [22] where a higher prevalence of 72% in milk collection centers was reported. Cross contamination and an increased number of milk handlers of milk while bulking could be the possible causes of increased prevalence in bulk milk in collection centers. Omore *et al.* [23] reported that contamination of milk increases with the number of agents handling milk before it reaches the final consumer. Higher prevalence of 48.2% in restaurants has been reported in Ethiopia [20] and their report showed an increase in the prevalence of *S. aureus* from farm bulk milk through milk shop to restaurants. Resistance at the farm level was higher than other sampling points. This suggests that there is frequent use of antimicrobial at the farm level, especially the treatment of mastitis which is a common infection in dairy animals. The results of the current study showed that the resistance along the production line was significantly positively correlated. This means that improper use of antimicrobial at the farm level could cause resistant strains to spread at other levels of production. This is a concern to the public health because handling and consumption of contaminated milk could provide a potential vehicle for transmission of resistant strain to human. Moreover, the results indicated that animal, human, and environmental sources could be involved in the contamination of dairy products along with their production chain.

In general, the resistance of *S. aureus* isolates to the antimicrobial tested was moderately high. Resistance to penicillin was more frequently observed than other drugs. This result is comparable with the study by Hamidi and Sylejmani [24], and Al-Thani and Al-Ali [25], who reported the resistance of 55.5% and 60%, respectively. Higher levels of resistance to penicillin have also been reported in studies by Gandhale *et al.* [26] and Ateba *et al.* [19] who reported the resistance of 91.5% and 100%, respectively. Resistance to erythromycin observed in this study is almost similar to that reported by Mohanta and Mazumder [27], who found resistance among 21.3% and 19.6% of isolates, respectively. Higher resistance of 61.5% was reported

Table-3: Frequency distribution of antibiotic resistance of *Staphylococcus aureus* isolates from cows' milk obtained from smallholder dairy farms in Mbeya and Mbozi districts, Tanzania (n=21).

District	Number of isolates (%) susceptible to all antibiotics tested	Number of isolates (%) resistant to 1-2 antibiotic	Number of isolates (%) resistant to 3-6 antibiotics
Mbeya	1 (4.8)	4 (19.0)	4 (19.0)
Mbozi	1 (4.8)	3 (14.3)	8 (38.1)
Total	2 (9.6)	7 (33.3)	12 (57.1)

The percentage presented in this table was obtained by dividing the number of antibiotic-resistant phenotype by the number of *Staphylococcus aureus* isolates tested

Table-4: The multiple antibiotic-resistant patterns for *Staphylococcus aureus* isolated from cows' milk obtained from smallholder farms in Mbeya and Mbozi districts in Tanzania.

Antibiotic pattern	Number of pattern (%)
Cloxa-Pen	1 (4.8)
Pen-Tetra	1 (4.8)
Chlora-Enr	1 (4.8)
Amp-Pen	1 (4.8)
Amp-Pen-Tetra	1 (4.8)
Amp-Chlora-Erythro-Pen-Sulf-Tetra	1 (4.8)
Amp-Chlora-Enr-Erythro-Pen	1 (4.8)
Amp-Erthro-Pen	2 (9.5)
Amp-Erythro-Pen-Tetra	2 (9.5)
Erythro-Pen	3 (14.3)

NB; Erythro=Erythromycin, Pen=Penicillin, Tetra=Tetracycline, Amp=Ampicillin, Enr=Enrofloxacin, Chloromp=Chloramphenicol and Sulf=Sulfamethoxazole. The percentage presented in this table was obtained by dividing the total number of multiple antibiotic resistant of a particular phenotype by the number of *Staphylococcus aureus* isolates tested

in Turkey [28]. However, lower resistances against chloramphenicol and tetracycline have been reported in Egypt [5] where 6.7% and 0% of *S. aureus* isolates were resistant to the respective drugs. In this study, lower resistance was observed against enrofloxacin, cloxacillin, and sulfamethoxazole. These results are in agreement with De Oliveira *et al.* [29] who reported that the level of resistance among *S. aureus* isolates was lower for these antimicrobial agents. The observed level of resistance against penicillin can be due to the fact that *S. aureus* is known to be naturally resistant to penicillin group of antibiotics due to β-lactamase production. The resistance against erythromycin can be due to the frequent use of this antibiotic in the study area.

Furthermore, the misuse of these drugs could be another reason since most animal keepers in the study area buy these medications and treat their animals without knowing the infection status of the herd or animal in question. The misuse of antibiotics may result in the building up of antimicrobial-resistant bacteria. Uncontrolled selling of antibiotics may be another factor which contributed to the problem because antibiotics were being sold in an open and unprotected environment with direct exposure to the sun. This may render the antimicrobials ineffective and, once used for the treatment, expose bacteria to suboptimal concentrations, thereby creating the conditions for the bacteria to develop resistance. It was found in the study area that livestock farmers keep the stock of expired antibiotics and use them to treat their animals (personal observation).

The study showed that none of the isolates were resistant to vancomycin. This finding is supported by an earlier report by Abo-Shama [5], who found that all *S. aureus* isolates were susceptible to vancomycin. The higher susceptibility shown to this drug could be due to the fact that vancomycin has not been used for the treatment of animals in the study area. On the other hand, isolates showed moderately low resistance to cloxacillin and ciprofloxacin. This is a concern because the resistant strain can be transmitted to human by consumption of milk and its products. Furthermore, the consumption of food carrying antibiotic-resistant bacteria can directly or indirectly result in the acquisition of antibiotic-resistant infections [7].

The frequency of MAR to three or more antibiotics was observed in more than half of the isolates tested. The results of this study are comparable with that reported in Brazil [30] and Ethiopia [31] where 64.4 and 45.1% of *S. aureus* isolates, respectively, resisted three or more antibiotics. In Ethiopia Firaol *et al.* [32] reported MAR of 87.6% which is higher compared to the result reported in this study. According to our results, penicillin, ampicillin, erythromycin, and tetracycline were the most frequently observed patterns. This reflects the use of these antibiotics in the study area, and it shows that *S. aureus* has been exposed to these drugs. Another possible reason for the observed pattern is the availability and price of these drugs. It was noticed that these drugs are widely available from agro-vet distributors and can be purchased easily without any prescription from an authorized facility.

Conclusion

S. aureus was isolated from milk samples collected in all milk marketing channels in the study area. This indicates poor hygiene in the milk marketing channel which is of great concern to the consumer's health. Moreover, isolates were resistant to the commonly used antibiotics tested. Education on the proper use, handling, and storage of antibiotics should be prioritized for livestock farmers and other drug users.

Authors' Contributions

HFM: Developed the concept, wrote the protocol, collected the data during fieldwork, drafted the manuscript, and final proofreading of the manuscript. RHM: Interpretation and description of analyzed data read and corrected the manuscript on technical and language part. LRK: Planned and coordination of the experiments during fieldwork and laboratory analysis and advised on the manuscript formatting. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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