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Research Article

Prevalence and antibiotic susceptibility pattern of methicillin-resistant *Staphylococcus aureus* isolated from bovine raw milk in Njombe region, Tanzania

George J. Sanga[1,2*], Athumani M. Lupindu[3] and Abubakar S. Hoza[1]

[1] Department of Veterinary Microbiology, Parasitology and Biotechnology, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P.O.BOX 3019, Chuo Kikuu, Morogoro, Tanzania
[2] SACIDS Foundation for One Health, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P.O.BOX 3015, Chuo Kikuu, Morogoro, Tanzania
[3] Department of Veterinary Medicine and Public health, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P.O.BOX 3021, Chuo Kikuu, Morogoro, Tanzania

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***Corresponding author:**
George J. Sanga
george.sanga@sua.ac.tz

Abstract

The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) creates a serious public health concern due to its ability to colonize and infect humans and animals. This cross-sectional study investigated the prevalence, antibiotic susceptibility pattern of *S. aureus* and MRSA isolated from bovine raw milk in the Njombe region, Tanzania. A total of 470 samples, including 389 raw milk samples collected at farm level, 57 raw milk samples from bulk milk-can at collection centers, and 24 swab samples from bulk milk cans. The samples were cultured on mannitol salt agar, presumptive colonies were sub-cultured onto blood agar for the isolation of *S. aureus* which was subsequently preliminarily confirmed using microbiological and biochemical tests. Further, confirmation of isolates was done using conventional PCR targeting the *gltB* gene for *S. aureus* and *mecA* gene for MRSA which was later sequenced. Isolates were tested for antibiotic susceptibility by using the disc agar diffusion method. The overall prevalence of *S. aureus* in the study was 22.6% (106/470), with 2.9% (14/470) being MRSA. Both *S. aureus* and MRSA showed high resistance to penicillin (74%, 8.5%) and ampicillin (78%, 11.3%), respectively. A total of 81 (77%) isolates were resistant to at least one antibiotic and 14 isolates (13.2%) showed multidrug-resistant (MDR); with frequent antibiotic resistance patterns being to cefoxitin, penicillin, ciprofloxacin, tetracycline, and erythromycin. In conclusion, the prevalence and the MDR patterns exhibited by *S. aureus* and MRSA observed in this study provide baseline data for planning mitigation measures to safeguard public health.

Keywords: Raw milk, Njombe, Tanzania, *mecA*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA)

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Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive zoonotic bacterium involved in a wide range of human and animal diseases. Its pathogenicity is particularly associated with an aggregate of genetic traits mediating virulence, invasive capacity, immune evasion, and antibiotic resistance (Chua et al., 2014). *S. aureus* is related to intramammary infections (IMI) in dairy ruminants, inflicting both clinical and subclinical forms of mastitis accompanied by economic losses due to reduced milk production and quality (Bergonier et al., 2003). Methicillin-resistant *S. aureus* (MRSA) are strains that are resistant to beta-lactam antibiotics conferred by the acquisition of a mobile genetic element, the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) carrying the *mecA* gene which encodes an altered penicillin-binding protein (PBP–PBP2A/PBP20) (Paterson et al., 2014).

The emergence of livestock-associated MRSA is increasingly reported worldwide, with rising concern for the risks of zoonotic transmission not only for people with occupational livestock exposure but also the introduction of the strains in the community through the food chain (Kluytmans, 2010). The resultant is higher treatment costs, longer treatment time, and higher rates of hospitalization and comorbidities (Haran et al., 2012). In Uganda, studies on MRSA contamination to milk and milk products have been done and high preva-

lence has been reported, such as a 9.6% prevalence of MRSA in raw milk and its products in cattle (Asiimwe et al., 2017). Similarly, a study in Kenya by (Omwenga et al., 2021) reported a 4.6% prevalence of MRSA in raw milk. Although dairy farming practices in Uganda, Kenya, and Tanzania are similar (Bingi and Tondel, 2015), a relatively low prevalence of 2.5% of MRSA in bovine raw milk is reported in Tanzania (Mohammed et al., 2018).

Nevertheless, there is limited data on the prevalence of MRSA along the bovine dairy supply chain, especially in the Njombe region. Therefore, the current study aimed to determine the prevalence and the antimicrobial susceptibility pattern of MRSA isolated from bovine raw milk and milk cans in the Njombe region, Tanzania.

Materials and Methods

Study location and design

A cross-sectional study was carried out in four districts of Njombe Town council, Makambako Town council, Njombe District Council, and Wanging'ombe District Council in Njombe region, Tanzania from November 2020 to March 2021 (Figure 1).

Ethical approval

This study was approved by the institutional review board of Sokoine University of Agriculture (Ref. SUA/DRPTC/R/186.VOL.III). Also, farmers gave verbal or written consent before sampling their cows.

Sample size and collection

A total of 470 samples were used in this study, including 389 milk samples collected at farm level, 57 milk samples collected from bulk milk cans at collection centers, and 24 swab samples collected from milk collection cans. The milk samples collected from the farms were aseptically pooled from all teats of individual lactating dairy cows to obtain one sample per farm. Briefly, the cow's udder was cleaned with warm water and dried with a clean cloth. The fore strips from each quarter were discarded, and 20 ml of milk were then collected from each lactating cow per farm to form composite milk from which 25 ml was collected into a sterile universal bottle to form one sample per farm.

At the milk collection centers constituting milk of several dairy farmers, cleaned milk collection cans were aseptically swabbed before the collection of milk (one swab was taken per each can), then one milk sample (25 ml) was collected from the bulk milk can using a sterile universal bottle. All samples were then packed in the cool box and transported to the laboratory for bacteriological culture.

Bacterial isolation and identification

For isolation of *S. aureus*, fresh milk samples were cultured on Mannitol salt agar (Liofilchem, Italy) and incubated at 37℃ for 24hrs. The characteristic yellow colonies grown on the Mannitol salt agar were subcultured onto the blood agar (Liofilchem, Italy) and incubated at 37℃ for another 24hrs to check for hemolysis. Identification of *Staphylococcus* species was preliminary done using colony morphology and Gram staining, followed by biochemical tests including catalase test using 3% hydrogen peroxide and a slide coagulase test using 1% rabbit plasma. Isolates showing a positive reaction to both catalase and coagulase tests were preliminarily considered as *S. aureus* and were stored in nutrient agar slants at -20℃ for further bacteriological and molecular analysis.

Antibiotic susceptibility testing (AST)

The *S. aureus* isolates that were confirmed biochemically were then screened for methicillin resistance by Kirby–Bauer disc diffusion method using cefoxitin antibiotic disc to identify the MRSA strains according to the Clinical and Laboratory Standards guidelines (CLSI, 2020). Isolates were further tested for susceptibility to commonly used antibiotics for veterinary and human medications in Tanzania (MHCDGEC, 2019). Antibiotic discs used were; cefoxitin (30 µg), penicillin (10 µg), clindamycin (2 µg), erythromycin (15 µg), ciprofloxacin (5 µg), tetracycline (30 µg), gentamicin (10 µg), ampicillin (10 µg) and trimethoprim/sulfamethoxazole SXT (1.25/23.75 µg) (Oxoid, Basingstoke, UK).

Briefly, two to three *S. aureus* colonies were added into sterile normal saline to form the suspension which was adjusted to achieve turbidity equivalent to 0.5 McFarland standard solution. The suspension was cultured by streaking onto the Mueller-Hinton (Liofilchem, Italy) agar plates followed by placing of antibiotic discs as described by Bonjean et al. (2016). Plates were incubated at 37°C for 16 to 18 hrs. *S. aureus* ATCC 25923 was used as a quality control strain. The zone of inhibition was measured in millimeters using a metric ruler and results were interpreted according to standard guidelines described in Clinical and Laboratory Standards Institute 30th Edition of 2020 (CLSI, 2020).

DNA extraction and molecular identification of MRSA

The *S. aureus* genomic DNA was extracted by boiling method as previously described (Zhang et al., 2004). Briefly, a loopful of *S. aureus* colonies was added into 1.5 ml Eppendorf tubes containing 500 µl nuclease-free water. The tubes were spined for 30 sec for thorough mixing, then boiled in a water bath at 95℃ for 10 min, followed by centrifugation at 15,000 ×g for 5 min. 3 µl of supernatant were used as a template in a 20 µl PCR mixture.

PCR confirmation of *S. aureus* and detection of *mecA*

PCR detection of *S. aureus* and MRSA was performed using primer sets coding for 108bp region of an enzyme glutamate synthase (*gltB*) and 147 bp region for *mecA* gene respectively (Macrogen Inc., Seoul, South Korea).

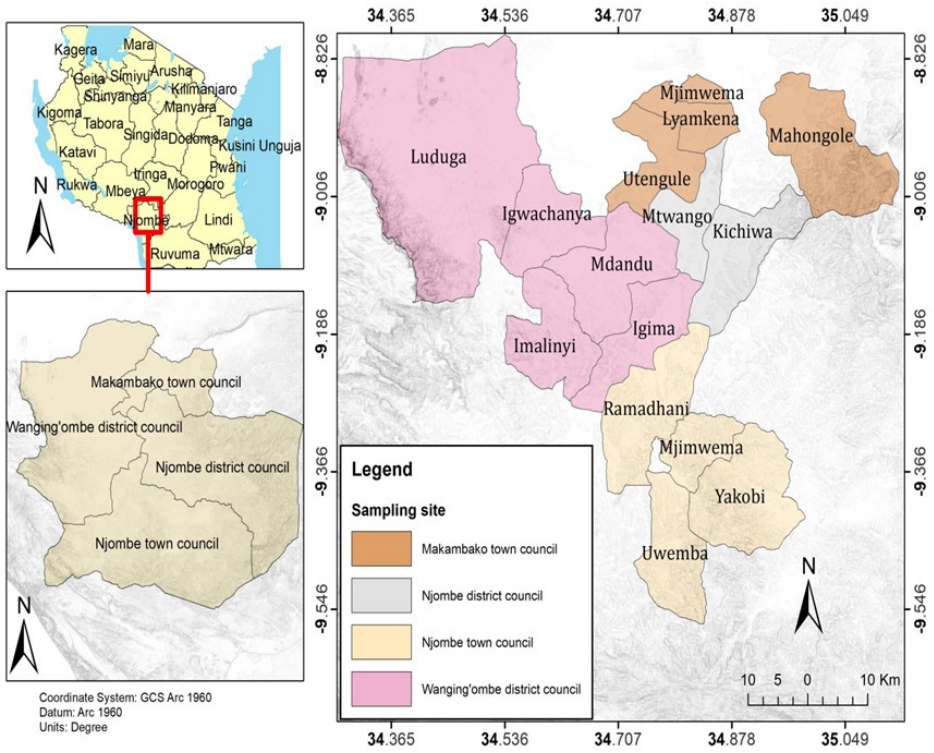

Figure 1: A map showing the study districts and their respective wards in Njombe region, Tanzania

The PCR mixture consisted of 10 µl of the master mix, 6 µl of nuclease-free water, 0.5 µl of each primer, and 3 µl of the genomic DNA template. Thermal cycling conditions for *S. aureus* (*gltB* gene) were; initial denaturation at 95℃ for 5 min, then 35 cycles of 95℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 30 sec, with a final extension at 72℃ for 5 min and hold at 4℃. In the case of *mecA* gene for MRSA identification, the thermal cycling conditions were; initial denaturation at 94℃ for 5 min then 30 cycles of 94℃ for 1 min, 50℃ for 1min, and 72℃ for 2min, with a final extension at 72℃ for 10 min and hold at 4℃ as previously described (Zhang et al., 2005).

PCR products were visualized using 1.5% Agarose gel electrophoresis containing GelRred (x10,000) in 1x Tris-Acetate- EDTA (TAE) buffer at 100V for 30 min as described by (Martineau et al., 1998) with minor modifications, as the used agarose gel concentration was 1.5% instead of 2%, Tris-acetate-EDTA used instead of Tris-borate EDTA, GelRed instead of Ethidium bromide and 100V was used for 30 min instead of 170V for 15min. DNA was visualized and documented using a Bio-Rad Gel Doc EZ Imaging system.

Sequencing of *mecA*

PCR products (15 µl for each *mecA* positive sample) were denatured at 95°C for 10 min, packed, and sent for sequencing (Macrogen Inc., Seoul, South Korea). PCR products were purified and sequenced using a cycle dideoxy nucleotide sequencer AB13710 (Applied Biosystems, Carlsbad, CA). The quality of the sequenced nucleotides was examined and edited by the Sequence Scanner software version 2 from Applied Biosystems biotechnology company. A reliable consensus sequence of the *mecA* gene was generated using Bio edit software 7.0.5.3. The consensus sequence was blasted in NCBI (Nucleotide blasting) GenBank to determine the homology with other *S. aureus* carrying *mecA* gene deposited sequences in the NCBI GenBank. The consensus sequence was further submitted into the ExPASy translation tool to determine the amino acids sequences. The amino acids sequence was blasted using NCBI Protein blast to determine the homologous protein from the GenBank.

Data analysis

Data were stored in a Microsoft Excel spreadsheet and analyzed using R 3.5.1 software. Briefly, R3.5.1 software was used for computing proportions and percentages of variables, also, for Chi-square and Logistic regression analysis to determine the association between the outcome variable (*S. aureus* occurrence) and explanatory variables (sample source, district). All results at $p<0.05$ were considered statistically significant.

Results and discussion

Out of 470 samples (raw milk and swab samples) collected in this study, 106 (22.6%) were PCR confirmed as *S. aureus* isolates (Figure 2). The prevalence observed in the current study is in agreement with a study from Ethiopia (23.4%) (Ayele et al., 2017), and higher than the prevalence reported in a study from Mbeya, Tanzania (16.7%) (Massawe et al., 2019). The current study has also shown a slightly lower contamination rate as compared to a prevalence of 49% at

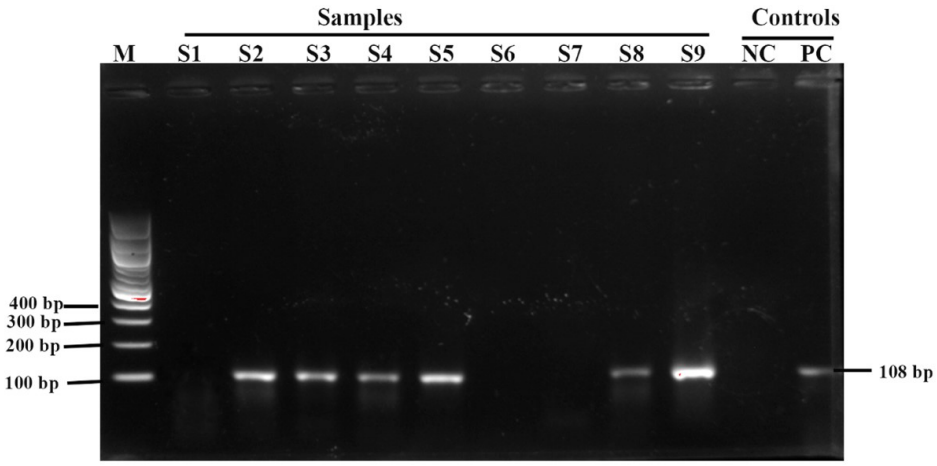

Figure 2: PCR detection of *S. aureus*, M- DNA ladder, samples S2, S3, S4, S5, S8, and S9 positive for *S. aureus* target gene (*gltB*) at 108 bp, while S6 and S7 negative for *gltB*, NC and PC negative and positive controls respectively.

Table 1: Antibiotic susceptibility pattern for *S. aureus* (n=106) and MRSA (n=14) isolates.

Antibiotics	**Susceptible (%)**	**Intermediate (%)**	**Resistant (%)**	***mecA* MRSA (%)**
Cefoxitin	88 (83)	0	18 (17)	6 (5.7)
Penicillin G	28 (26)	0	78 (74)	12 (11.3)
Clindamycin	85 (80)	13 (12)	8 (8)	1 (0.9)
Ciprofloxacin	99 (93.3)	1 (1)	6 (5.7)	2 (1.9)
Tetracycline	79 (74.5)	6 (5.7)	21 (19.8)	2 (1.9)
Erythromycin	86 (81.2)	10 (9.4)	10 (9.4)	0
Gentamicin	106 (100)	0	0	0
Ampicillin	23 (22)	0	83 (78)	9 (8.5)
Trimethoprim-sulfamethoxazole	103 (97)	0	3 (3)	0

Sokoine University of Agriculture dairy farms, Tanzania (Kashoma et al., 2015), 41% in local markets Morogoro Municipality, Tanzania (Mohammed et al., 2018), 32% in selected dairies in Algeria (Matallah et al., 2019) and 31.2% in dairy and pastoral farms in Morogoro, Tanzania (Kalee et al., 2021). The observed *S. aureus* prevalence in the current study can be due to the presence of subclinically infected cows, unhygienic milking and milk handling practices.

Nevertheless, the study found a 20/57 (35%) prevalence of *S. aureus* in bulk can milk at collection centers which is slightly higher than 28.9% in a similar study in China (Zhao et al., 2021) and slightly lower than 37.32% in a study in Turkey (Keyvan et al., 2020). Cross-contamination and increased number of handlers of milk while bulking could also be contributing factors of increased *S. aureus* prevalence in this study. Research shows that contamination increases with the number of agents handling the milk before it reaches the final consumer (Omore et al., 2004).

Furthermore, findings from this study revealed that 25% of milk collection cans were contaminated with *S. aureus* before milk collection. This may be due to improper washing of the milk cans or the use of unpotable water before and after milk collection resulting in a high prevalence observed at milk collection centers. Additionally, there was a statistically significant association between *S. aureus* isolation and the sources of milk samples such that, the chance of isolating *S. aureus* from milk samples collected from milk collection centers was two times higher than that from farm level ($p<0.04$).

With regards to MRSA, a total of 18/106 (17%) *S. aureus* isolates were resistant to cefoxitin hence phenotypically identified as MRSA (Table 1). However, PCR confirmed 14/106 (13.2%) *S. aureus* isolates that

Table 2: Multidrug-resistant patterns of *S. aureus* and MRSA.

Antibiotic pattern[1]	Number (%) of *S. aureus* isolates	Number of MRSA	Number of antibiotic classes
Pen-Cip-Ery	1 (7.14)	0	3
Cef-Pen-Clin	1 (7.14)	1	3
Clin-Ery-Amp	1 (7.14)	0	3
Pen-Cip-Tet	1 (7.14)	1	3
Cef-Pen-SXT	1 (7.14)	0	3
Pen-Clin-SXT	1 (7.14)	0	3
Pen-Tet-Ery	2 (14.3)	0	3
Cef-Pen-Tet	1 (7.14)	1	3
Cef-Pen-Clin, Tet	1 (7.14)	0	4
Cef-Pen-Cip-Ery	1 (7.14)	1	4
Cef-Pen-Cip-Tet	2 (14.3)	1	4
Cef-Clin-Tet-Amp	1 (7.14)	0	4
Total	14 (100)	5	–

[1]Pen=Penicillin G, Cip=Ciprofloxacin, Ery=Erythromycin, Cef=Cefoxitin, Clin=Clindamycin, Amp=Ampicillin, Tet=Tetracycline, SXT=Sulfamethoxazole/Trimethoprim.

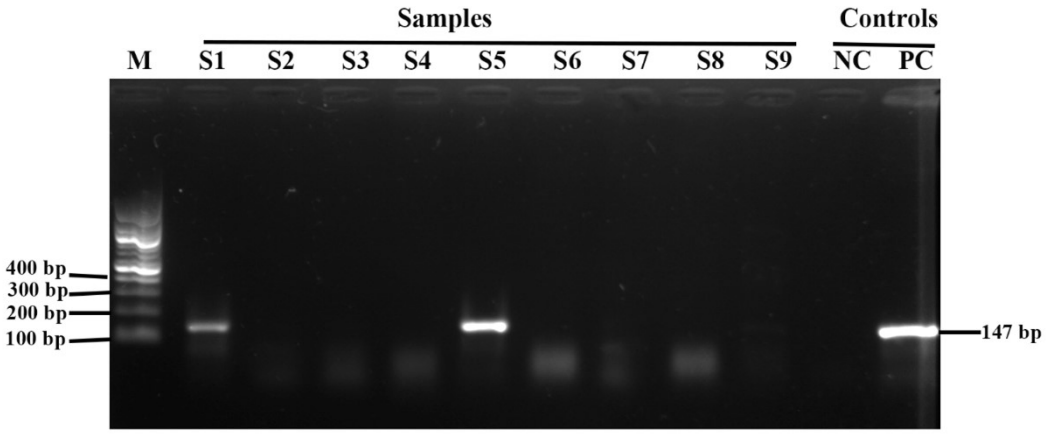

Figure 3: PCR detection of *mecA* gene, M- DNA ladder S2, S3, S4, S6, S7, S8, and S9 negative for *mecA*; S1 and S5 positive for *mecA* at 147 bp, NC and PC negative and positive controls respectively.

were harboring the *mecA* gene (Figure 3). Only 6/106 (5.7%) *S. aureus* isolates were resistant to cefoxitin, and they were carrying the *mecA* gene as well (Table 1). This finding shows that majority of phenotypic methicillin resistance *S. aureus* isolates were not carrying the *mecA* gene. It is not surprising because there are other mechanisms (non–*mecA* gene mechanisms) that are mediating methicillin resistance which may account for the observed scenario (Peacock and Paterson, 2015). Therefore, it should be well understood that the overall resistance level in a population of MRSA depends on the efficient production of PBP-2a which is modulated by a variety of chromosomal and extra-chromosomal factors (Appelbaum, 2007).

MRSA has increasingly been recognized in farm animal populations throughout recent years (Vanderhaeghen et al., 2010). In this study, out of 470 of the collected samples, 14 (2.9%) showed MRSA contamination. Our results indicated a low prevalence of MRSA in bovine raw milk and milk cans, which concurred with the previous investigations, e.g. 1.7% by Riva et al. (2015); 2% by Jamali et al. (2015); Parisi et al. (2016), and Mohammed et al. (2018). However, Asiimwe et al. (2017) and Omwenga et al. (2021), reported a high-level prevalence of 9.6% in raw milk from the pastoral community in South-West Uganda and 4.6% in bovine raw milk from Northern, Kenya respectively. The lower MRSA prevalence in this study is due to the fact that the majority of the *S. aureus* isolates were not carrying the *mecA* gene.

Sequencing analysis was done for further confirmation of *mecA* gene carriage by the *S. aureus* isolates, a blast analysis revealed that nucleotides for *S. aureus* isolated from bovine raw milk in Njombe region was 96.60% identical to *S. aureus* strain DUVASU/MRSA-10 (MH113821) and *S. aureus* strain DUVASU/MRSA-9 (MH113820) deposited in the National Center for Biotechnology Information (NCBI) sequence database. Similarly, the nucleotides yielded amino acids sequence which upon blasting was 100.00%

homology to methicillin resistance protein subunit A (ASR 18389.1) and penicillin-binding protein 2A (QCTC24437.1) from the GenBank. This result confirms the presence of the *mecA* gene in *S. aureus* isolates from bovine milk.

Results of antibiotic susceptibility testing in this study revealed that both *S. aureus* and MRSA isolates showed high resistance rates to ampicillin (78%, 8.5%) and penicillin (74%, 11.3%) respectively (Table 2). These findings are in agreement with previous studies in South Africa (Schmidt, 2011), Northern Ethiopia (Haftu et al., 2012), and Tanzania (Mohammed et al., 2018; Massawe et al., 2019) who reported high resistance of *S. aureus* including MRSA strains to ampicillin and penicillin. This can be explained by the fact that *S. aureus* is known to be naturally resistant to the penicillin group of antibiotics due to β-lactamase production, also frequent use of these antibiotics for treatment regimes in the region could be a plausible cause of high resistant rates in Njombe. Also, the current study revealed low resistance of *S. aureus* to sulphamethoxazole/trimethoprim (SXT) (3%) and ciprofloxacin (5.7%) which compare with the findings from the studies done by Schmidt (2011) and Massawe et al. (2019) who reported a low prevalence of *S. aureus* against SXT and ciprofloxacin respectively.

The frequency of resistance to three or more antibiotics (MDR) was observed in 14/106 (13.2%) *S. aureus* isolates tested (Table 2). The results of this study are comparable with those reported by Jamali et al. (2015) and Mohammed et al. (2018), where 15.4% and 26.1% of *S. aureus* isolates, respectively were resistant to three and more antibiotics. According to the findings from this study, the most frequent antibiotic resistance pattern exhibited by *S. aureus* isolates was that of cefoxitin, penicillin, ciprofloxacin, tetracycline, and erythromycin. This reflects the frequent use of these antibiotics in animals and humans in the study area, the finding which is in agreement with studies by Kimera et al. (2020) and Sindato et al. (2020).

Conclusion

The prevalence, antibiotic resistance patterns of *S. aureus* and MRSA observed in this study have several implications including (i) contamination of milk with MRSA (ii) lack of awareness on antimicrobial resistance among dairy farmers, and (iii) improper use of antibiotics in dairy farms. Therefore, we recommend providing training on good hygiene practices as well as antimicrobial resistance awareness campaigns especially on the proper use of antimicrobials to be provided to the stakeholders in the dairy supply chain. Further studies should be performed to characterize the MRSA isolates obtained in the current study to add information on clonal relatedness, phylogenetic pathways, and genetic diversity along with tracking the spread of MRSA infections, which are imperative for active surveillance, and controlling of the spread of MRSA.

Article Information

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Conflict of Interest. The authors have no conflict of interest to declare.

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