Prevalence, Associated Risk Factors and Drug Susceptible Pattern of *Staphylococcus aureus* from Bovine Milk in Assosa Town, Benishangul Gumuz Regional State

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Abstract: *Staphylococcus aureus* is pathogenic bacterium contaminating milk and milk products causing food poisoning primarily due to its enterotoxins. A cross - sectional study design was conducted from November 2023 to May 2024 in Assosa administrative town, Benishangul Gumuz Region, Ethiopia, to estimate prevalence, risk factors, public health significance and antimicrobial susceptibility pattern of *S.aureus* from cows' milk. A cross-sectional study was employed to collect a total of 384 samples; of this 134 milk sample, 134 udder swabs, 52 hand swabs, and 64 container swabs were included. Multivariable binary Logistic regression analysis of the effect of different risk factors on the prevalence of *S.aureus* was performed. Isolation and identification of *S. aureus* were carried out following standard microbiological techniques. There was statistically a significant difference ($P < 0.05$) in isolation of *S.aureus* among isolates from different sources and factors (udder swab, parity, age, BCS, udder washing, previous udder treatment and drainage). The study has also showed relatively a higher contamination rate of *S.aureus* from udder swab. From the total of 384 samples examined, the overall prevalence of *S. aureus* was 73/384 (19.01%). The prevalence of *S. aureus* in cow milk, udder swab, hand swab and container swab were 10.47%, 24.63%, 23.08% and 15.62% respectively. Based on availability of antibiotic disc out of 73 identified *S.aureus*, 36 isolates were subjected to antimicrobial susceptibility tests for seven selected antibiotic discs available in the market. The isolates were highly susceptible to Ciprofloxacin (100%) and Gentamicin (100%) followed by Cefoxitin (94%) and Chloramphenicol (94%). However, they were highly resistant to penicillin (100%) and Amoxicillin (92%). Lack of stringent regulation and monitoring in the dispensing and use of antimicrobials in the area might contribute to the occurrence of high antimicrobial resistance to these drugs. An attempt was made to assess the milk handling practices and consumption behavior of actors using semi structured questionnaire survey that include farmers, consumers and hotel/café owners. In general, the study has revealed the possibility of public health risk posed by *S.aureus* in Assosa town. Creation of public awareness about good milk handling practices, pasteurization or boiling of milk prior to consumption, rational use of drugs and periodic assessment of the antimicrobial sensitivity of drugs prior to use is recommended. [Fesseha Kelemework **Prevalence, Associated Risk Factors and Drug Susceptible Pattern of *Staphylococcus aureus* from Bovine Milk in Assosa Town, Benishangul Gumuz Regional State.**
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1. INTRODUCTION

1.1. Background of the Study

In developing countries, food-borne infections constitute the major cause of sickness and death. Food-related illnesses are caused by changes in eating patterns, mass catering, and improper food true in developing countries like Ethiopia, where the production of milk and various dairy products often occurs under unsanitary conditions and the consumption of raw milk is common. *Staphylococcus aureus* is pathogenic bacterium-contaminating milk and milk products causing food poisoning primarily due to its enterotoxins. Milk is one of the most important foods for human beings and also universally recognized as a complete diet due to its essential components (Getachew, 2018). However, health risk to consumers can be associated with milk, due to the presence of zoonotic pathogens and antimicrobial drug residues (Regasa, 2019). Milk is an excellent bacteriological medium for a large number of microorganisms, including *Staphylococcus aureus*. When milk is drawn from the udder of a healthy animal, it contains organisms that have entered the teat canal through its opening. *S.aureus* bacteria are mechanically flushed out during milking. The presence of foodborne pathogens in milk and milk product is due to direct contact with contaminated sources in the dairy farm environment, contaminated milking equipment, the hands of the milker's and excretion from the udder of an infected animal. The contamination of milk with pathogenic bacteria has

been known to occur mainly due to unhygienic way of handling and processing (Megersa, 2021).

Staphylococcus aureus in raw milk comes from cows with mastitis, from handlers or from deficient hygiene. When found in milk, high levels of contamination can be reached quickly under favorable conditions. Its presence in foods can be a risk to human health, causing a public health problem, as these bacteria produces toxins that can cause toxic food infections. The capacity to coagulate plasma, the principal characteristic of the *S.aureus*, is highly correlated to the capacity to produce enterotoxins harmful to the tissues of the contaminated host (Tassew *et al*., 2017).

Staphylococcus aureus mastitis is a serious problem in dairy production and infected animals may contaminate bulk milk. *S.aureus* is still an important cause of food borne intoxications worldwide. The ability of *S.aureus* to grow and produce *staphylococcal* enterotoxins (SEs) under a wide range of conditions is evident from the variety of foods implicated in *staphylococcal* food poisoning (SFP). SFP is suspected when the symptoms including nausea, violent vomiting, abdominal cramps and diarrhea affect the patients between 1 and 8 h after food consumption. In addition, the prevalence of mastitis and its associated pathogens in animals can be reduced by improving on the farm management techniques within the dairy industry. It is thus of paramount importance to ensure that proper hygienic practices are enforced in both the area where the animals are kept and the milking environment. Although it is difficult to control mastitis caused by *S.aureus* with antibiotics only, various antimicrobial agents antifungals are constantly being used to treat this disease in cattle. This practice results in the development of antibiotic-resistant strains. The usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains (Tarekegne, 2016).

The prevalence of antibiotic resistance usually varies between isolates from the different sampled stations and even between isolates from different herds on the same farm. Consequently, the good quality milk is a challenge that can be overcome provided that basic care is taken at the source of production. The importance of microorganisms in the milk means that their microbial contamination index can be used to judge the quality, as well as the sanitary conditions of its production and the health of the herd (Abebe, 2013).

1.2. Statement of the Problem

The risk factor and antimicrobial resistance of *S.aureus* poses a severe challenge to both veterinary and health professions and dairy cattle producers because of their negative impact on therapy (Megersa, 2021). Therefore, determination of levels of *S.aureus* and an evaluation of the antibiotic-resistant phenotypes of the isolates could serve as a tool for determining the hygiene standards implemented during milking. Data on antibiotic resistance could also be used to characterize these opportunistic pathogens, which may further limit the risks associated with the consumption of contaminated milk and its products. There is few published data about *S.aureus* isolated from Mastitis Lactating Cows of 151/384(39.32 %) 85/384(22.14%) *S.aureus* were isolated and identified. The proportional prevalence of *S.aureus* species was 85/151(56.29%) in and around Assosa Administrative town. From a total of 85 isolates of *S.aureus* obtained from the study antimicrobial susceptibility tests were performed on 63 isolates. 77.19 % of the *S.aureus* was found to be resistance to Cefoxitin, which shows the prevalence of MRSA. The resistance profile of Penicillin G, Tetracycline, Streptomycin, Gentamycin, Vancomycin, Clindamycin and Bacitracin were 95.55%, 63.4%, 60.78%, 59.37%, 56.75%, 54.35 and 53.65 %, respectively. *S.aureus* were found to be highly susceptible to Chloramphenicol (77.27%), Cloxacillin (70.58%), Trimethoprim-sulfamethoxazole (65.0%) and followed by Kanamycin (58.62%) (Tassew *et al*., 2017). However, there is no previous work done on the prevalence and susceptibility assay specifically of *S.aureus* from lactating dairy cows' raw milk in Assosa Administrative town. So, the study will carried out to estimate the Prevalence, Public Health Significance, Associated Risk Factors and Antimicrobial susceptibility Profile of *S.aureus* from Cows' Milk could play significant role in the control and prevention of pathogen in Assosa Administrative Town.

1.3. Basic Research Questions

- What is the prevalence of *S.aureus* in Assosa town from bovine milk?
- What are the potential risk factors of diseases and Public health significance?
- What is status of drug susceptibility pattern of *S.aureus?*

1.4. Objectives of the Study

1.4.1. General objective

Estimate of Prevalence, Associated Risk Factors and Antimicrobial Susceptibility Profile of *Staphylococcus aureus* Isolated from Cows' Milk with Public Health Significance in Assosa Administrative Town, Benishangul Gumuz Regional State, Ethiopia

1.4.2. Specific objectives

- To estimate the prevalence of *S.aureus* from cows' milk in Assosa town.
- To assess associated risk factors for occurrence of *S.aureus* with Public Health Significance in the study area.
- To determine the antimicrobial susceptibility profile of *S.aureus* in the study area.

2. MATERIAL AND METHODS

2.1. Description of the Study Area

The study was conducted from November 2023 to May 2024 in Assosa administrative town. Assosa is the capital city of Benishangul Gumuz Regional State which is located at 8°30′ and 40°27′ N latitude and 34°21′ and 39°1′ E longitude, 687 km western part of Ethiopia (CSA, 2021). It has an altitude ranges from 580 to over 1,560 m above sea level with the average annual rainfall of the town is 850–1316 mm with unimodal type of rainfall that occurs between April and October. The area is characterized by low land plane agro-ecology with mean annual temperature ranges between 16.75°C and 36°C which has 'kola' micro climate with land coverage of 2,317 km^2 areas (NMSA, 2014). The estimated total human population of the town had 66,062 of whom 33,947 were men and 32,115 were women (CSA, 2021). The town total Cattle population of 1,200 that supply milk and meat (Source: Assosa town Agricultural Directive, 2023).

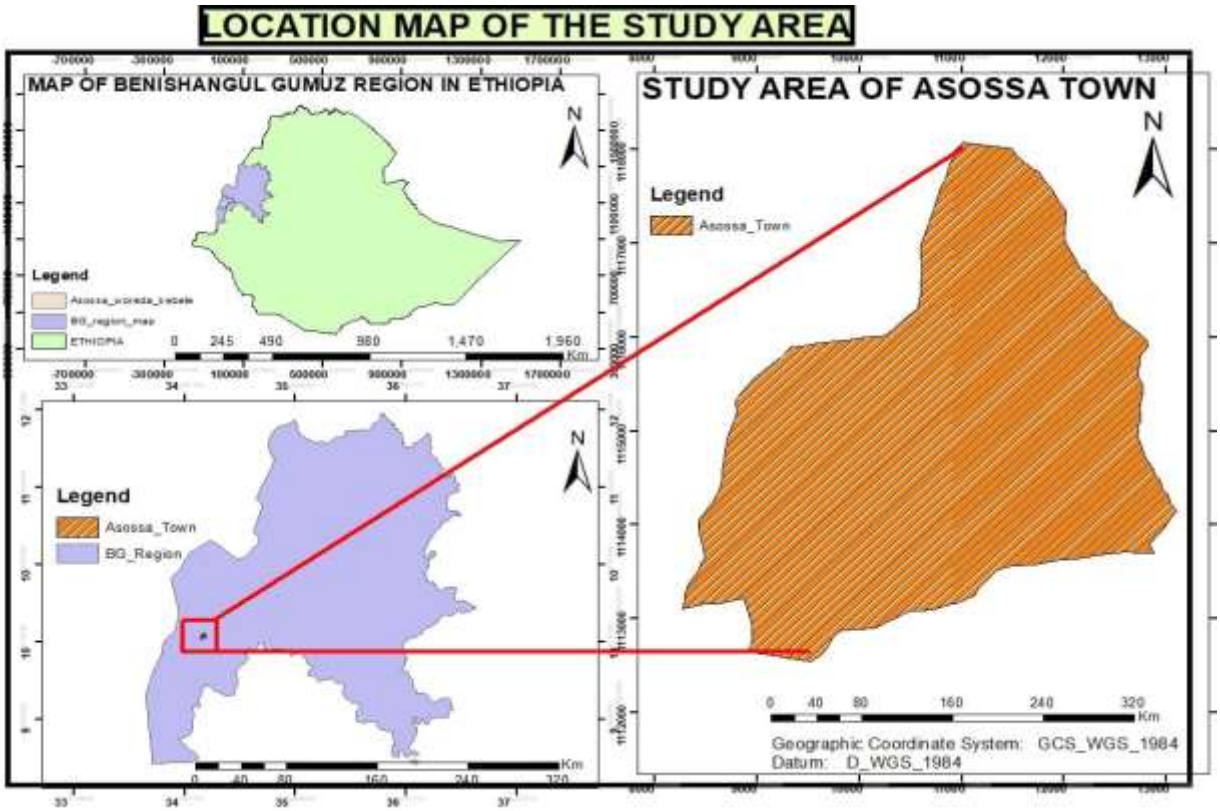

Figure : Map of the study area of Assosa Administrative town

2.2. Study Animals

The study animals were apparently healthy lactating dairy cows of cross-breed kept under intensive production system and milking personnel on dairy farms in Assosa administrative town. The age of the study animals were determined based on birth records from the farm, and dentition characteristics and categorized as young (3-5 years), adult (6-9 years), and old (>9 years). Parity numbers were categorized as (1-2 calves), (3-6 calves), and (> 6 calves). The lactation stage was grouped as early (< 3 months), medium (3-6 months), and late (>6 months) (Markos *et al.*, 2023*).*

2.3. Study Design and Study Period

A cross -sectional study design was conducted from November 2023 to May 2024 to estimate Prevalence, Associated Risk Factors and Antimicrobial Susceptibility Profile of *Staphylococcus aureus* Isolated from Cows' Milk with Public Health Significance in Assosa Administrative Town.

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2.4. Sample Size Determination

The total sample size was assigned according to statistical formula. A 5% absolute precision at 95% confidence interval was used during determining the sample size. The expected prevalence was taken as 50% according to Thrusfield formula (Thrusfield, 2018), since there is no previous work done on the prevalence. Therefore, the total sample size for the study was calculated as follows:

$$n = \frac{(1.96)^2 x\ P\ (1-P)}{d^2}$$

Where: n = the total sample size, P = expected prevalence (50%), d = desired absolute precision (5%), (0.05) at 95% CI.

n = (1.96) x (1.96) x (0.5) x (1-0.5)/ (0.05) x (0.05) = 384; Based on the equation, the calculated total sample size was 384. Therefore, based on equal proportion 134 lactating dairy cow's milk, 134 Swabs of udder and based on the milkers' and milk container available in the farm 52 Swabs of milkers' hands and 64 Swabs of milk container were included that making the total sample size 384.

2.5. Samples and Sampling Technique

Simple random sampling technique was used to select kebeles and dairy farms in two districts of Assosa administrative town. Accordingly, Seven 'kebeles' was selected from two districts of the town; woreda 1 (kebele 1, 4 and 5) woreda 2 (kebele 1, 3, 4, and 5) based on number of dairy farms owned by each kebeles in Assosa administrative town. Lottery method were used to collect samples from both small scale (≤ 5) and large scale (>5) dairy farms in the study area (Markos *et al.,* 2023). Hence from the total sample size of 384, 134 milk samples from lactating dairy cows, 134 udder swabs , 52 milkers' hands Swabs and 64 milk container Swabs were collected. Prior to collet sample information like age, parity, lactation stage, animal body condition score, teat lesion, floor type; previous udder treatment and udder washing before milking were gathered to determine the potential risk factors for *S.aureus* (Appendix II**)**. A total of 60 respondents were interviewed, (24 from farm owners or workers, 10 from hotel or cafe owner and 26 from consumer in the town) using semi structured and pre tested questionnaire survey to determine the possibility of public health significance of *Staphylococcus aureus (*Appendix I**)**.

2.6. Sample Collection Procedure and Transportation

2.6.1. Milk sample and swab sample collection

Milk sample was collected according to National mastitis council (2004). Udder of the cow was properly washed with tap water and soap. Then teat and udder was swabbed with cotton soaked in 70% alcohol. Approximately, 5 ml of milk was then collected aseptically from cow udder into sterile tubes after discarding the first three milking streams. A sample from each quarter was pooled together.

Udder, milkers′ hands and milk container swabs were taken with sterile swab before milking. Prior to sampling swabs, swab tips were moistening with Buffer peptone water (BPW) (Himedia M021, India). Then, the moistened swab was thoroughly rotated over the surfaces (udder, milker hand and container). The swabs were placed inside a tube containing Buffer peptone water, capped, labeled, kept in an icebox with ice pack and transported to Assosa regional microbiology laboratory where it was stored at 4 ^{0}C until bacteriological analysis was done (Quinn *et al*., 2004).

2.6.2. Questionnaires survey

Semi structured and pre-tested questionnaires which prepared in English languages was used to obtain information on which form of milk consumed, ill after consuming raw milk, milk borne diseases, clinical signs, milking equipment, way of management and storage temperature (Appendix I). All respondents of

questionnaire survey were selected based on their voluntariness; thus, a total of 60 respondents were interviewed by translating English language to Amharic.

2.7. Isolation and Identification of *Staphylococcus aureus*

The collected milk samples and swabs were streaked on to selective media of Mannitol salt Agar (Alpha Chemika, MS160) and incubated at 37 ^{0}C for 24 hrs for growth and change in the color of the medium. The presence of growth and change of PH in the media (red to yellow color) were regarded as identification of the salt tolerant *Staphylococci*. Phenol red PH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *S.aureus* causes yellow discoloration of the medium (Quinn *et al*., 2004). The colonies grown on Mannitol Salt Agar (Alpha Chemika, MS160) were taken and culture on nutrient agar plates (Alpha Chemika, NA181) and incubated at 37 ^{0}C for 24 hrs. *S.aureus* colonies' morphology on NA was circular, smooth, convex, and opaque and produces golden yellow pigment observed and recorded. For Gram stain the suspected colonies of *Staphylococcus aureus* from nutrient agar were picked and smeared on labeled clean glass slide. The smeared slides were stained using gram stain technique. Once stained, the smear was examined using the oil immersion lens. The slides were evaluated for the presence of bacterial cells as well as the Gram reaction (color); cocci shape and occur as clusters appearing like a bunch of grapes as Quinn, *et al.* (2004). The colonies were taken from nutrient agar (Alpha Chemika, NA181) with wire loop and inoculated onto sterile blood agar plates (BAP) (Micro-master, India) enriched with 7% heparinized sheep blood and then incubated at 37^{0}C for 24 hours under aerobic culture conditions and showed zones of clear beta-hemolysis. Pure colonies from NA were preserved and maintained on nutrient broth (NB) (Himedia M002, India) for further characterization of the isolates. Further isolation and identification was done by conducting biochemical tests such as catalase test, oxidase test, coagulase, Oxidative-Fermentative (O-F) test, methyl red (MR) and Voges-Proskauer (VP) test (Himedia M070, India) (Quinn *et al*., 2004).

2.8. Antimicrobial Susceptibility Testing of *Staphylococcus aureus*

Antibacterial susceptibility testing of *S.aureus* isolates was evaluated against seven different antibiotics; Cefoxitin (Ck-30µg), Gentamycin (Gen-10µg), Penicillin (P-10µg), Sulfonamide (S3-300µg), Ciprofloxacin (Cip-10µg), Chloramphenicol (C-30µg), Amoxicillin (Amx-10µg)) by using Kibry-Bauer disk diffusion method following Clinical and Laboratory Standards Institute guidelines (CLSI, 2023). 36 *S.aureus* isolates from the total isolates were selected and tested against different antibiotics because of shortage antibiotic discs. For this testing, two plastic Petri dish sized of 85 mm was used to place antibiotic discs (each takes 3-4 discs) placed at 24mm apart. From each isolate, 3-5 biochemically confirmed well-isolated colonies grown on nutrient agar (Alpha Chemika, NA181) was transferred into tubes containing 5 ml of nutrient broth (Himedia M002, India). The broth culture was adjusted to a turbidity equivalent to a 0.5 McFarland standards. Sterile cotton swab was dipped into the suspension and the bacteria were swabbed uniformly over the surface of Muller-Hinton agar plate (Himedia M173, India). Then, the antibiotic discs were placed on the agar plate using sterile forceps and pressed gently to ensure complete contact with the agar surface. The plates were read 24 hours after incubation at 37 ^{0}C under aerobic conditions. The diameter of the zone of complete inhibition was measured in millimeters, interpreted and classified as susceptible, intermediate, or resistant according to procedures established by CLSI (Table 3).

Table 1: Standards of Diameter of the inhibition zones of *S.aureus*

Antimicrobials	**Drug concentration/disc**	**Diameter of the inhibition zones of *S.aureus* according to CLSI (mm)**		
		S	**I**	**R**
Cefoxitin	CK 30 µg	≥ 22	–	≤ 21
Amoxicillin	Amx 10 µg	≥ 22	–	≤ 21
Sulphonamide	S3-300	≥ 17	13-16	≤ 12
Penicillin	P-10 µg	≥ 29	–	≤ 28
Chloramphenicol	C-30 µg	≥ 18	13-17	≤ 12
Gentamicin	Gen-10 µg	≥ 15	13-14	≤ 12

Ciprofloxacin	cip-5 µg	≥ 21	16-20	≤ 15

Key: S = Susceptible, I = Intermediate, R = Resistant

2.9. Data Quality Control, Data Management and Statistical Analysis

Samples were collected following strictly aseptic conditions of sample collection procedure for microbial analysis to avoid cross contamination. Laboratory result was evaluated for their consistency with standard using manuals or positive/negative control isolates. Data validation was performed during data entry process for its consistency, accuracy and missing value before analysis. Each steps of the research was conducted strictly following the data collection, analysis and presentation procedures. Communication to dairy farm owners before the commencement of sample collection and involving voluntary owners, confidentiality on the information and disseminating the result was considered concerning the ethical issues. Collected data was entered in to Microsoft excel spread sheet and checked for accuracy. After validation, coded and analyzing was done by computer software STATA version 17. Multivariable binary Logistic regression test was used when appropriate to analyze the proportions of categorical data. Odd ratio and 95% CI was computed, the 95% confidence level was used, and results were considered significant at P value (< 0.05).

3. RESULTS

3.1. Prevalence of *S. aureus*

For prevalence of *S.aureus* to isolate Mannitol Salt Agar were used as selective culture media and the following biochemical tests (mannitol, catalase, oxidase, coagulase, hemolysis, methyl red (MR), Voges-Proskauer (VP) and oxidative-fermentative (O-F) test) were also used for identification of *S.aureus*. From four sample type, 134 milk sample, 134 udder swab, 52 milkers hand swab and 64 milk container swab totally 384 samples were initially screened on Mannitol salt agar and after incubation at 37°C colonies that showed yellow color were selected for further biochemical tests. Among the total samples 73 were found to be positive for *S.aureus*.

Table 2: Descriptive Statistics of Sample type

Sample type	Total sample examined	Number of positive	Prevalence %
Milk sample	134	18	13.4
Udder swab	134	33	24.63
Hand swab	52	12	23.08
Container swab	64	10	15.63
Total	384	73	19.01

Pearson Chi^2 = 6.49 Prob. = 0.0901

Although the highest prevalence of *S.aureus* was attained in udder swab, there was no a statistically significant association/preference of *S.aureus* to the sample type (X^2= 6.49, p-value=0.0901). The overall prevalence of the *S.aureus* in the current study is 19.01% (CI: 15.07, 22.95).

Table 3: Prevalence Estimations of *S.aureus*

S.aureus	Frequency (%)	Std.err	[95% conf. interval]	
Negative	311(80.99%)	2	77.05	84.93
Positive	73(19.01%)	2	15.07	22.95

3.2. Risk factors associated with the prevalence of *S.aureus* isolates

As the multivariable binary logistic regression shown that majority of risk factors such as age, body condition score and parity status of dairy cows, the sample type, udder washing practice, presence of teat lesion, drainage of the floor, and previous treatment practices have a significant association with the prevalence of *S.aureus*.

As to the sample type, udder swab is 3.4 times more likely to harbor *S.aureus* organisms as opposed to milk sample (adj. OR =3.44 [CI: 1.505, 7.865], P-value = 0.003). Dairy cows which did not receive udder washing practices are 4.7 times more prone to *S.aureus* caused infections (adj. OR: 4.274 [CI: 1.85, 9.872]). Udder washing practices of dairy farm owner help to reduce the likely hood prevalence of *S.aureus* organisms as opposed to those not practiced udder washing activity. Therefore, the organisms have a statistically significant for no udder washing (p-value=0.001).

Table 4: Multivariable binary Logistic regression analysis of associated risk factors with the prevalence of *S.aureus* isolates

S. aureus	**Adjusted Odds ratio**	**St. Err.**	**t-value**	**p-value**	**[95% Conf. Interval]**		**Sig**
Sample type							
Milk	Ref						
Udder swab	3.44	1.451	2.93	0.003	1.505	7.865	***
Hand swab	1.316	0.684	0.53	0.597	0.475	3.643	
Container swab	1.316	0.676	0.53	0.594	0.48	3.603	
Type of farms							
Small	Ref						
Large	0.651	0.239	-1.17	0.242	0.317	1.336	
Parity							
Few (1-2)	Ref						
Moderate (3-6)	1.764	1.016	0.99	0.324	0.571	5.452	
Many (> 6)	4.784	2.766	2.71	0.007	1.541	14.857	***
Lactation stage							
Early (< 3)	Ref						
Medium (3-6)	1.003	0.49	0.01	0.995	0.385	2.615	
Late (> 6)	1.667	0.817	1.04	0.297	0.638	4.358	
Age							
Young (3-5)	Ref						
Adults (6-9)	0.361	0.181	-2.03	0.042	0.135	0.963	**
Old (>9)	0.629	0.298	-0.98	0.327	0.249	1.59	
BCS							
Good	Ref						
Medium	2.618	1.062	2.37	0.018	1.182	5.799	**
Poor	2.464	1.087	2.04	0.041	1.038	5.852	**
Teat's lesion							
Yes	Ref						
No	1.071	0.441	0.17	0.867	0.478	2.4	
Udder washing:							
Yes	Ref						
No	4.274	1.826	3.4	0.001	1.85	9.872	***

Type of floor:							
Muddy	Ref						
Concrete	1.589	0.825	0.89	0.372	0.575	4.395	
Previous udder treatment							
Yes	Ref						
No	0.284	0.117	-3.06	0.002	0.127	0.636	***
Drainage:							
Yes	Ref						
No	4.93	2.508	3.14	0.002	1.819	13.362	***
Constant	0.021	0.026	-3.08	0.002	0.002	0.243	***

Number of observation: 384 Ref=Reference Chi-square: 106.519

Prob > chi^2: 0.000 *** p < .01, ** p < .05

In the current study, adult cows (6-9 years of age) are less likely to contract/ affected by *S.aureus* organisms than younger cows (adj. OR = 0.36 [CI: 0.135, 0.963]), and therefore, the organisms have a statistically significant preference to younger cows (p-value=0.042). Additionally, the results revealed that positive with *S.aureus* was less likely to occur in cows with no previous udder treatment than cows with previously treated (adj. OR=0.28 [CI: 0.127, 0.636], p-value=0.002). The occurrence of positive *S.aureus* was 4.9 times more likely to occur in the cow house of with no drainage than the house having drainage (OR=4.93, [CI: 1.819, 13.362], P-value = .002).

The results revealed that infection with *S.aureus* was more likely to occur in cows with higher parities than cows with few parities (OR=4.784, [CI: 1.541, 14.857], P-value = 0.007) and cows with medium and poor body condition were more likely to be positive to *S.aureus* than cows in good body condition (OR=2.618, [CI: 1.182, 5.799], P-value = 0.018) of medium and for poor body condition (OR=2.464, [CI: 1.038, 5.852], P-value = 0.041).

3.3. Results of Biochemical Tests on *S.aureus* Isolates

A diverse range of biochemical tests are known for the identification and differentiation of *S.aureus*. A conventional biochemical test is often used to identify microorganisms; the results were observed by color change and confirmation was made based on the reaction of an enzyme with a specific substrate of *S.aureus* was identified. Using the following biochemical (mannitol, catalase, oxidase, coagulase, hemolysis, methyl red (MR), Voges-Proskauer (VP) and oxidative-fermentative (O-F) test) yielded the results as indicated in (Table 7) and all result for 73 isolates of *S.aureus* were in (Appendix I).

Table 5: Results of various biochemical tests performed on *S.aureus* isolates

Samples	Mannitol	Catalase	Oxidase	Coagulase	Hemolysis	MR	VP	O-F	Isolated Bacteria
03M	+	+	-	+	Beta (+)	+	+	Fermentative	*S.aureus*
07HS	+	+	-	+	Beta (+)	+	+	Fermentative	*S.aureus*
125M	+	+	-	+	Beta (+)	+	+	Fermentative	*S.aureus*
13US	+	+	-	+	Beta (+)	+	+	Fermentative	*S.aureus*
21HS	+	+	-	+	Beta (+)	+	+	Fermentative	*S.aureus*
26CS	+	+	-	+	Beta (+)	+	+	Fermentative	*S.aureus*
30US	+	+	-	+	Beta (+)	+	+	Fermentative	*S.aureus*

Key: (MR) Methyl Red, (VP) Voges-Proskauer, (+) Positive reaction, (-) Negative reaction, (O-F) Oxidative-Fermentative, (M) Milk, (US) Udder swab, (HS) Hand swab, (CS) Container swab.

3.4. Antimicrobial Susceptibility Profiles of *S. aureus*

Out of 73 *S.aureus* identified, 36 isolates were subjected to antimicrobial susceptibility tests. Amoxicillin and Penicillin were drugs to which a large proportion of *S.aureus* isolates were resistant. As it is indicated in Table 8, most isolates (94% and 100%) were resistant to these two drugs respectively. *S.aureus* isolates were also 61.1% resistant to Sulphonamide. All 36 tested species of *S.aureus* were highly susceptible to Ciprofloxacin (100%) and Gentamicin (100%) followed by Cefoxitin (94%) and Chloramphenicol (94%).

Table 6: Antimicrobial susceptibility pattern of *S. aureus* isolates

Antimicrobials	**Drug concentration/disc**	**Number of isolates (n=36)**		
		S (%)	**I (%)**	**R (%)**
Cefoxitin	CK 30 µg	34 (94)	0 (0.0)	2 (6)
Amoxicillin	Amx 10 µg	3 (8)	0 (0.0)	33 (92)
Sulphonamide	S3-300	4 (11)	11 (31)	21 (58)
Penicillin	P-10 µg	0 (0.0)	0 (0.0)	36 (100)
Chloramphenicol	C-30 µg	34 (94)	1(3)	1 (3)
Gentamicin	Gen-10 µg	36 (100)	0 (0.0)	0 (0.0)
Ciprofloxacin	cip-5 µg	36 (100)	0 (0.0)	0 (0.0)

Key: % = Percent, S = Susceptible, I = Intermediate, R = Resistant

3.4.1. Multidrug resistance of *S.aureus*

In this study 13 isolates of *S.aureus* showed resistance to two classes of antimicrobial drugs. From this isolates eleven for Amoxicillin and Penicillin, one for Amoxicillin and Cefoxitin and one for Penicillin and Sulfonamide. Of the 20 isolates of *S.aureus* 18 isolates were resistance to three classes of antimicrobial (Amoxicillin, Penicillin and Sulfonamides); 2 isolates were each for (Chloramphenicol, Penicillin and Sulfonamides) and (Amoxicillin, Penicillin and Cefoxitin). The maximum multiple drug resistance registered for 2 isolates were resistance to four classes of antimicrobials as indicated in (Table 9).

Table 7: Multidrug resistance (MDR) patterns among *S.aureus* isolates

No. of antimicrobial resistance	**Antimicrobial resistance patterns**	**Number of isolates**	**Number of isolates (%)**
Two	S3,PG	1	1(2.8)
	AMX,PG	11	11(30.6)
	AMX,FOX	1	1(2.8)
Three	CHL,PG,S3	1	1(2.8)
	AMX,PG,S3	18	18(50)
	AMX,PG,FOX	1	1(2.8)
Four	AMX,PG,S3,CHL	1	1(2.8)
	AMX,PG,S3,FOX	1	1(2.8)

Key: No=Number, %=Percent, AMX=Amoxicillin, PG=Penicillin, S3=Sulfonamides, CHL=Chloramphenicol, FOX=Cefoxitin

3.5. Results of the Questionnaire Survey on public health impact

The issues of public health significance arising from *S.aureus* and possible sources of milk, udder, milkers' hand and milk container contamination with *S.aureus* were assessed using semi-structured questionnaire survey on 24 farm owners, 26 consumers and 10 hotel and cafe owners, specifically prepared for each type of respondent accordingly and a total of 60 respondents were participated on questionnaire survey.

In this study in addition to risk factors for prevalence of *S.aureus* were assessed by questionnaire for public health significance like milk consumption, form of milk consumption, acquiring illness due to consuming milk, clinical Signs of illness showed for milk born disease, awareness on milk born disease and *Staphylococcal* food poisoning, frequency of milk consumption and other question raised (Table 9). Among the total of 24 interviewed dairy farmers, 83.3% of them consume milk. From those consume milk, 20% of them consume in the form of raw milk. The consumption of raw milk is relatively higher among uneducated dairy farmers (50%, 2/4) than those who can at least read and write (25%, 1/4). Only (37.5%, 9/24) of the dairy farmers were aware of the occurrence of foodborne diseases due to raw milk consumption, and 4.2% of them have aware of *staphylococcal* food poisoning associated with consumption of raw milk and milk products. Out of 20 farmers how consume milk 25.00% (5/20) acquiring illness and 75% (15/20) were no acquiring illness. Of the 24 farmers, 16.7%, 33.3% and 50% of them practiced cleaning of dairy house three times per day, twice per day and one times per day, respectively, however, 33.3% (8/24) didn't wash udder and 58.3% did wash their hands before milking. On the other hand, all of them practiced washing of dairy equipment with detergents before milking. 87.5% of the farmers used plastic containers for milking and 91.7% of them not use refrigerator (Table 10).

Table 8: Results of questionnaire survey at farm level on public health significance of *S.aureus*

	Value	**Frequency**	**Percentage (n=24)**
Public health significance issues raised for farm owners/workers			
Milk consumption	Yes	20	83.30%
	No	4	16.70%
Form of milk consumption	Raw	4	20.00%
	Ergo	6	30.00%
	Ayib	2	10.00%
	Boiled	8	40.00%
Acquiring illness	Yes	5	25.00%
	No	15	75.00%
Signs of illness showed	Diarrhea	2	40.00%
	Vomiting	1	20.00%
	Diarrhea and Vomiting	1	20.00%
	Abdominal pain and Cramp	1	20.00%
Awareness on milk borne illness	Yes	9	37.50%
	No	15	62.50%
Awareness about Staphylococcal food poisoning	Yes	1	4.20%
	No	23	95.80%
Feeding habit	Rarely	4	20.00%
	Frequently	8	40.00%
	As a common diet	5	25.00%
	As required	3	15.00%

Time gap between taking and using the milk	Yes	16	66.70%
	No	8	33.30%
Average time gap between taking and using the milk	1-2hrs	9	37.50%
	2-8hrs	11	45.80%
	8-12hrs	4	16.70%
	>12hrs	0	0.00%
Use of refrigeration	Yes	2	8.30%
	No	22	91.70%
Level of education those who consume raw milk	Read and write	1	25.00%
	High school	1	25.00%
	College	0	0.00%
	No education	2	50.00%
Floor type living area	Concrete ground	21	87.50%
	Natural floor	2	8.30%
	Stone and Sand	1	4.20%
	Made from wood	0	0.00%
Floor type milking area	Concrete ground	21	87.50%
	Natural floor	2	8.30%
	Stone and Sand	1	4.20%
	Made from wood	0	0.00%
Time length of cleaning dairy house	Three times a day	4	16.70%
	Two times a day	8	33.30%
	One times a day	12	50.00%
Time length of cleaning milking cows udder	No cleaning	8	33.30%
	Only before milking	16	66.70%
	Only after milking	0	0.00%
	Before and after milking	0	0.00%
Equipment used for milking	Aluminum cans	3	12.50%
	Plastic can	21	87.50%
	Clay pot	0	0.00%
	other traditional	0	0.00%
Use of Soap, Detergent to clean milk container	Yes	24	100.00%
	No	0	0.00%
Hand washing before milking	Yes	14	58.30%
	No	10	41.70%

Among the 26 consumers, 53.8% (14/26) of the interviewed consumers drink boiled milk while 23% consume Yoghurt/Ergo and 15.4% consume Cheese/Ayib. 57.7% of them (15/26) had no aware of milk borne disease associated with drinking raw milk and 7.7% of the respondents had knowledge about staphylococcal food poisoning. Out of 26 consuming milk in different forms 34.62% (9/26) were acquiring illness; and 65.38% (17/26) no acquiring illness. Of the consumers, 53.8%, 30.8% and 15.4% of them purchased milk from farms, Milk selling center and from Hotel/cafe

respectively. 77% (20/26) of them used plastic containers while the rest 23% used metallic containers to transport milk to their homes. 69.2% (18/26) of them kept milk in a refrigerator while 30.8% of them kept milk at room temperature (Table 11).

Table 9: Results of questionnaire survey at consumer level on public health significance of *S.aureus*

	Value	**Frequency**	**Percentage (n=26)**
Public health significance issues raised for milk consumer			
Form of milk consumption	Boiled milk	14	53.80%
	Yoghurt/Ergo	6	23.00%
	Cheese/Ayib	4	15.40%
	Raw milk	2	7.70%
Awareness about milk born disease	Yes	11	42.30%
	No	15	57.70%
Awareness about Staphylococcus food born disease	Yes	2	7.70%
	No	24	92.30%
Acquiring illness after consuming milk and milk product	Yes	9	34.60%
	No	17	65.40%
Knowledge on Signs of illness	Diarrhea	4	44.40%
	Vomiting	2	22.20%
	Diarrhea and Vomiting	1	11.20%
	Stomach cramp	2	22.20%
Where do you purchase milk	Farm	14	53.80%
	Milk selling center	8	30.80%
	Hotel /cafe	4	15.40%
Type of container do you use to collect milk	Plastic	20	77.00%
	Metallic	6	23.00%
Duration of milk stay at home prior consumption	<1hr	5	19.20%
	1-2hr	7	27.00%
	>2hr	14	53.80%
Temperature of milk storage	< $4o^c$ /Refrigerator	18	69.20%
	Room temperature	8	30.80%

60% and 40% of the interviewed hotels/cafes purchased raw milk from farms and milk selling centers respectively. Among them, 70% used plastic container while 30% used metallic containers for milk transportation. On the other hand, the respondents (hotels/cafes owners) indicated that they used different methods of quality assessments like boiling (20%) and organoleptic test of visualizing and smelling (80%) before purchasing milk. Milk was found to be kept in a refrigerator by all hotels/cafes until consumption. 80% of them had aware of the occurrence of milk borne diseases associated with drinking raw milk and none of the respondents had aware of staphylococcal food poisoning (Table 12).

Table 10: Results of questionnaire survey at hotel and cafe level on public health significance of *S.aureus*

	Value	Frequency	Percentage (n=10)
Public health significance issues raised for hotel/café owners			
Where do you purchase raw milk	Farm	6	60.00%
	Milk selling centers	4	40.00%
	Others source	0	0.00%
Type of container usually used to collect milk	Plastic	7	70.00%
	Metallic	3	30.00%
	other	0	0.00%
Type quality assessment usually used before purchasing the milk	Alcohol test	0	0.00%
	Lactometer reading	0	0.00%
	Organoleptic test (smell, test, visualization)	8	80.00%
Milk product do you serve for customer	Boiling	2	20.00%
	Yoghurt	2	20.00%
	Boiled milk	7	70.00%
	Cheese	1	10.00%
	Raw milk	0	0.00%
Awareness about food born disease associated with milk	Yes	8	80.00%
	No	2	20.00%
Awareness about staphylococcal food born disease	Yes	0	0.00%
	No	10	100.00%
Time gap between purchasing and serving the milk	Yes	9	90.00%
	No	1	10.00%
How much is the average time gap	1-4hrs	6	66.60%
	4-10hrs	2	22.20%
	10-16hrs	1	11.10%
	more than 16hrs	0	0.00%
Temperature of kept milk	<4 o^c (refrigerator)	10	100.00%
	Room temperature	0	0.00%

4. DISCUSSION

The current study showed a 19.01 % overall prevalence of *S. aureus* in the lactating dairy cows in the study area, which is slightly higher than 15.6 % prevalence reported from Ambo and Bako towns in the West Shoa Zone of the Oromia Regional state, Ethiopia (Bizunesh *et al.*, (2023), but lower when compared with 24.6% the reports of Lema *et al.*, (2021) in Addis Ababa and 21.46% of Tibebu *et al.*, (2021) Bishoftu, Ethiopia. The variable prevalence of *S. aureus* in lactating dairy cows across different reports may be attributed to differences in farm management practices, environmental conditions, and awareness of disease transmission. *S. aureus* is a contagious pathogen that spreads from infected cows to healthy ones during unhygienic milking practices and contact with animals. Multivariable binary logistic regression analysis revealed that the prevalence of *S.aureus* isolates were significantly different among higher

parity groups. A similar result of a significant association of higher parity with the prevalence of *S.aureus* isolates. Cows with higher parity had 4.78 times more risk of infection with *S.aureus* as compared to the cows with low parity. This might be due to the increased opportunity of infection with time and the prolonged duration of infection (Markos *et al.,* 2023).

The current result revealed that cows with no udder washing were 4.27 times more likely infected as compared with cows of udder washing practiced. This result was found consistent with 2.76 times more likely infected than cows of udder washing previously reports of Bizunesh *et al.,* (2022) in Ambo and Bako towns in the West Shoa Zone of the Oromia Region. A significantly higher risk ($p<0.05$) was observed in the sample of Udder swab (OR=3.44, [CI: 1.505, 7.865], P-value = 0.003), than milk sample. This result disagreed with the finding of Tibebu *et al*., (2021) who reported less risk in the udder swab 10% with insignificant (P-value = 0.123) from Bishoftu town, central high lands of Ethiopia. This study revealed that dairy cow house with poor drainage was 4.93 times more likely to be harbor *S.aureus* than well drainage housing systems. The association can be attributed to poor sanitation practices and the housing of cows in dirty and muddy common barns with bedding materials that promote the survival and transmission of pathogens (Bizunesh *et al.,* 2022).

Antimicrobial susceptibility tests of *S.aureus* isolates revealed that the highest rate of susceptibility among the isolates was recorded against Ciprofloxacin and Gentamicin. The results agree with the finding of Tibebu *et al*., (2021) who reported susceptibility to *S.aureus* to Ciprofloxacin and Gentamicin. On the other hand, *S.aureus* isolates showed the highest resistance to Penicillin- G (100%) and followed by Amoxicillin (94%). The current investigation was in agreement with the report of Tsige, (2018) who reported the resistance of *S.aureus* to Penicillin (100%). Moreover, the present report was comparable with the result of Markos *et al.,* (2023) in Shinshicho town recording 100% for both Penicillin and Amoxicillin. This is supported by the findings of Endrias *et al.,* (2022) from Holeta who reported *S.aureus* resistant to Amoxicillin (95%) and Oxacillin (87.50%). Similarly, *S.aureus* resistance to penicillin (94%), were reported from Bishoftu (Tibebu *et al*., 2021).

The variability in susceptibility results could partly arise from how frequently a drug was in use for dairy cows treatment in the study area. The resistance of *S.aureus* isolates to penicillin-G may be attributed to the production of beta-lactamase enzyme that inactivates penicillin and closely related antibiotics. Resistant to penicillin-G is used as a marker to assess the susceptibility of *S.aureus* isolates against other β – lactam antibiotics (Markos *et al.,* (2023*).* The current study has demonstrated the existence of alarming levels of resistance to *S.aureus* to commonly used antimicrobials (including penicillin-G and amoxicillin) in the study farms. Antibiotic-resistant *S.aureus* isolates has been a challenge to both animal and public health. Multiple antibiotic-resistant in this study was 30.6 % of the isolates develop against penicillin-G and amoxicillin. This result was higher than the finding of Lemma *et al.,* (2021) who reported 13.5 % of multiple drug-resistant *S.aureus* isolated from cow milk in Addis Ababa. This might be due to the variation in the type and frequency of use of these antibiotics for the treatment and prevention of prevailing bacterial diseases. Multiple antibiotic-resistant *S.aureus* strains have been isolated from milk obtained from dairy animals in many parts of the world (Lemma *et al.,* 2021*).*

The consumption of raw milk and its different forms of product is common in Ethiopia, which is not safe from consumer‘s health point of view as it may lead to transmission of various diseases. It may be contaminated at the site of production and during processing, the cow itself, unclean milk containers and the milk handlers. The hygienic condition or quality of milk has serous implication on public health safety. The questionnaire result mainly gave broad understanding of the milking and hygienic practice in the study area. In this study among the farmers, 20 % had a habit of drinking raw milk and 62.50% of them didn‘t have awareness about food borne diseases associated with consumption of raw milk. This result is agree to a study done by Tsige, (2018) around Arsi Negelle town, which is 21.7 % of raw milk consumption and 62.9 % of them have no awareness about milk borne disease among farmers. Though the results showed relatively a lower percentage of raw milk consumption, still these individuals are at a greater risk of contracting food born intoxication or infection than those who do not consume raw milk. Similar to the farmers, 30.7 % consumer drink raw milk and raw milk products like yogurt 92.3 % of them have no awareness of milk borne diseases. Consumers are the last group of the food chain and therefore they are at risk of any mal-practice occurring in the chain. Also 30.8 % of the consumers and 91.7 % of farmers kept milk at room temperature. This Lack of

refrigeration facilities at farm and household level with high ambient temperature implies that raw milk will easily be spoiled during storage and transportation (Tsige, 2018)

5. CONCLUSION AND RECOMMENDATIONS

Present study has shown that *Staphylococcus aureus* was widely prevalent in udder swab, milk and milk products in Assosa town. Udder washing and drainage are the determinants of *S.aureus* milk contamination. In addition, hands of milkers' and milk containers were found to be the potential sources of milk contamination with this pathogen. The prevalence of *S.aureus* indicates the higher public health risk due to the widespread consumption of raw milk and its products in Ethiopia. The study also revealed inadequate knowledge of milk borne disease and occurrence of antimicrobials resistant *S.aureus*. Moreover, the large proportion of MDR *S.aureus* isolates may impede effective control of *S.aureus* udder infection in cows as well as it present a public health risk due to the spread of drug-resistant zoonotic *S.aureus*. It was observed that *S.aureus* isolates were highly sensitive to Ciprofloxacin and Gentamicin. Whereas, the highest rate of resistance among the isolates was against Penicillin followed by Amoxicillin. In general, the study has revealed the possibility of the public health risk posed by *S.aureus* in Assosa town. Educational programs to increase knowledge and raise awareness of farm workers, milk product handlers, and milk selling centers on the importance of good hygiene help to increase the good practices of food handlers, which could significantly reduce contamination levels. So based on this conclusion the following recommendations are forwarded;

- Raw milk intended for human consumption must be subjected to pasteurization or heat treatment at least equivalent to pasteurization temperature.
- Hands of milkers' and milk containers should be effectively cleaned with detergent and soap.
- Udder washing should be practiced before milking.
- Milk should be maintained in a cold chain starting from production until consumption.
- Awareness should be created among the community for the implementation of better control and subsequent reduction of SFP.
- Future studies should consider further investigation and designing of cost effective preventive method and control options that would enable to reduce milk contamination by *S.aureus* and there by the associated public health risks.
- In addition monitoring, rational use of drugs and periodic assessment of the antimicrobial sensitivity of drugs prior to use are recommended.

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